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INTERACTION OF ACIDIC LIPOSOMES WITH RED BLOOD CELLS

INDUCTION OF ENDOCYTOSIS AND SHEDDING OF PARTICLES

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Calcium ions induced tight binding of massive amounts of liposomes containing cardiolipin, phosphatidylethanolamine and phosphatidylcholine to erythrocytes. Initially, liposome-liposome fusion occurred and only subsequently the resulting large structures adhered to cells. Large clumps composed of liposomes and cells were formed. Upon prolonged incubation, the clumps were dissipated spontaneously and excess liposomes were released. A constant amount of phospholipid (15–25 nmol/10^8 cells) were incorporated into the cell membranes. Upon disaggregation, the cells shed erythrocyte particles. The latter were isolated and shown to contain lipids from both cellular and liposomal origin. The particles lacked spectrins and contained variable amounts of band 3 content. Liposomes induced endocytosis in reticulocytes but not in mature erythrocytes. In most cases, the liposomes themselves were not engulfed by the cells and remained attached to their surface. The relevance of this phenomenon to delivery of liposome contents into cells is discussed.

Introduction

Liposomes have attracted attention as potential delivery vehicles into cells, with which the hydrophobic barrier posed by plasma membranes can be circumvented. The main modes of liposome-cell interaction are transient or tight adsorption of vesicles to cells, fusion of liposome membranes with cellular ones and endocytosis (for review, see Refs. 1 and 2). Accordingly, the liposome lipid may be adsorbed to cell surface, become part of the plasma membrane or end up in endocytotic vesicles. In addition, transfer and/or exchange of individual phospholipid molecules has been demonstrated. The vesicle contents either remain in adsorbed vesicles, are injected into cell cytoplasm or are internalized by endocytosis. In addition it is possible that they leak into the cytoplasm from adsorbed liposomes or from endocytotic vesicles.

Delivery of drugs and especially enzymes into the endocytotic and digestive systems of the cells seems a promising therapeutic approach to genetic storage diseases [3,4]. On the other hand, delivery of drugs and nucleic acids into cell cytoplasm would allow manipulation of cell metabolism and hereditary material [5,6]. Various nucleotides, drugs and fluorescent markers have been injected into cell cytoplasm, presumably by fusion of vesicles with cells [7,8]. Recently viral RNA and DNA have een injected into cells and were expressed in the cells [9,10]. these experiments provided a reliable assay allowing distinction between injection into cell cytoplasm and absorption. They have demonstrated that previous reports of efficient liposome-cell fusion were greatly exaggerated and most liposomes are either absorbed to the cells or endocytosized by them. Clearly, better understanding of the phenomenology of liposome-cell interaction is required.

We have chosen to study liposome-erythrocyte

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interaction since the latter allows easy detection of vesicle adsorption and endocytosis. The following intriguing features have been observed: (a) tight binding of liposomes which is spontaneously reversed upon prolonged incubation, (b) liposomes induced shedding of erythrocyte particles, and (c) liposomes induced endocytosis in reticulocytes and not in mature erythrocytes.

Materials and Methods

Phosphatidylcholine and cardiolipin were purchased from Sigma, St. Louis. Phosphatidylethanolamine was prepared from soybean lipid extract according to published procedures [11]. ^{32}P-labelled phospholipids were prepared essentially according to the procedure of Johnson and Zilversmit [12]. [^{32}P]Phosphoric acid (5 mCi) was neutralized by addition of sodium phosphate (0.2 M, pH 7.3, 1.0 ml) and injected into the peritoneal cavity of a young rat. The next day, the liver was removed and extracted. The total radioactivity amounted to 50 μCi and the specific activity was 0.5 μCi/μmol. Purified radioactive phospholipids were prepared from the liver by isolation of mitochondrial fraction. The latter was extracted and the various phospholipids were separated by thin-layer chromatography on silicic acid-coated plates with chloroform/methanol/water (65:25:4, v/v) as eluent. The spots were located by autoradiography, scraped and extracted. The specific activities of phosphatidylcholine, phosphatidylethanolamine and cardiolipin were 1.0, 0.8 and 0.05 μCi/μmol, respectively.

Blood was drawn from the ear of a young rabbit into a beaker containing a few grains of heparin dissolved in 10 ml Hepes (20 mM, pH 7.6) and NaCl (160 mM). The cells were washed three times by centrifugation for 5 min at $1000 \times g$ and resuspended in the same buffer. Finally the cells were resuspended at 40% hematocrit in the buffer used throughout the present work containing Hepes (20 mM, pH 7.6), KCl (128 mM) and NaCl (32 mM). The cells were used immediately. When needed, rabbit blood was enriched with reticulocytes by bleedings of 10–15 ml every other day for two weeks prior to the experiment.

Liposomes were prepared by drying down the appropriate amounts of various phospholipids under a stream of nitrogen gas, redissolving them in ether (3 ml) and drying then down again. Buffer was added to a final lipid concentration of 3% (w/v). After 5 min incubation at room temperature, the sample was sonicated to clarity with a bath-type sonicator (80 kHz, Laboratory Supplies, Long Island, NY). Liposomes were kept on ice until used the same day.

Erythrocytes were incubated at room temperature with liposomes in scintillation vials precoated with silicone. To ensure equilibration with air oxygen, incubation volume was limited to 2 ml and the vials were agitated at 100 rpm. The incubation buffer contained glucose (10 mM). Cell samples were withdrawn, mixed with EDTA (25 mM), incubated for 5 min at room temperature and passed through a 5 ml Ficoll (20% in buffer) by centrifugation for 5 min at $3000 \times g$. The upper layer containing excess liposomes and in some cases, erythrocyte particles (see below) was collected. The erythrocyte pellet was washed with buffer and assayed. Erythrocyte particles were isolated by centrifugation of the cell supernatant sample into a continuous 2 to 40% Ficoll gradient in buffer at $100000 \times g$ for 2 h. The particles' fraction was diluted with buffer and pelleted by centrifugation at $150000 \times g$ for 1 h.

