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CYTOCHEMICAL STUDY OF LIPOSOME AND LIPID VESICLE PHAGOCYTOSIS *

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Electron microscopy cytochemistry has been used to study the cytoplasmic location of liposomes and lipid vesicles following specific antibody-dependent phagocytosis. The vesicle compositions were 94–99 mol% 'fluid' lipid (egg phosphatidylcholine or dimyristoylphosphatidylcholine at 37°C or 'solid' lipid (dipalmitoylphosphatidylcholine at 37°C). In some cases, 4 mol% phosphatidylserine was included in the vesicle membrane so as to vary the surface charge density. These vesicles undergo specific antibody-dependent phagocytosis by RAW264 macrophages when the lipid membranes contain 1–2 mol% dinitrophenyl lipid hapten in the presence of rabbit anti-dinitrophenyl IgG antibody. Internalized lipid vesicles can be visualized with the electron microscope when ferritin is trapped in the internal aqueous compartments prior to internalization. The lipid vesicles were demonstrated to be internal to the macrophage plasma membranes by selectively staining the plasma membranes with Ruthenium red. The cytoplasmic location of vesicles and liposomes was studied by electron microscopic staining for activities of the following enzymes: (1) acid phosphatase; (2) inorganic trimetaphosphatase; (3) adenosine triphosphatase; and (4) glucose-6-phosphatase. The first two enzymatic activities were found in association with ferritin-containing vesicles after antibody-dependent phagocytosis, showing the formation of vesicle-containing phagolysosomes. Adenosine triphosphatase and glucose-6-phosphatase were primary not associated with the vesicles, suggesting a minimal association of vesicles with plasma membrane, Golgi, endoplasmic reticulum and perinuclear cisternae. Phagosome-lysosome fusion did not appear to depend on the type of target lipid vesicle or liposome, on the 'fluidity' of the target membrane, or the presence of phosphatidylserine in the target membrane.

Introduction

Antigens or lipid haptens incorporated into the bilayer membranes of vesicles or liposomes have been used as targets and/or stimulators of afferent and/or efferent immune responses, in vivo and in vitro [1–4]. In addition, vesicles and liposomes have been used to deliver molecules such as macrophages activation factor and interferon to phagocytic cells [5,6]. In recent work, we [7–11] and others [12–15] have studied the antibody-dependent and -independent phagocytosis of phospholipid vesicles and liposomes. Our work has focused upon kinetics of macrophage-vesicle binding [8], stimulation of the respiratory burst [9], cell-surface receptors [7], proteins [11] and surface morphology [10].

The present work was undertaken to define

* This paper is dedicated to Norman Davidson on the occasion of his 65th birthday.

Abbreviations: ATPase, adenosine triphosphatase; DMPC, dimyristoylphosphatidylcholine; DPPC, diplamitoylphosphatidylcholine; DNP-cap-PE, dinitrophenyl-ε-aminocaproylphosphatidylethanolamine; PS, phosphatidylserine; egg PC, egg phosphatidylcholine.

further the cytoplasmic location of internalized vesicles or liposomes following phagocytosis. We were particularly interested in determining the state of association of vesicles with phagolysosomes, since this should have a bearing on the fate of substances encapsulated within these lipid membranes. It was also of interest to know whether or not target-membrane 'fluidity' played any role in cytoplasmic localization, since fluidity does appear to play a significant role in the kinetics of specific antibody-dependent vesicle-macrophage binding, and triggering of the respiratory burst [8,9]. In the present work, a 'fluid' lipid membrane is defined as one in which the lateral diffusion coefficients of lipids and spectroscopic lipid probes are at least 10^{-8}–10^{-7} cm^2/s, and a 'solid' lipid membrane is defined as one in which these lateral diffusion coefficients are at least 10^{-10} cm^2/s. Previous studies have shown that antibodies specifically bound to lipid haptens have the same lateral diffusion coefficients as the lipids, and lipid haptens, in the absence of antibodies [16].

