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LEVELS AND DISTRIBUTIONS OF PHOSPHOLIPIDS AND CHOLESTEROL IN THE PLASMA MEMBRANE OF NEUROBLASTOMA CELLS

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Summary

Murine neuroblastoma cells (clone N-2A) grown in suspension (spinner cells) or attached on a plastic surface (monolayer cells) were used in studies of the phospholipid and cholesterol composition of whole cells, primary plasma membranes, plasma membranes internalized during phagocytosis of polystyrene latex beads, mitochondria and microsomes.

Monolayer cells contained higher concentrations of total phospholipid, phosphatidylserine and phosphatidylcholine, and lower concentration of phosphatidylethanolamine than spinner cells. The cholesterol levels and the relative proportions of the various phospholipids were similar in both cell types except phosphatidylethanolamine and sphingomyelin whose proportions were lower in monolayer cells.

The primary plasma membranes of the two cell types differed significantly in the relative proportions of all phospholipids, except sphingomyelin, and the phospholipid to protein and the cholesterol to protein ratios were all higher in the membranes of spinner cells. In contrast to these results, all the phospholipid to protein and the cholesterol to protein ratios of the internalized plasma membranes were higher in monolayer than in spinner cells, and the proportions of all phospholipids, except phosphatidylethanolamine, were similar in both cell types.

The membrane distributions of individual phospholipids and cholesterol were inferred from comparison of the phospholipid and cholesterol compositions of primary plasma membranes and plasma membranes internalized during phagocytosis of polystyrene beads. The results are consistent with a non-random distribution of most phospholipids in both spinner and monolayer cells, but the patterns of these distributions were different in the two cell types. With regard to cholesterol the results are compatible with a random or a heterogeneous distribution.

All the phospholipid to protein ratios of the mitochondrial fraction of both cell types were lower than those of the plasma membranes. However, these ratios of the microsomal fraction were higher than those of the plasma membranes of monolayer cells, whereas they were comparable, with a few exceptions, to those of spinner cell membranes. The cholesterol to phospholipid molar ratios of plasma membranes were 6.4 and 4.3 fold greater than those of the mitochondrial and microsomal fractions, respectively.

Introduction

It was earlier shown that the organization and dynamics of the plasma membrane of the C1300 murine neuroblastoma cells (clone N-2A) vary depending on whether the cells are grown in suspension (spinner cells) or attached on an appropriate plastic surface (monolayer cells). This conclusion was based on studies of the distribution of polypeptides, enzymes, and concanavalin A receptors along the plasma membrane bilayer of these cells [1,2]. It was also shown that the kinetics of interaction of concanavalin A with its cell surface receptors were different in spinner and monolayer neuroblastoma cells [3].

The present studies were undertaken to investigate whether any differences exist in the plasma membrane levels and distributions of major phospholipids and cholesterol between spinner and monolayer neuroblastoma cells. The phospholipid and cholesterol composition of the mitochondrial and microsomal fractions of these cells was also determined and compared with that of the plasma membranes.

Methods and Materials

Cell cultures. The N-2A clone of the C1300 murine neuroblastoma (CCL 131) obtained from the American Type Culture Collection, Rockville, MD, was grown in suspension and as monolayers according to procedures described previously [1]. The growth medium contained the following antibiotics (mg/ml): penicillin g sodium salt, 0.05; streptomycin sulfate, 0.1; kanamycin, 0.1. The cell density of spinner cultures was maintained between 1.2 and 6.0 · 10^5 cells/ml. At stationary phase of growth the cell density was 1.9—2.0 · 10^6 cells/ml. In the case of monolayer cultures the cell density ranged between 2 and 12 · 10^6 cells/culture bottle (75 cm^2 of growth area and 17 ml of growth medium). Confluent cultures contained 34—35 · 10^6 cells. All experiments were performed with cells harvested at late logarithmic phase of growth at a cell density of 6—6.5 · 10^5 cells/ml for spinner and 12—12.1 · 10^6 cells/bottle for monolayer cultures. All cultures were routinely checked for absence of contamination by bacteria, fungi, and mycoplasma [4]. Cell viability measured by exclusion of trypan blue was greater than 97%.