SDS gel electrophoresis of protein samples was carried out according to Laemmli [13]. ATP was determined with the firefly tail assay [14]. Phospholipids were analyzed by extraction with chloroform and methanol and chromatography with chloroform/methanol/water (65:24:4, v/v).

Electron microscopy. Negative staining. Samples were fixed with cold OsO_4 (1% in sodium phosphate buffer, pH 7.4, 0.2 M) for 1 h. Drops were layered on collodion films. Excess medium was drained and the samples were stained with aqueous uranyl acetate (2%). Thin sectioning. Cells were washed twice with buffer, once with sodium cacodylate (0.2 M, pH 8.0) and fixed for 2 h at 0°C with glutaraldehyde (2.5% in sodium cacodylate). The pellets were washed twice with sodium phosphate (0.2 M, pH 7.3) and fixed overnight with OsO_4 (1% in phosphate buffer). The pellets were washed three times with NaCl (155 mM), dehydrated rapidly in cold acetone solutions and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

Results

In the presence of calcium ions, liposomes containing phosphatidylethanolamine, cardiolipin and phosphatidylcholine became tightly adsorbed to erythrocytes (Fig. 1). Large visible clumps containing liposomes and cells were formed. Within minutes, about 90% of added liposomes were trapped in these clumps. The amount of liposomes bound to the cells seemed unlimited (Fig. 2a). The aggregation was dependent on calcium and could be stopped by sequestering the calcium with a chelating agent (Fig. 1). Similar aggregation was induced by magnesium (40 mM) or lanthanum ions (0.5 mM, data not shown). Once formed, the aggregates were not affected by chelating agents.

However, on further incubation, the aggregated dispersed spontaneously (Fig. 1). This occurred even in the presence of calcium ions. After about 2 h, most of the liposomes' phospholipids were easily separated from the cells. Disaggregation and release of excess liposomes was prevented by incubation at 0°C but not by fixation with glutaraldehyde (data not shown).

The residual amount of liposomes, bound to the cells was constant and amounted to about 20 nmol bound to 10^8 cells, corresponding to about 20% of cell phospholipids (Fig. 2b). This residual amount was not removed upon repeated washes or prolonged incubation even in the presence of chelating agents. The ratio of the various liposome phospholipids remaining in the cells was similar to that in the original liposomes (Table I). The aggregation was dependent on liposome participation and in their absence, calcium ions caused no change in the cells' shape or aggregation state. Calcium induced rapid clumping of cells incubated with liposomes composed of pure cardiolipin, but these clumps did not undergo spontaneous disaggregation even upon incubation of more than 24 h. Calcium did not induce aggregation of cells in presence of liposomes containing either phosphatidylcholine or phosphatidylcholine and cardiolipin (70 and 30 mol%, respectively). Only small amounts of the latter liposomes became attached to the cells (Table II).

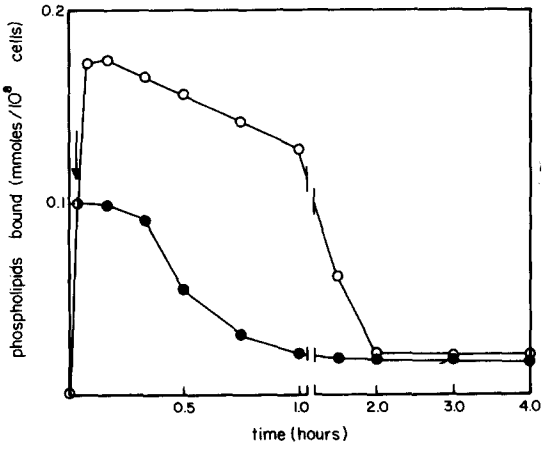


Fig. 1. Time-course of liposomes association with erythrocytes. Liposomes containing radioactive cardiolipin, phosphatidylethanolamine and phosphatidylcholine (30, 60 and 10 mol%, respectively, 1 mM, specific activity of 0.05 μCi/μmol) were incubated with washed erothrocytes (10^8 cells/ml) in presence of $CaCl_2$ (20 mM) (○——○). After 2 min, EDTA (30 mM) was added to part of the cells (●——●) and all cells were further incubated. Samples were withdrawn, treated with EDTA, washed and tested for radioactivity.

TABLE I

INCORPORATION OF PHOSPHOLIPIDS INTO ERYTHROCYTES AND INTO PARTICLES

Liposomes (1 mM) containing phosphatidylcholine, cardiolipin and phosphatidylethanolamine (10, 30, 60 mol%, respectively, total specific activity 0.5 μCi/μmol) were incubated with washed erythrocytes (10^8 cells/ml) in presence of $CaCl_2$ (20 mM) for 3 h. The cells were treated with EDTA and separated from excess liposomes and released particles by passage through a Ficoll cushion. Particles were isolated from the supernatant as described in legend to Fig. 8. The radioactivity and phospholipid contents were determined and the specific activity was calculated. Phospholipids were extracted from the cells and the particles, separated by thin-layer chromatography and the radioactivity of the various spots, was determined after scraping and extraction. The radioactivity of the various phospholipids are presented as percent of total radioactivity in the same sample.