Materials and Methods

Materials. Trimetaphosphate, β-glycerophosphate, glucose 6-phosphate and horse-spleen ferritin were obtained from Sigma Chemical Co. (St. Louis, MO). Glutaraldehyde, cacodylic acid, ruthenium red and OsO_4, were obtained from Polysciences, Inc. (Warrington, PA). Cell buffer was composed of Hanks' balanced salts solution (GIBCO, Santa Clara, CA) and 0.1% ovalbumin (Sigma).

RAW264 macrophages. The murine RAW264 macrophage cell line [17] was the generous gift of Dr. Peter Ralph, Sloan-Kettering Institute for Cancer Research, Rye, NY. The RAW264 macrophages were grown as adherent cells, as previously described [8]. The cells were grown in RPMI 1640 medium with 10 mM Hepes buffer (GIBCO), 10% fetal calf serum and 40 μg/ml gentamycin sulfate. The adherent RAW264 macrophages were removed by incubation in Earle's balanced salts solution without Ca^{2+} or Mg^{2+} (GIBCO) containing 2 mM EDTA for 30 min at room temperature.

Phospholipid vesicles and liposomes. Ferritin-containing, unilamellar vesicles of approx. 1 μm were prepared by a modification of the ether injection method of Deamer and Bangham, or a freon injection technique [18]. Vesicle lipid composition was 94–99 mol% dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), or egg phosphatidylcholine (prepared as described [18]) and 1–2 mol% dinitrophenyl-ε-aminocaproyl-phosphatidylethanolamine (DNP-cap-PE). In selected cases, 4 mol% phosphatidylserine (PS) was included. Ferritin was trapped within the vesicles by inclusion of 20–50 mg/ml ferritin in the phophate-buffered saline (0.01 M sodium phosphate/0.15 M NaCl (pH 7.4)) injection buffer. To remove unentrapped ferritin, the vesicles were washed four times by centrifugation ($12000 \times g$ for 30 min). Electron micrographs of vesicles prepared in this fashion have been previously presented [18].

Liposomes (multilamellar, approx. 0.5–5 μm diameter) were prepared as previously described [16]. Briefly, the lipids of compositions described above were dried under vacuum. Following hydration in the presence of ferritin, the container was vigorously vortexed for 1 min. This preparation was washed four times by centrifugation.

Antihapten antibody. Rabbit anti-DNP antiserum was obtained from Miles Laboratories (Elkhart, IN). The anti-DNP IgG was fractionated from serum by affinity chromatography using protein A coupled to cyanogen bromide-activated Serpharose. In all experiments the anti-DNP was centrifuged at $110000 \times g$ for 15 min in a Beckman airfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) to remove aggregates. The antibody dilution employed in these experiments was 1 : 5. This was chosen because: (i) vesicles were not agglutinated; and (ii) substantial internalization of vesicles and loss of Fc receptor activity took place [7,8].

Phagocytosis of vesicle and liposomes. To 1.2 × 5.0 cm polypropylene vials (E&K Scientific Products, Inc., Saratoga, CA), 100 μl vesicles (0.05 μmol total lipid, suspended in phosphate-buffered saline), and antibody diluted into 100 l of phosphate-buffered saline were added and thoroughly mixed. $1 \cdot 10^6$ macrophages were added in 300 μl of cell buffer. The mixtures were incubated at 37°C in gyratory water-bath shaker (model G76,

New Brunswick Scientific Co., Edison, NJ). After 1 h, the vials were transferred to an ice bucket.

Fixation. Cell buffer was removed by centrifugation twice and washing with fixation buffer. With the exception of ATPase samples, the cells were fixed in suspension with 1% cold glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4)/2% sucrose. The trimetaphosphatase, acid phosphatase, glucose-6-phosphatase and Ruthenium red samples were fixed for 1 h. The cells were then washed four times with cacodylate buffer plus 5% sucrose, this being following by the cytochemical methods described below. The ATPase samples were fixed with 1% cold glutaraldehyde in 0.1 M Tris-maleate (pH 7.0)/2% sucrose for 1 h. The cells were then thoroughly washed in Tris-maleate buffer containing 5% sucrose.