Isolation of plasma membranes. The primary plasma membranes and the plasma membranes internalized during phagocytosis of polystyrene latex beads (0.794 μm in diameter) were isolated as described previously [2]. Appropriate aliquots from each membrane preparation were used in the determination of protein and in assays of the following enzymatic activities chosen to assess

possible contamination of the membranes by mitochondria, microsomes, and lysosomes. For mitochondria: succinate dehydrogenase, rotenone-sensitive NADH-cytochrome *c* reductase, and monoamine oxidase. In addition several membrane preparations were examined by difference spectrophotometry (reduced minus oxidized spectra) at liquid nitrogen temperature for the presence of cytochromes aa_3, c_1, *c* and *b*. For microsomes, NADPH-cytochrome *c* reductase. For lysosomes: acid phosphatase, aryl sulfatase, and *p*-nitrophenyl-*N*-acetylglucosaminidase. The above assays were performed as indicated previously [2] under conditions that detect mitochondrial or microsomal protein contaminants amounting to at least 1% of the plasma membrane protein, as calculated from the specific activities of the marker enzymes in isolated mitochondria and microsomes. The assays for the lysosomal enzymes could detect 0.5% of the respective activity of whole homogenates. From this and the low lysosomal protein content of these cells (less than 8% of the cell proteins) it was estimated that the lowest detectable level of lysosomal protein contaminants amounted to less than 0.6% of the plasma membrane proteins. The primary plasma membranes were also analyzed for their enrichment in $(Na^+ + K^+)$-activated ATPase (ATPase), adenylate cyclase, and acetylcholinesterase activities. In neuroblastoma cells ATPase and adenylate cyclase activities are found only in the plasma membranes, whereas acetylcholinesterase is present also in other subcellular fractions [2].

These studies have shown that both the primary and the internalized plasma membranes of both cell types were free of contamination by nuclei (assessed by phase microscopy), mitochondria, microsomes, and lysosomes. In order to further assess the purity of the internalized plasma membranes they were put through the purification procedure used in the isolation of the primary plasma membranes starting with the step where the preparation is treated with the two phase polymer system [2]. The specific activities of 5′-nucleotidase, $(Na^+ + K^+)$-activated ATPase (both plasma membrane markers [2]), and acetylcholinesterase were compared before and after this additional purification. In four such experiments (two for each of spinner and monolayer cells) it was found that the specific activities before and after this additional purification varied by less than ±7%, and were the same as reported earlier (Table V of Ref. 2).

The yield of the primary plasma membranes from both cell types was 52 ± 1% (mean of 52 preparations ± S.E.) based on the recoveries of $(Na^+ + K^+)$-activated ATPase and adenylate cyclase activities. The yield of the internalized plasma membranes was 92 ± 1.5% (mean of 70 preparations ± S.E.). This was derived by comparing the amount of polystyrene latex beads taken up by the cells with the amount of beads recovered in the isolated phagocytic vacuoles (the plasma membrane-enclosed beads) from which the surrounding plasma membranes were obtained in 99% yield based on protein recovery.

Isolation of mitochondria and microsomes. The isolation of these fractions and their content of several enzymatic activities were performed as described previously [2]. In agreement with the earlier findings both of these fractions were free of contamination by plasma membranes as judged by the absence of $(Na^+ + K^+)$-activated ATPase, adenylate cyclase and 5′-nucleotidase activities. The mitochondrial fraction was free of NADPH-cytochrome *c* reductase activity (a microsomal marker), and the microsomes were free of succinate dehydro-

genase, rotenone-sensitive NADH-cytochrome *c* reductase, monoamine oxidase activities and cytochromes aa_3, *b*, *c*, and c_1, (all mitochondrial markers).

Neither aryl sulfatase nor *p*-nitrophenyl-*N*-acetylglucosaminidase activity was demonstrable in any of the mitochondrial and microsomal fractions. However both fractions exhibited low levels of acid phosphatase activity. The specific activity of this enzyme in mitochondria was 15—17 and in microsomes 10—12, as compared with 18—22 in cell homogenates. Thus, of the three lysosomal markers only acid phosphatase was present in the mitochondrial and microsomal fractions. It is not clear whether these fractions are contaminated by lysosomes or whether the acid phosphatase was released from lysosomes and was then trapped in the mitochondrial and microsomal fractions. In all of the above assays the lowest detectable level of any one contaminant amounted to 1% of the total mitochondrial or microsomal proteins. The specific activities of the microsomal and mitochondrial enzyme markers in microsomes and mitochondria, respectively, were in excellent agreement with the values reported earlier [2].

Enzymatic assays. The following enzymatic activities were assayed as previously described [2]: $(Na^+ + K^+)$-activated ATPase (EC 3.6.1.3), 5′-nucleotidase (EC 3.1.3.5), acetylcholinesterase (EC 3.1.1.7), NADPH-cytochrome *c* reductase (EC 1.6.2.4), NADH-cytochrome *c* reductase (EC 1.6.99.3) and rotenone-insensitive NADH-cytochrome *c* reductase, succinate dehydrogenase (EC 1.3.99.1), acid phosphatase (EC 3.1.3.2), aryl sulfatase (EC 3.1.6.1), and *p*-nitrophenyl-*N*-acetylglucosaminidase (EC 3.2.1.30). Monoamine oxidase (EC 1.4.3.4) was assayed by the method of Chuang et al. [5] and adenylate cyclase (EC 4.6.1.1) by the method of Londos and Rodbell [6] using [α-^{32}P]-ATP as substrate.