	Liposomes	Erythrocytes	Particles
Specific activity Phospholipids (μCi/μmol)	0.5	0.11	0.18
Phosphatidylcholine (%)	(10)	10.5	9.8
Phosphatidylethanolamine (%)	(60)	59.2	62.3
Cardiolipin (%)	(30)	32.1	30.4

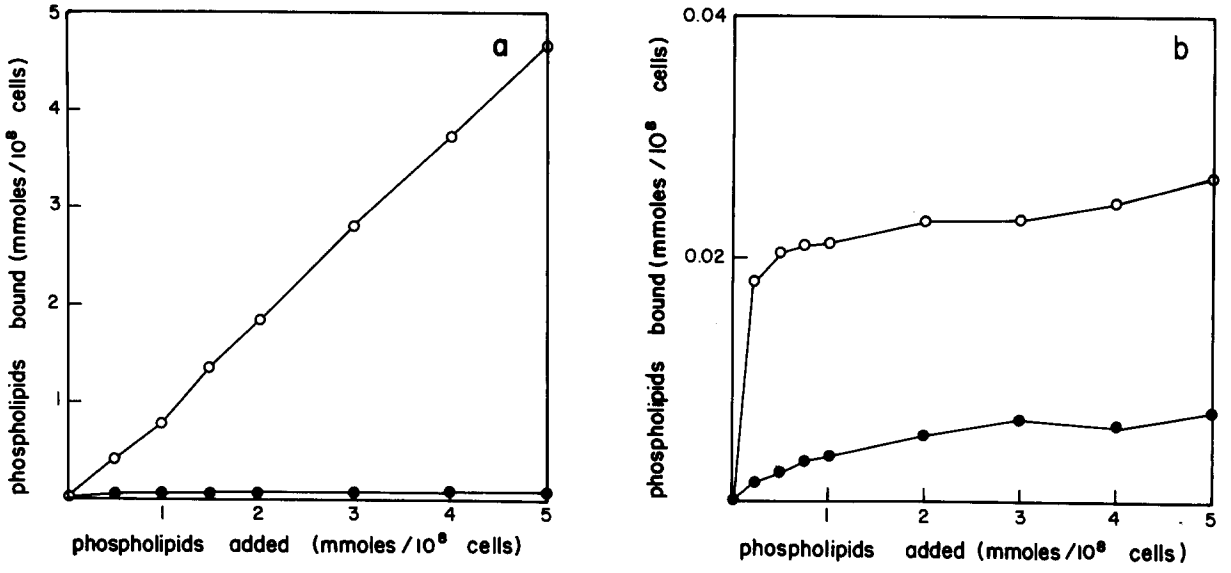


Fig. 2. Effect of liposome concentration on their association with erythrocytes. Liposomes containing radioactive cardiolipin, phosphatidylethanolamine and phosphatidylcholine (30, 60 and 10 mol%, respectively, spec. act. 0.05 μCi/μmol) were prepared as described under Materials and Methods. Various liposome concentrations were incubated with washed erythrocytes (10^8 cells/ml) either in presence (O——O) or absence of $CaCl_2$ (20 mM, ●——●). Samples were withdrawn after either 30 min (part a) or 3 h (part b). The cells were treated with EDTA, washed and tested for radioactivity.

Electron microscopy

Better insight into the nature of the aggregation and spontaneous disaggregation phenomena was obtained by electron microscopic examination of the cells. At low liposome to cell ratio, the clumps contained deformed cells held together by monolamellar lipid vesicles. The vesicle size varied between 50 and 500 nm (Fig. 3). Since the diameter of the original liposomes varied between 20 and 50 nm [15], the lipid vesicles present in the clumps must have been the product of limited fusion of liposomes. At higher liposome to cell ratio, larger

TABLE II

EFFECTS OF LIPOSOME COMPOSITION AND CATIONIC IONS ON RELEASE OF PARTICLES

Liposomes of various lipid compositions were prepared with addition of radioactive phosphatidylcholine (final specific activity 0.5 μCi/μmol) except for the sample containing dimyristoylphosphatidylcholine. Washed erythrocytes (10^8 cells/ml) were incubated with liposomes (0.1 mM) for 3 h at 30°C in the absence or presence of either $CaCl_2$ (20 mM) or $MgCl_2$ (40 mM). The cells were treated with EDTA and their radioactivity content was determined. Particles were isolated and their hemoglobin content was determined. The hemoglobin content of the particle fraction was assumed to express the particles' amount.

	Bound phosphatidylcholine (% of added)			Hemoglobin in particles (% of erythrocyte content)		
	–	$CaCl_2$	$MgCl_2$	–	$CaCl_2$	$MgCl_2$
Phosphatidylethanolamine/egg phosphatidylcholine/ cardiolipin (6 : 1 : 3, mol%)	3.6	21.2	18.3	0.0	0.02	0.018
Egg phosphatidylcholine/cardiolipin (7 : 3, mol%)	3.7	5.1	4.7	0.0	0.0	0.0
Egg phosphatidylcholine	4.5	4.3	3.9	0.0	0.0	0.0
Dimyristoylphosphatidylcholine	–	–	–	0.025	0.021	0.028