Cytochemical procedures. The cell surface was stained with Ruthenium red as previously described [19]. Briefly, the fixed cells were incubated in cacodylate buffer plus 0.1% Ruthenium red for 30–60 min at room temperature. The cells were pelleted, then resuspended in cacodylate buffer containing 0.1% Ruthenium red and 1% osmium tetroxide for 1 h at room temperature.

Acid phosphatase was stained by the modified Gormori's medium consisting of 13.9 mM sodium β-glycerophosphate, 1 mM $Pb(NO_3)_2$, 0.05 M acetate buffer (pH 5.4), 0.08% $CaCl_2$ and 5% sucrose. Incubation was carried out at 37°C with agitation for 30–60 min. The trimetaphosphatase experiments were conducted with Berg's trimetaphosphate medium as described by Doty et al. [20]. Briefly, 9 mg of trimetaphosphate were dissolved in 2.25 ml 0.1 M acetic acid. 25 ml of H_2O and 5 ml of 1.5% Pb acetate were added to this mixture. The pH was adjusted to 3.9 and the final volume was 50 ml. The incubation was carried out at 37°C for 30–60 min with agitation.

Macrophages were stained for ATPase with Wachstein-Meisel's medium as previously described [21,22]. The cells were incubated in 0.1 M Tris-maleate buffer (pH 7.0)/1 mM ATP/10 mM $MgCl_2$/3.6 mM $Pb(NO_3)_2$/2% sucrose for 1 h at 37°C with agitation. The cytochemical procedure for the localization of glucose-6-phosphatase was performed as previously described [23]. The incubation was carried out in Tris-maleate buffer (pH 7.0)/17 mM glucose 6-phosphate/0.2% $Pb(NO_3)_2$/2% sucrose for 1 h at 37°C with agitation. These samples were then washed four times by centrifugation with 0.1 M Tris-maleate buffer contaning 5% sucrose.

All reaction mixtures were routinely filtered before use to insure clarity. Following incubation for trimetaphosphatase, the cells were washed four times with buffer followed by treatment with 1% $(NH_4)_2S$ for 1 min. This procedure was optional for the acid phosphatase, ATPase and glucose-6-phosphatase stains. This treatment was immediately followed by four additional washes. Cytochemical controls were performed as described above (including $(NH_4)_2S$ treatment) except for the omission of either substrate or lead.

Transmission electron microscopy. Cells were post-fixed with 1% OsO_4 in 0.1 M cacodylate buffer for 1 h at room temperature. The samples were dehydrated in ethanol and embedded in Spurr's resin [24]. The blocks were thin-sectioned and examined in a Hitachi HU-11E electron microscope. The thin-sections were not counterstained to enhance the contrast between the cytoplasm and ferritin; the contrast of the cell cytoplasm is, therefore, lower.

Results

Fig. 1 shows a representative electron micrograph of a Ruthenium red-stained RAW264 macrophage following antibody-dependent uptake of ferritin-containing lipid vesicles. The ruthenium red reacts only with cell-surface carbohydrates [19,25]. As can be seen in Fig. 1, this stain is only on the outer surface of the plasma membrane. The stain is not associated in any way with the ferritin-containing vesicles. Thus, the vesicles are not in communication with the extracellular medium in any plane-of -section. All recognizable ferritin-containing vesicles are internal to the plasma membrane – they were phagocytosed. Apparently, few vesicles or liposomes were surface-bound subsequent to the incubation period. However, this could be attributable to the washing prior to fixation or the dehydration in ethanol (solubilization of some lipids) preceding plastic embedding. In either or both cases, ferritin could be removed from the cell periphery. Therefore,