Extraction of lipids. The lipids of cells and subcellular fractions were extracted with methanol/chloroform (1 : 1, v/v) under argon by the five step procedure of Weinstein et al. [7]. The amount of solvent used in each step was 2 ml in the case of the primary and the internalized plasma membranes isolated from 1.5 to 2.0 · 10^8 cells, and 5 vols. in the case of whole cells and subcellular fractions. The combined lipid extract of each sample was dried at room temperature under a stream of argon in the dark, redissolved in 2 ml of the above solvent, and washed with 0.4 ml of cold water to remove non-lipid material. Aliquots from the chloroform phase were used in the determination of total phospholipid and total cholesterol (free plus esterified), and in the separation of the major phospholipids by thin-layer chromatography. The antioxidant 4-methyl-2,6-di-*tert*-butylphenol was added to the samples at 0.05% final concentration prior to the lipid extraction. It was omitted when cholesterol was to be measured since it interferes with the cholesterol colorimetric assay.

Separation and quantitation of major phospholipids. An aliquot of the lipid extract containing 8—10 μg of organic phosphate was applied to a silica gel HR plate (20 × 20 cm, 250 μm thick) along with standard phospholipids, and the chromatoplate was developed in the appropriate solvent as follows: the phospholipids derived from whole cells, the primary, and the internalized plasma membranes, were separated by one-dimensional chromatography in chloroform/methanol/acetic acid/water (25 : 15 : 4 : 1.9, v/v) [8]. The phospholipids

derived from the mitochondrial and microsomal fractions were separated by two-dimensional chromatography [9]. The developing solvent in the first direction was chloroform/methanol/acetic acid/water (50 : 20 : 7 : 3, v/v), and in the second direction it was chloroform/methanol/40% aqueous methylamine/water (13 : 7 : 1 : 1, v/v). Phospholipid spots were detected by exposure to iodine vapor and identified by comparison with standards. The spots were scraped into pyrex tubes and elution of the phospholipids was carried out by the method of Skipski and Barclay [10]. The eluates were dried under a stream of argon and were dissolved in chloroform. Duplicate aliquots from each sample were analyzed for inorganic phosphate and total phosphate after digestion. The difference between inorganic and total phosphate represents the phospholipid phosphorus. The inorganic phosphate was less than 0.5% of the total phospholipid phosphorus of each eluted sample. The recoveries of standard phospholipids and of the experimental extracts, chromatographed and eluted as described above, ranged between 98 and 101%. Control areas of the chromatoplates not exposed to phospholipids were also eluted and digested in a similar fashion. They contained traces of inorganic phosphate and no additional phosphate was formed after digestion.

The one-dimensional chromatographic procedure gives excellent and reproducible separation of phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine. Phosphatidic acid, cardiolipin and neutral lipids move together near the solvent front. The amount of organic phosphate in this area of the chromatograms was less than 0.5% of the total phospholipid phosphorus of extracts derived from the primary or the internalized plasma membranes, and 1.6—2.0% in the case of the lipid extracts of whole cells. Phosphatidylglycerol, which in this solvent system has an R_F value similar to that of phosphatidylethanolamine, was not demonstrable in any of the lipid extracts by thin-layer chromatography in solvent system I of Skipski et al. [11]. The two-dimensional chromatographic system gives excellent and reproducible separation not only of the phospholipids mentioned above but also of phosphatidic acid, cardiolipin, phosphatidylglycerol and lysophosphatidylethanolamine.

Analytical methods. Inorganic phosphate was measured by the method of Fiske and SubbaRow [12]. Digestion of phospholipids to inorganic phosphate was carried out by the method of Ames and Dubin [13] and the inorganic phosphate was measured by the ascorbate-molybdate method of Chen et al. [14]. This procedure gives 100% digestion of individual or mixtures of standard phospholipids. The amount (in nmol) of either the total or the individual phospholipids shown in Results was obtained from the respective amount (in nmol) of organic phosphate, assuming 1 mol of phosphate/mol of phospholipid, except for cardiolipin where 2 mol of phosphate/mol of cardiolipin was assumed. Total cholesterol (free plus esterified) was measured by the ferric chloride-sulfuric acid colorimetric method [15], and protein by the method of Lowry et al. [16]. The amount of ingested polystyrene latex beads was determined as described previously [17].