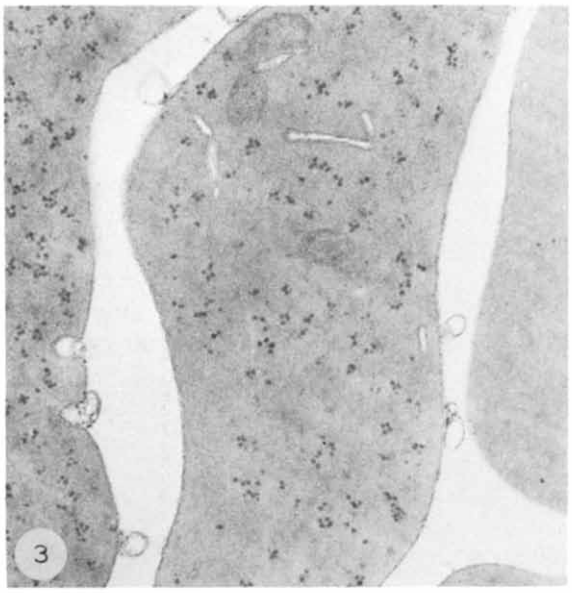
and more complex lipid structures were observed adsorbed to the cells (Figs. 4 and 5). At high liposome to cell ratios, very large complex structures composed of dense multilamellar formations and paracrystalline hexagonal ones were seen adsorbed and bridging between the cells (Fig. 6). Similar structures were observed when liposomes were treated with calcium ions in absence of cells [15]. Initially, the liposomes had fused with each other and only subsequently they became attached to the cells. Lipid structures associated with the cells after 1 min incubation appeared similar to those observed after 30 min. Only the amount of lipid structures increased. After incubation periods longer than 2 h neither cell aggregates nor lipid structures associated with them were observed. Upon release from the clumps, the cells appeared as spheroechinocytes and shed massive amounts of particles (Fig. 7). After further incubation the cells assumed the spherocyte shape and many erythrocyte particles were observed free in the medium.

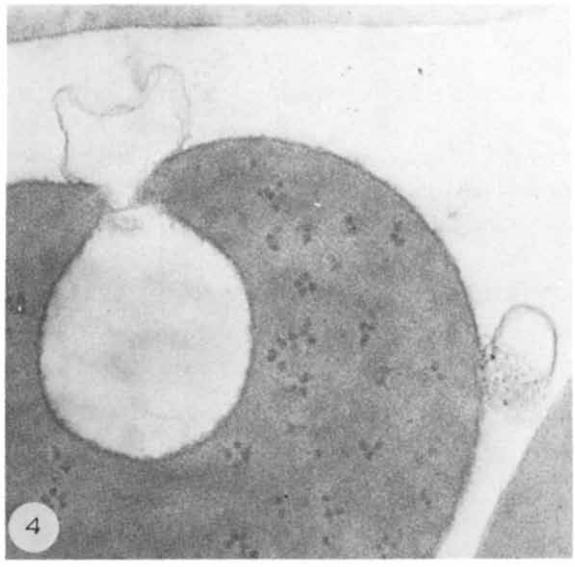
Liposome-induced endocytosis in reticulocytes

Reticulocytes were recognized by polyribosomes present in their matrix (Refs. 16, 17 and Figs. 3, 4, 6). Liposomes, in the presence of calcium ions, induced extensive endocytotic activity in erythrocytes. A seemingly surprising feature of the endocytosis was that although it was induced by liposomes, most of the internalized vesicles did not contain lipid structures. This phenomenon could be explained by the following observation. As shown in Figs. 3, 4, and 5, the endocytotic invaginations were associated with liposomes adsorbed to the cells. However, the latter were not engulfed by the cells. Thus, lipid structures induced formation of strings of seemingly empty endocytotic vesicles. The highest amount of endocytotic activity occurred in the clumps. Upon release of adsorbed lipid structures no endocytotic activity was observed and the number of internalized vesicles decreased, presumably by fusion with the plasma membranes. In absence of either calcium ions or liposomes, reticulocytes contained only a few internal vesicles. Liposomes containing phosphatidylcholine or phosphatidylcholine and cardiolipin did not enhance endocytosis.

Erythrocyte particles

As mentioned above, upon disaggregation, cells shed particles. The latter were easily isolated by





Figs. 3–6. Electron microscopy of liposome association with erythrocytes. Liposomes were preapred as described in legend to Fig. 1, except that they were not radioactive. Red blood cells, enriched in reticulocytes (10^8 cells/ml) were incubated with 0.05 mM (Fig. 3), 0.25 mM (Figs. 4 and 5) and 1 mM (Fig. 6) liposomes. After 10 min, samples were withdrawn and processed for electron microscopy as described under Materials and Methods. Magnification: Fig. 3, ×35000; Fig. 4, ×58500; Fig. 5, ×65000; Fig. 6, ×100000.