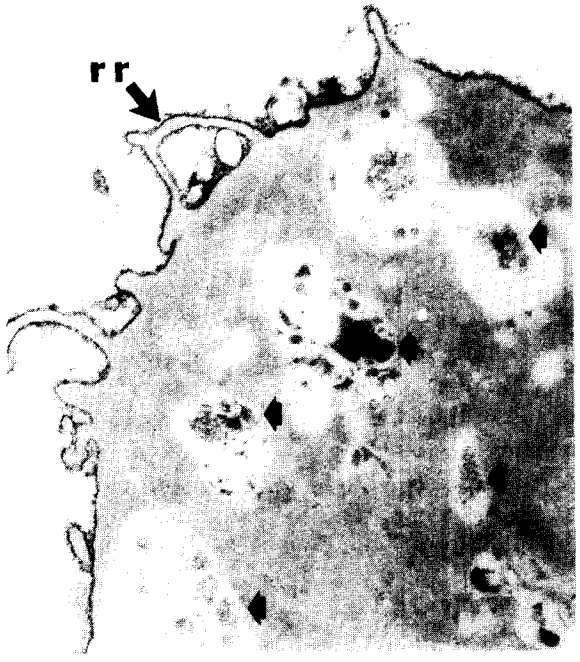


Fig. 1. Thin-section transmission electron micrograph of a RAW264 macrophage following the antibody-dependent phagocytosis of lipid-hapten and ferritin-containing liposomes. After glutaraldeyde fixation, the cells were stained with Ruthenium red (indicated with rr). Numerous ferritin liposomes can be seen throughout the cytoplasm (indicated with small arrows). However, the Ruthenium red, which is specific for the plasma membrane, does not stain the ferritin liposomes. This indicates that the vesicles are not in communication with the cell surface, i.e., they have been phagocytosed.

electron microscopy is not unequivocal regarding vesicles which are simply bound to the surface. In a few cases (less than 5%; data not shown) ferritin could be observed on the cell surface; this probably represents an underestimation of vesicle binding. As an additional consequence, kinetic studies such as those previously given [7–10] primarily show fewer internal ferritin vesicles or liposomes, providing few data on binding per se.

We have employed electron microscopic cytochemistry to study the intracellular fate of phagocytosed model membranes. Both fluid (DMPC and egg PC at 37°C) and solid (DPPC at 37°C) lipid targets containing 1–2 mol% lipid hapten underwent specific antibody-dependent phagocytosis. The cells were then stained for the enzymes acid phosphatase, trimetaphosphatase, adenosine triphosphatase and glucose-6-phosphatase (see Table I).

A series of experiments utilizing ferritin-containing vesicles is shown in Fig. 2, panels A and B. A representative micrograph of a DMPC vesicle is given in panel A. In panel B is shown an experiment using egg PC vesicles following reaction for the same cellular compartment as the ferritin. Similar results were obtained with DPPC vesicles which had been stained for the enzyme acid phosphatase or trimetaphosphatase. These results indicate phagolysosome formation.

In Fig. 2, panels C and D, we show phagocytosed ferritin-containing liposomes. Pancel C depicts a typical ferritin-containing liposome within a RAW264 macrophage. The ferritin-containing vesicular structures can be seen throughout the cytoplasm. The partially aggregated ferritin may be due to the fixation with glutaraldehyde, as previously suggested. Panel B shows the trimetaphosphatase reaction pattern of egg PC liposomes which contain 4 mol% PS and ferritin. In this case, the reaction product of trimetaphosphatase can be found in the vicinity of ferritin within vesicular structures. Similar results were found with DPPC liposomes. This demonstrates that lysosomal enzymes may be found associated with the phagocytosed target membranes.

A similar series of experients utilizing ferritin-containing vesicles is shown in Fig. 2, panels C and D. A representative micrograph of a ferritin-containing vesicle (DMPC) is given in panel C. Panel D shows a DPPC ferritin-containing vesicle (no PS) which has been stained for the enzyme trimetaphosphatase. It is clear that reaction product is in the same cellular compartment as the ferritin.In panel D is shown a similar experiment using egg PC ferritin-containing vesicles following reaction for the enzyme acid phosphatase. Similar distributions of reaction product are found, indicating phagolysosome formation.