Materials. These were purchased from the following sources: polystyrene latex beads (0.794 μm in diameter) were from Dow Chemical, Midland, MI; dextran T-500 was from Pharmacia Fine Chemicals; polyethylene glycol

(Carbowax 6000) was from Fisher Scientific; phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, cardiolipin, spingomyelin, lysophosphatidylethanolamine, lysophosphatidylcholine and phosphatidic acid were from P-L Biochemicals, Inc., Milwaukee, WI; methanol and chloroform, 'Spectranalyzed' grades were from Fisher Scientific Co., U.S.A. Silica gel HR chromatoplates (20 × 20 cm, 250 μm thick) were from Analtech Inc., Newark, DE; the antioxidant, 4-methyl-2,6-di-*tert*-butylphenol was obtained from Aldrich Chemical Co., Milwaukee, WI, and was added as preservative to the reference phospholipids at 0.005% concentration; phosphatidylglycerol, and all the chemicals used in the various enzymatic assays were from Sigma [2]; cyclic [^{3}H]AMP and [α-^{32}P]ATP were from New England Nuclear.

Statistics. Comparisons between any two sets of data were made with Student's *t*-test [18].

Results

Phospholipid and cholesterol composition of cells, primary and internalized plasma membranes

Table I shows that monolayer cells were richer in total phospholipid, phosphatidylserine, and phosphatidylcholine, and poorer in phosphatidylethanolamine. In both cell types phosphatidylcholine accounted for approximately half of the total phospholipid, but the relative proportions of phosphatidylethanolamine and sphingomyelin were lower in monolayer than in spinner cells. It should be noted that the protein content of both cell types was 290 ± 10 μg/1 · 10^6 cells [2].

TABLE I

PHOSPHOLIPID AND CHOLESTEROL COMPOSITION OF N-2A NEUROBLASTOMA CELLS GROWN IN SUSPENSION (SPINNER) OR ATTACHED (MONOLAYER)

The values of the phospholipids were derived from the nmol of organic phosphate obtained after digestion of the lipid extracts, assuming 1 mol of phosphate/mol of phospholipid. The values for the 'area near the solvent front' are nmol of organic phosphate derived, very likely, from phosphatidic acid or cardiolipin or both [8]. The cholesterol values represent the sum of free plus esterified forms. The values are the means of four experiments with deviations from the mean of less than ± 6%.

Lipid	Spinner		Monolayer	
	nmol/mg of protein	% of total phospholipid	nmol/mg of protein	% of total phospholipid
Total phospholipid	128	100.0	149 *	100.0
Phosphatidylethanolamine	25	19.5	19 *	12.8 *
Phosphatidylserine	19	14.8	23 *	15.4
Phosphatidylinositol	13	10.2	16	10.7
Phosphatidylcholine	64	50.0	85 *	57.1
Sphingomyelin	4	3.1	3	2.0 *
Lysophosphatidylcholine	none	0.0	none	0.0
Area near solvent front	2	1.6	3	2.0
Cholesterol	29		33	
Phospholipid recovery (%)	99.2		100.0	
Cholesterol/phospholipid	0.23		0.22	

* Significantly different from the values of spinner cells, $P < 0.001$.

TABLE II

PHOSPHOLIPID AND CHOLESTEROL COMPOSITION OF THE PRIMARY PLASMA MEMBRANES OF N-2A NEUROBLASTOMA CELLS GROWN IN SUSPENSION (SPINNER) OR ATTACHED (MONOLAYER)

The conditions are as described in Table I. The values are the means of four experiments with deviations from the mean of less than ± 6%.

Lipid	Spinner		Monolayer	
	nmol/mg of protein	% of total phospholipid	nmol/mg of protein	% of total phospholipid
Total phospholipid	693	100.0	340 *	100.0
Phosphatidylethanolamine	120	17.3	104 *	30.6 *
Phosphatidylserine	68	9.8	48 *	14.1 *
Phosphatidylinositol	65	9.4	23 *	6.7 *
Phosphatidylcholine	382	55.1	138 *	40.6 *
Sphingomyelin	50	7.2	28 *	8.2
Lysophosphatidylcholine	3	0.4	2	0.6
Cholesterol	353		177 *	
Phospholipid recovery (%)	99.3		100.9	
Cholesterol/phospholipid	0.51		0.52	

* Significantly different from the corresponding values of the membranes of spinner cells, $P < 0.001$.