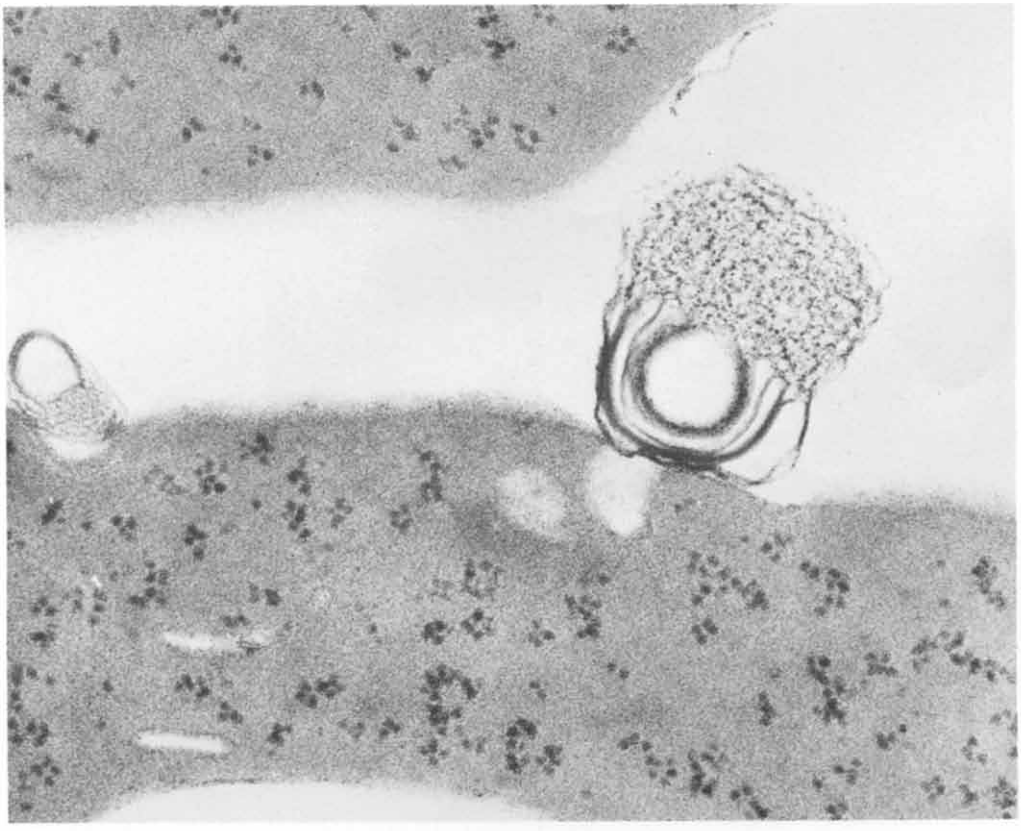


Fig. 5.

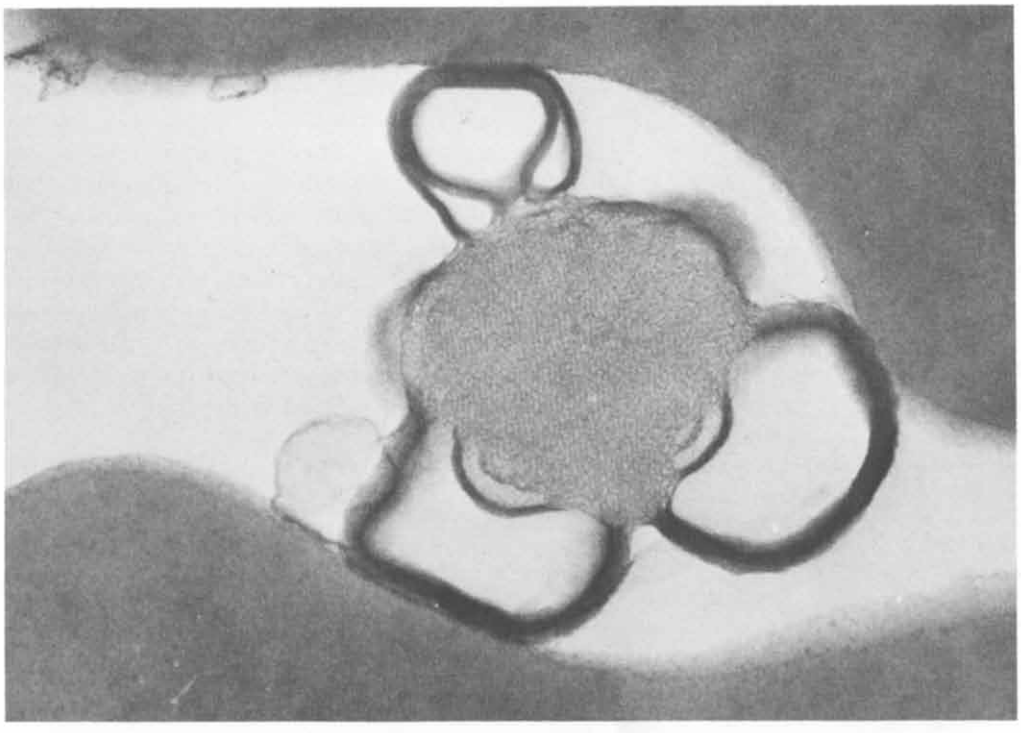


Fig. 6.