Table II shows that the phospholipid to protein ratios for all the phospholipids and the cholesterol to protein ratios were significantly higher in the plasma membranes of spinner cells. Furthermore, the membranes of the two cell types differed significantly in the relative proportions of all the phospholipids except sphingomyelin. The molar ratio of cholesterol to phospholipid

TABLE III

PHOSPHOLIPID AND CHOLESTEROL COMPOSITION OF THE INTERNALIZED PLASMA MEMBRANES OF N-2A NEUROBLASTOMA CELLS GROWN IN SUSPENSION (SPINNER) OR ATTACHED (MONOLAYER)

The plasma membranes internalized during phagocytosis of polystyrene latex beads (0.794 μm in diameter) were isolated as described in the text. All other conditions are as described in Table I. The values are the means of four experiments with deviations from the mean of less than ±6%.

Lipid	Spinner		Monolayer	
	nmol/mg of protein	% of total phospholipid	nmol/mg of protein	% of total phospholipid
Total phospholipid	425	100.0	743 *	100.0
Phosphatidylethanolamine	122	28.7	171 *	23.0 *
Phosphatidylserine	50	11.8	98 *	13.2
Phosphatidylinositol	76	17.9	156 *	21.0
Phosphatidylcholine	122	28.7	213 *	28.7
Sphingomyelin	56	13.2	104 *	14.0
Lysophosphatidylcholine	none **		none **	
Cholesterol	459		648 *	
Phospholipid recovery (%)	100.2		99.9	
Cholesterol/phospholipid	1.08		0.87	

* Significantly different from the corresponding values of the membranes of spinner cells, $P < 0.001$.
** Less than 0.5 nmol/mg of protein.

was similar in both types of plasma membranes, and was more than twice that of whole cells.

In marked contrast to the results obtained with the primary plasma membranes, the phospholipid to protein ratios for all the phospholipids and the cholesterol to protein ratio were significantly higher in the internalized plasma membranes of monolayer cells as compared with those in the membranes of spinner cells (Table III). Moreover, the relative proportions of the individual phospholipids in these membranes were comparable in both cell types except phosphatidylethanolamine which comprised a higher percentage of total phospholipid in the membranes of spinner cells. The cholesterol to phospholipid ratio was somewhat higher in the membranes of spinner cells as compared with that of monolayer cells.

The differences in the lipid to protein ratios of the internalized plasma membranes between spinner and monolayer cells can result from differences in either the lipid or the protein (or both) content. A distinction between these alternatives is possible by calculating the concentrations of phospholipids, cholesterol, and protein on the basis of the amount of latex beads of the phagocytic vacuoles from which these membranes were derived. This calculation revealed that the membranes of both cell types contain similar concentrations of protein, namely, 24.5 ± 0.7 and 25.6 ± 0.6 μg/mg of latex beads, respectively (means of 70 preparations ± S.E.). Hence, the differences in the lipid to protein ratios of these membranes between the two cell types reflect differences in the amount of the lipid component.

Plasma membrane distributions of phospholipids and cholesterol

The internalized plasma membranes represent those areas of the primary plasma membrane interiorized during phagocytosis of latex beads (phago-

TABLE IV

INFERENCE OF THE PLASMA MEMBRANE DISTRIBUTIONS OF INDIVIDUAL PHOSPHOLIPIDS AND CHOLESTEROL FROM DIFFERENCES IN THE PHOSPHOLIPID AND CHOLESTEROL COMPOSITION BETWEEN PRIMARY AND INTERNALIZED PLASMA MEMBRANES

Inferred membrane distributions of membrane components		Observed differences in the phospholipid (or cholesterol) to protein ratios between primary and internalized plasma membranes	Observed differences in the proportions of the various phospholipids between primary and internalized plasma membranes
Protein	Phospholipid or cholesterol		
1. Random	random	none	none
2. Non-random	random	similar (uniform) differences for all phospholipids and cholesterol	
3. Random	non-random	dissimilar (non-uniform) differences for the various phospholipids, depending on whether the phospholipid is concentrated in or excluded from the internalized sites of the plasma membrane	dissimilar (non-uniform) differences for the various phospholipids
4. Non-random	non-random	same as in case 3	same as in case 3

TABLE V

COMPARISON OF THE PHOSPHOLIPID AND CHOLESTEROL COMPOSITION BETWEEN PRIMARY AND INTERNALIZED PLASMA MEMBRANES OF SPINNER AND MONOLAYER CELLS

The values were computed from the data of Tables II and III, and are the means ± S.E. (a) Ratios of the phospholipid and cholesterol concentrations (nmol/mg of protein) of the internalized plasma membranes to those of the primary plasma membranes. (b) Ratios of the relative proportions (percentages of total phospholipid) of the various phospholipids of the internalized plasma membranes to those of the primary plasma membranes.