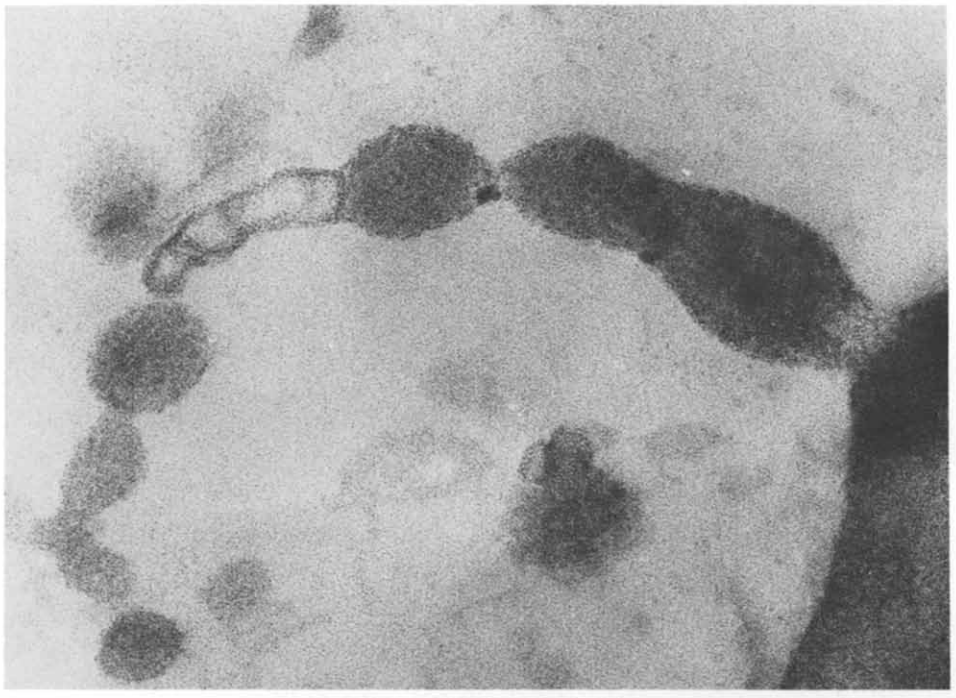


Fig. 7. Shedding of erythrocyte particles. Erythrocytes (10^8 cells/ml) were incubated with liposomes as described in the legend to Fig. 4. After 2 h incubation at room temperature, a sample was processed for electron-microscopy. Magnification $\times 100000$.

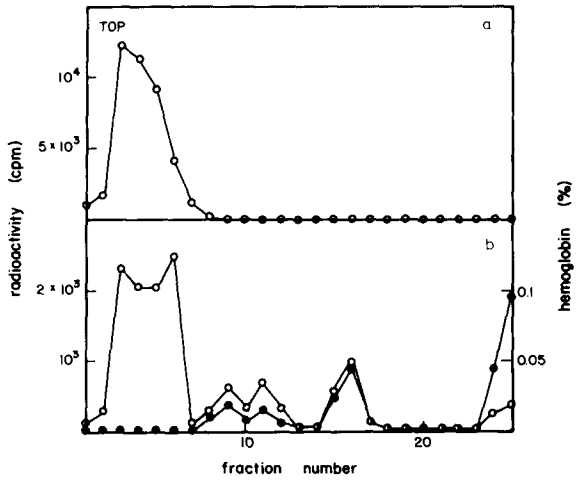


Fig. 8. Isolation of erythrocyte particles. Radioactive liposomes (0.2 mM, 0.5 μCi/μmol) containing cardiolipin, phosphatidylethanolamine and phosphatidylcholine (30, 60 and 10 mol%, respectively) were incubated in presence (part b) or absence (part a) of erythrocytes (10^8 cells/ml). After 1 h in presence of 20 mM $CaCl_2$, the samples were centrifuged through a Ficoll cushion. Supernatant samples (0.1 and 1.0 in parts a and b, respectively) were centrifuged into continuous Ficoll gradients as described under Materials and Methods. Gradient fractions were collected and assayed for phospholipid radioactivity (○——○) and hemoglobin content (●——●). Examination of the various fractions revealed in part b, lipid structures in fractions 3–6, erythrocyte particles mixed with empty membrane structures in fractions 8–12, erythrocyte particles in fractions 15, 16 and erythrocytes at the bottom of the gradient. Fractions 15, 16 are referred to as 'erythrocyte particles'.

centrifugation into a Ficoll gradient. Two particle populations were obtained, a heavier one containing only particles and a lighter one containing also empty vesicles (Fig. 8). After negative staining, the particles appeared as vesicles and myelin bodies (Fig. 9a). Thin-sectioning of the heavier particle fraction showed a rather homogeneous population of particles with a mean diameter of about 0.1 μm (Fig. 9b). No empty vesicles or multilamellar structures were observed in this fraction indicating that it was not contaminated with liposomes or complex structures formed by liposome-liposome fusion. Under similar conditions, liposomes or products of liposome-liposome fusion, remained near the top of the Ficoll gradients (Fig. 8a).

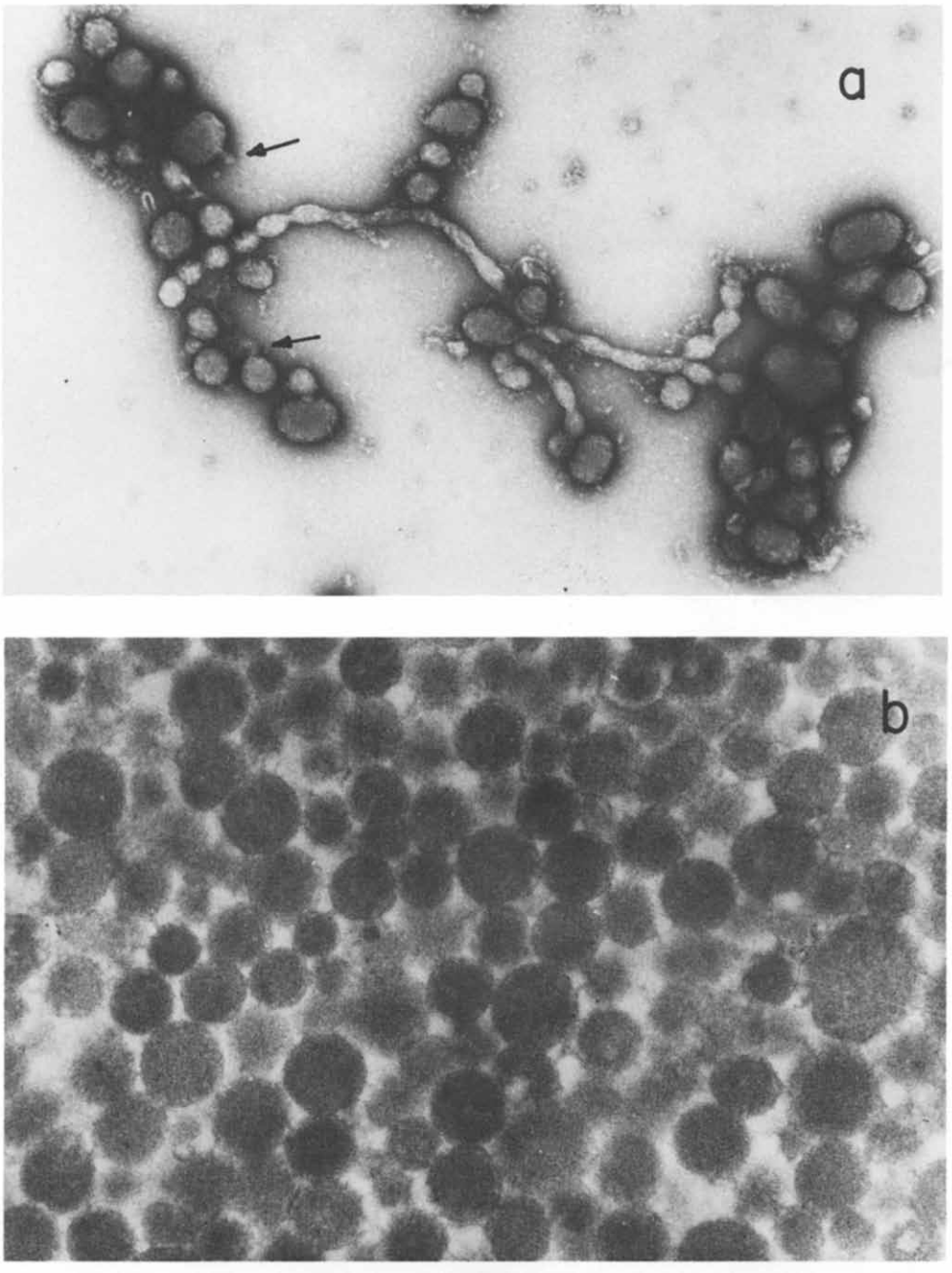


Fig. 9. Erythrocyte particles. Erythrocyte particles were isolated as described in legend to Fig. 8. A sample was processed for negative staining (part a) and another was washed by dilution of the Ficoll with buffer and centrifugation at 100000× *g* for 2 h. The pellet was processed for thin sectioning (part b). Arrows mark particle 'tails'. Magnification part a, ×40000; part b, ×58000.

After various incubation periods, particles were isolated as above and the hemoglobin content of the particle fraction was determined as an assay for amount of particles released. Most of the particles were shed during or immediately following disaggregation of the cells (Fig. 10). During the incubation period, the ATP content of the cells was not reduced appreciably (Fig. 10). Particle

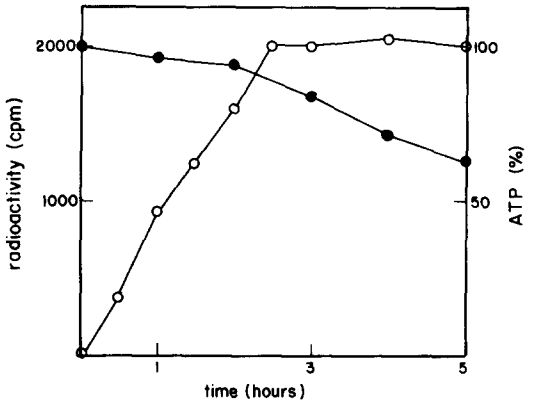


Fig. 10. Time-course of erythrocyte particle shedding. Erythrocytes were incubated with liposomes as described in legend to Fig. 8. At various times, samples were withdrawn and assayed for ATP content. Erythrocyte particles were isolated as described in the legend to Fig. 8 and their radioactivity (○——○) and ATP content (●——●) were determined. The ATP content of control cells was 0.9 μmol/ml packed cells.

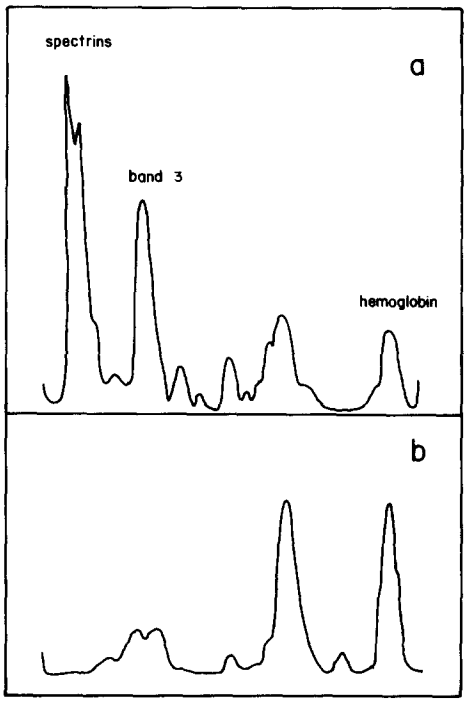


Fig. 11. Polypeptide pattern of erythrocyte particles. Erythrocytes were incubated with liposomes and released particles were isolated as described in the legend to Fig. 8. Hemoglobin-free ghosts werre prepared from non-treated cells. Particle (b) and ghost (a) samples containing 20 μg organic phosphate were denatured with SDS and analyzed by polyacrylamide gel electrophoresis.

ejection was dependent both on divalent cations and liposome composition (Table II). Calcium ions as well as magnesium ions induced clumping-disaggregation of the cells and shedding of particles. Of various liposome compositions tested, only ones containing phosphatidylethanolamine, phosphatidylcholine and cardiolipin induced both clumping-disaggregation and particle shedding. Neither acidic liposomes containing cardiolipin and phosphatidylcholine nor neutral liposomes induced particle shedding. It has been reported that dimyristoylphosphatidylcholine liposomes exchange phospholipids with erythrocytes and induce release of particles from them [19–21]. We have confirmed this result. The latter phenomenon was not dependent on divalent ions and occurred only with dimyristoylphosphatidylcholine and not with non-saturated phosphatidylcholine such as the egg one (Table II).