Lipid	Spinner		Monolayer	
	a	b	a	b
Total phospholipid	0.6 ± 0.04	—	2.2 ± 0.10	—
Phosphatidylethanolamine	1.0 ± 0.02	1.7 ± 0.07	1.6 ± 0.06	0.8 ± 0.02
Phosphatidylserine	0.7 ± 0.03	1.2 ± 0.02	2.0 ± 0.09	0.9 ± 0.02
Phosphatidylinositol	1.2 ± 0.02	1.9 ± 0.10	6.8 ± 0.14	3.1 ± 0.11
Phosphatidylcholine	0.3 ± 0.02	0.5 ± 0.03	1.5 ± 0.05	0.7 ± 0.04
Sphingomyelin	1.1 ± 0.04	1.8 ± 0.05	3.7 ± 0.17	1.7 ± 0.06
Cholesterol	1.3 ± 0.03	—	3.7 ± 0.20	—

cytic sites). Table IV shows how the membrane distributions of phospholipids and cholesterol can be inferred unambiguously from the observed differences in the phospholipid and cholesterol composition between primary and internalized plasma membranes. The only situation which is difficult to interpret is when the amounts of protein and phospholipid within the phagocytic areas of the plasma membrane may change by the same factor. In such a case the phospholipid to protein ratios will not be altered even though the distribution of the phospholipid along the plasma membrane is non-random. However, such a case is likely to be a rare occurrence.

In order to simplify the comparison of the phospholipid and cholesterol compositions between primary and internalized plasma membranes the results are expressed as the ratios of the values obtained with the internalized membranes (Table III) to the corresponding values of the primary plasma membranes (Table II). A value of 1.0 indicates that the phospholipid to protein ratios in the two types of membranes are the same. Table V shows that in spinner cells the values for all the phospholipids and cholesterol, except phosphatidylethanolamine and sphingomyelin differed from 1.0 by varying amounts, indicating that the phospholipid to protein ratios as well as the proportions of these phospholipids of the internalized membranes were significantly different from those of the primary plasma membranes ($P < 0.001$). Particularly impressive was the very low value for phosphatidylcholine. In monolayer cells all the phospholipids and cholesterol had values greater than 1.0. These differences between internalized and primary plasma membranes of monolayer cells were all statistically significant ($P < 0.001$), with the exception of phosphatidylserine whose proportion was comparable ($P < 0.05$) in both types of membranes. These results support the conclusion that in both cell types the membrane distributions of most of the phospholipids are non-random, and that the patterns of these distributions are different in the two cell types ($P < 0.001$). With regard to the distribution of phosphatidylethanolamine and

sphingomyelin in the membranes of spinner cells and of cholesterol in the membranes of both cell types the results are compatible with a random distribution or a heterogenous distribution in conjunction with a heterogeneous distribution of the membrane protein.

Phospholipid, cholesterol, and protein composition of internalized membranes at different intervals of phagocytosis

A concern of particular importance for the interpretation of the data is the possibility that the levels of phospholipids and cholesterol of the phagocytic sites (the membrane areas internalized during phagocytosis) could have been altered through enzymatic action. Such alteration could occur at the level of the primary plasma membrane or the phagocytic vacuoles (the membrane-bound ingested beads). In addition, if the phagocytic sites of the plasma membrane were regenerated following their internalization during phagocytosis (secondary phagocytic sites) and their phospholipid and cholesterol make-up was different from that of the original phagocytic sites, engagement of secondary phagocytic sites in a second cycle of phagocytosis would give rise to phagocytic vacuoles the membranes of which would exhibit different phospholipid and cholesterol composition than those derived from the original phagocytic sites.

In order to test for these possibilities experiments were carried out in which the phagocytic vacuoles were isolated at different time intervals of phagocytosis, and the phospholipid, cholesterol, and protein concentrations of their membranes were determined. It was reasoned that the operation of any of the

TABLE VI

PHOSPHOLIPID, CHOLESTEROL, AND PROTEIN COMPOSITION OF INTERNALIZED PLASMA MEMBRANES ISOLATED AT DIFFERENT TIME INTERVALS DURING PHAGOCYTOSIS

The cells were allowed to phagocytize polystyrene latex beads for 5, 15, and 30 min, and the plasma membranes internalized during these periods were isolated and their protein, phospholipid, and cholesterol content was determined as described in the text. The values (means of two experiments) represent nmol of lipid or μg of protein/mg of latex beads of the isolated phagocytic vacuoles. Deviations of individual values from the mean were less than ±6%. Other conditions are as described in Table I.