Particles released by cells incubated with acidic liposome lacked extrinsic proteins such as spectrins (Fig. 11) and were enriched in other proteins. Their content of band 3 varied from one experiment to the other. An example of particles with low band 3 content is presented in Fig. 11.

The particles contained lipids of both cellular and liposome origin. When the cells had been incubated with liposomes composed of radioactive phospholipids, the ratio of the latter in the particles was similar to that observed in the liposomes (Table II). The specific radioactivity of the liposome phospholipids was diluted about 5-fold in the cells and particles (Table II) indicating that these phospholipids constituted about 20% of the total phospholipids in the cells and in the particles.

Analysis of particles' lipid composition revealed a composition similar to that of the cells after incubation with liposomes. They contained lipids characteristic of cells with the addition of phospholipids donated by the liposomes (data not shown).

Discussion

The first event in calcium -induced interaction of liposomes with cells is liposome-liposome fusion concomitant with destruction of their vesicular

nature. This is more evident at high liposome to cell ratios where dense multilammelar and paracrystalline structures with no apparent trapped volume are formed. Under these experimental conditions, the potential of liposomes as delivery vehicles of vesicle contents into cells is very limited. At low lipid to cell ratio the liposomes seem to retain better their vesicular structure and might possess a better chance of eventually delivering their contents into cells.

Liposomes containing phosphatidylethanolamine and cardiolipin adsorb tightly to cells in the presence of divalent cations. The intriguing feature of this phenomenon is that although the adsorbed vesicles cannot be removed by either chelating agents or repeated washes they are released spontaneously upon incubation at room temperature. Freshly added liposomes are not adsorbed to cells previously incubated with liposomes. Thus a change must occur in the cell membrane leading to ineffectiveness of the sites involved in binding of acidic liposomes. This change is temperature-dependent but not inhibited by fixation with glutaraldehyde. The main event occurring in the clumps is incorporation of liposome phospholipids into cell membranes. It is not evident how this affects the adsorption of vesicles to the cells.

In the liposome-cell clumps, a constant amount of liposome phospholipids is incorporated into the cell membranes. After prolonged incubation, adsorbed liposomes are released, no liposomes are seen bound to the cells and particles released from the cells contain incorporated phospholipids. The incorporation occurs either by fusion of liposomes with cells or by transfer/exchange of individual phospholipids. The former possibility seems more applicable here since the ratio of the various phospholipids incorporated into the cells remains similar to that of the liposomes. There is no reason to expect equal transfer rates of the various phospholipids. Struck and Pagano have shown that the rate of transfer/exchange depends on the nature of phospholipid headgroup and is not inhibited at 2°C [22]. Proteoliposomes of similar lipid composition were shown to fuse with erythrocytes leading to incorporation of mitochondrial cytochrome oxidase and skeletal Ca^{2+}-ATPase into cell membranes [23,24]. There too the incorporation was dependent on divalent ions and the amount of phospholipids incorporated was constant at about 20% of cellular phospholipids.

Liposomes induce endocytosis in reticulocytes and not in mature erythrocytes. A similar phenomenon has been observed with concanavalin A [25,26]. It induces endocytosis in neonatal human erythrocytes and rabbit reticulocytes but not in mature erythrocytes. Spectrin network decreases membrane mobility in mature cells and limits endocytosis. An interesting feature of liposome-induced endocytosis is that in most cases the inducing liposomes are not engulfed by the cells, but remain attached to the surface. This raises the possibility that under certain conditions liposome contents might be endocytocized without the liposome membranes being internalized. Contact of cells and liposomes causes leakage of the latter contents [27]. Massive endocytosis next to a leaking liposome may well lead to endocytosis of liposome contents.

In the presence of divalent cations, liposomes containing phosphatidylethanolamine and cardiolipin induced released of erythrocyte particles. Erythrocytes were observed to shed particles upon aging in vitro, ATP depletion and introduction of calcium ions into cells [18,28–30]. ATP depletion seems to interfere with cytoskeletal function while internal calcium ions activate phospholipase *c* which produces diacylglycerol. Particle release observed in the present work seems not to be due to these causes. Interaction with liposomes did not interfere with cell ATP metabolism. The cells remained intact and calcium measured with radioactive isotope did not penetrate into them (data not shown). Moreover, magnesium ions were efficient too in inducing particle release. Recently, it has been observed that dimyristoylphosphatidylcholine liposomes induced released of erythrocyte particles [19–21]. Ott et al. [19] have suggested that for particle shedding to occur, phospholipid molecules have to be inserted into both leaflets of cell membranes. They presumed that phospholipids were transferred individually into the outer leaflet of the membranes and from there, they moved by flip-flop mechanism into the inner membrane leaflet. Introduction of agents into the outer monolayer has been shown to cause crenation of cells [31,32] and insertion of phospholipids was supposed to allow membrane fusion necessary for

release of particles. We have confirmed that dimyristoylphosphatidylcholine induced release of particles and have shown that it is limited to saturated phosphatidylcholines. the calcium and acidic liposome-induced release of particles could have been due to introduction of lipids into both membrane leaflets by fusion of the liposomes with the cells. The particles observed in this work had a morphology similar to that described before, including 'tails' similar to those isolated from particles produced by ATP-depletion or introduction of calcium into cells. The particles lack spectrin, as do other particle preparations, but they have reduced content of band 3 in contrast to other particle preparations which are enriched in it [18,19].

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