Compound measured	Interval of phagocytosis of latex beads (min)					
	Spinner cells			Monolayer cells		
	0—5	0—15	0—30	0—5	0—15	0—30
Total phospholipid	10.4	9.9	10.3	19.3	19.0	19.3
Phosphatidylethanolamine	3.0	2.9	3.1	4.4	4.5	4.2
Phosphatidylserine	1.2	1.1	1.2	2.5	2.3	2.4
Phosphatidylinositol	1.9	1.8	1.7	3.9	3.7	3.9
Phosphatidylcholine	3.0	3.0	3.1	5.4	5.5	5.5
Sphingomyelin	1.3	1.4	1.3	2.7	2.9	3.0
Lysophosphatidylcholine	none	none	none	0.3	none	none
Cholesterol	11.2	11.6	11.0	16.9	15.9	16.5
Phospholipid recovery (%)	100.0	103.0	101.0	99.5	99.5	98.5
Cholesterol/phospholipid	1.08	1.18	1.07	0.88	0.84	0.85
Protein	24.5	24.6	24.0	25.7	25.1	26.0

none, less than 0.1 nmol/mg of latex beads.

metabolic processes mentioned above will be reflected in changes of the concentrations of these constituents of the internalized membranes during the time course of phagocytosis. Table VI clearly shows that the phospholipid, cholesterol, and protein levels of the internalized plasma membranes of both cell types remain remarkably constant during the entire period of phagocytosis. These results strongly suggest that: (a) the phospholipid, cholesterol, and protein composition of the phagocytic sites of the plasma membrane was not altered during phagocytosis either before or after their internalization, and (b) if secondary phagocytic sites were generated and participated in phagocysotis, their phospholipid, cholesterol, and protein make-up must have been similar to that of the original phagocytic sites.

Phospholipid and cholesterol composition of mitochondria and microsomes

Table VII shows the composition of the mitochondrial and microsomal fractions of spinner cells. Similar data were obtained with these fractions isolated from monolayer cells (data not shown). The values of both fractions are in good agreement with those reported for mitochondria and microsomes from a variety of tissues [19]. The cholesterol to phospholipid molar ratio in mitochondria was 0.08 as compared with 0.12 in microsomes. Previously reported values of this ratio for mitochondria and microsomes isolated from a variety of tissues show considerable variation: 0.03—0.3 for mitochondria [20—26] and 0.04—0.5 for microsomes [20,21,23,26—29]. As expected, cardiolipin was a mitochondrial constituent and accounted for 11% of the total phospholipid. The phospholipid to protein and the cholesterol to protein ratios were higher in

TABLE VII

PHOSPHOLIPID AND CHOLESTEROL COMPOSITION OF THE MITOCHONDRIAL AND MICROSOMAL FRACTIONS OF SPINNER CELLS

The values are the means of two experiments with deviations from the mean of less than ±7%. Other conditions are as described in Table I.

Lipid	Mitochondria		Microsomes	
	nmol/mg of protein	% of total phospholipid	nmol/mg of protein	% of total phospholipid
Total phospholipid	250	100.0	520	100.0
Phosphatidylethanolamine	83	33.2	146	28.0
Phosphatidylserine	15	6.0	62	11.9
Phosphatidylinositol	14	5.6	26	5.0
Phosphatidylcholine	100	40.0	223	42.9
Sphingomyelin	9	3.6	57	11.0
Lysophosphatidylcholine	1	0.4	none	
Lysophosphatidylethanolamine	none		none	
Phosphatidic acid	1	0.4	none	
Cardiolipin	28	11.2	none	
Cholesterol	20		62	
Phospholipid recovery (%)	100		98.8	
Cholesterol/phospholipid	0.08		0.12	

none, less than 0.5 nmol/mg of protein.

microsomes as were the proportions of phosphatidylserine and sphingomyelin.

All the phospholipid to protein ratios of the plasma membranes of both cell types were significantly higher than those of the mitochondrial fraction ($P < 0.001$). However, the differences between plasma membranes and the microsomal fraction were not uniform. Thus, compared with the plasma membranes of monolayer cells, all the phospholipid to protein ratios, except that of phosphatidylinositol, were significantly higher in the microsomal fraction ($P < 0.001$), whereas compared with the plasma membranes of spinner cells, only the phosphatidylinositol and phosphatidylcholine to protein ratios were significantly lower in the microsomal fraction ($P < 0.001$). The cholesterol to phospholipid molar ratios of the plasma membranes were 6.4 and 4.3 fold higher than those of the mitochondrial and microsomal fractions, respectively. Previously reported values of this ratio for plasma membranes of various mammalian cells ranged between 0.6 and 0.76 [7,24,26,30—33].

Discussion

The plasma membrane distributions of the major phospholipids and cholesterol were inferred from a comparison of the phospholipid and cholesterol composition of plasma membranes and plasma membranes internalized during phagocytosis of latex beads. Based on the finding that the phospholipid to protein ratios as well as the proportions of the phospholipids were significantly different in the two types of membranes for both spinner and monolayer cells, it was concluded that: (a) the distributions along the plasma membrane bilayer of most, if not all, of the phospholipids are non-random, with membrane domains exhibiting heterogeneity in their phospholipid composition; (b) the plasma membrane distribution patterns of the phospholipids and cholesterol are different in the two cell types, and (c) the above differences between primary and internalized plasma membranes are either independent of, or could not have arisen entirely from a presumed heterogeneous distribution of membrane protein.

In reaching the above conclusions the following points have been taken into consideration: (1) the purity of the plasma membrane fractions. Both the primary and the internalized plasma membrane fractions were free of contamination by nuclei, mitochondria, microsomes, and lysosomes. The specific activities of $(Na^+ + K^+)$-activated ATPase (ATPase), adenylate cyclase, and acetylcholinesterase in the membranes of spinner cells were 63, 15, and 17, respectively, and 132, 18, and 28, respectively, in the membranes of monolayer cells. From these values and the corresponding specific activities in cell homogenates it was calculated that the plasma membranes of both cell types were enriched in ATPase and adenylate cyclase by a factor of 12 ± 1 (mean of 70 preparations ± S.E.) and 12.6 ± 1 (mean of 52 preparations ± S.E.), respectively, while acetylcholinesterase was enriched by a factor of 2.4 ± 0.5 (mean of 70 preparations ± S.E.). Since the plasma membrane distribution of ATPase is non-random in both cell types while that of acetylcholinesterase is random in spinner and non-random in monolayer cells, the constancy of these enrichment factors indicates that the plasma membrane fractions were of equal purity, and that in each case the same membrane fraction was being analyzed and com-

pared. Furthermore, electron microscopic studies of these cells showed no detectable fusion of phagocytic vacuoles with themselves or with lysosomes during 45 min of phagocytosis of polystyrene latex beads (Graham, D.I., Charalampous, F.C., and Gonatas, N.K., unpublished observations). (2) The phospholipid and protein composition of those areas of the plasma membrane internalized during phagocytosis could have been altered through enzymatic attack (or as a result of other metabolic processes) either before or after their internalization during the course of phagocytosis. Evidence against this was the finding that the phospholipid, cholesterol and protein composition of the internalized membranes remained constant throughout the course of phagocytosis (between 5 and 30 min). The possibility that membrane alterations occurred only in the first 5 min of phagocytosis and thus escaped detection is unlikely, although not excluded. In support of this premise was the demonstration that degradation products of phospholipids such as lysophosphatidylcholine or phosphatidic acid were either not demonstrable in the primary and internalized plasma membrane fractions or, when present, they amounted to less than 0.6% of the total membrane phospholipid. No measurements were made on the fatty acid composition of the phospholipid fraction of the internalized membranes, and therefore it is not known whether the acyl portion of the phospholipids was altered during phagocytosis through the combined action of deacylases and acylases. Throughout this paper the phospholipid data concern the type of phospholipid without consideration of the makeup of their acyl portions. (3) In each case the internalized plasma membrane fraction was a representative sample from all of the cells since more than 97% of the cells employed in an experiment had ingested beads as monitored by phase microscopy [2].

The question of whether the heterogeneous distributions of the plasma membrane phospholipids represent an inherent property of the plasma membrane of these cells, or whether they were induced during the internalization of the latex beads, remains to be answered. However, it is unlikely that the differences in the membrane distributions of these lipids between monolayer and spinner cells were generated by either contact of the cells with the beads or by phagocytosis since both cell types were treated with beads under similar conditions.

The results presented here are in agreement with previous reports on the non-random clustering of phosphatidylserine and phosphatidylethanolamine in erythrocyte membranes inferred from studies with cross-linking bifunctional reagents [34–36], and extend earlier observations which suggest lateral phase separation of phospholipids in artificial lipid membranes [37–41] and in bacterial [42,43] and mammalian [44–46] cell membranes. More recently phagocytosis of polystyrene latex beads was employed to show transbilayer asymmetry in the distribution of phosphatidylethanolamine, sphingomyelin and fatty acids in the plasma membranes of mouse LM cells [47].

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