In Fig. 3 we show a RAW264 macrophage which has been stained for the enzyme ATPase after phagocytosis of ferritin-containing liposomes (egg PC). This enzyme is associated with the Golgi apparatus (Fig. 3, insert A) and the plama membrane. Most liposomes were not associated with this enzyme. However, some phagosomes (approx. 15%) contained both the ferritin label and enzyme

TABLE I

Experiments were performed in duplicate 2–7 times. GERL, Golgi endoplasmic reticulum lysosomes; RER, rough endoplasmic reticulum.

	Cell surface	Lysosomes	Golgi/ GERL	RER	Perinuclear cisternae	'Fluid' target [a,c]	'Solid' target [b,c]
Ruthenium red	+	−	−	−	−	−	−
Trimetaphosphatase	−	+	−	−	−	+	+
Acid phosphatase	−	+	+	−	−	+	+
Glucose-6-phosphatase	−	weak	−	+	+	weak	weak
ATPase	+	−	+	−	−	weak	weak

[a] Liposomes or vesicles primarily composed of egg PC or DMPC with ferritin trapped in the internal aqueous volume.

[b] Liposomes or vesicles primarily composed of DPPC with ferritin as a marker for the internal volume of the targets.

[c] In some experiments, a negatively charged lipid, phosphatidylserine, was included (4 mol%) in addition to 1–2 mol% of DNP-cap-PE. No difference could be observed in any of these cases.

reaction product. An example of this is shown in insert B of Fig. 3. This may originate from plasma membrane ATPase.

We have also examined the localization of the enzyme glucose-6-phosphatase in macrophages following antibody-dependent phagocytosis. In Fig. 4 we show a representative cell stained for this enzyme. Reaction product is primarily associated with the rough endoplasmic reticulum and perinuclear cisternae; a fraction of lysosomal bodies were stained by this procedure. In many cases, staining was not associated with ferritin. However, in some cases we have observed a positive reaction. An example is shown in the insert of Fig. 4. The infrequent positive reaction is probably a manifestation of the infrequent positive reaction of lysosomes. The absence of substantial deposition of reaction product in association with ferritin suggests hat components of the rough endoplasmic reticulum and perinuclear cisternae are not made available to the internalized model membranes.

Discussion

We have examined the association of four RAW264 macrophage cytoplasmic enzymes with ferritin- and lipid hapten-containing liposomes and lipid vesicles after specific antibody-dependent phagocytosis. Previous studies from several research groups have shown that the uptake of antibody- or carbohydrate-coated liposomes or lipid vesicles primarily occurs via phagocytosis [8, 14, 15, 29–31], although unmodified vesicles may be taken up via fusion with the plasma membrane [32]. In the present work, we are primarily interested in the intracellular disposition of these model membranes. Electron microscopic cytochemistry has been used to locate enzymatic activities within macrophages. Previous biochemical and cytochemical studies have identified enzymatic activities of macrophage lysosomes [26]. The present study has shown that the lysosomal enzymes acid phosphatase and trimetaphosphatase may be found in the same cellular compartment as ferritin, the internal volume marker of the target membranes (Table I). This indicates that lysosomal enzymes became available to the phagocytosed liposomes and lipid vesicles, presumably by fusion of lysosomes and phagosomes. The Ruthenium red/OsO_4-staining reagent demonstrates unequivocally that the lipid model membranes were phagocytosed. Earlier studies [19] have shown that the Ruthenium red technique is definitive in this respect.

Previous studies have shown that the kinetics of antibody-dependent binding of lipid hapten-containing vesicles to macrophages is stongly dependent on target membrane fluidity [8]. The enhanced rate of binding of fluid vesicles has been attributed to the rapid lateral diffusion of surface-bound antibodies on the target vesicles, which facilitates binding to macrophage surface Fc receptors. On the other hand, per vesicle bound, the solid targets are significantly more effective in triggering the macrophage respiratory burst [9].

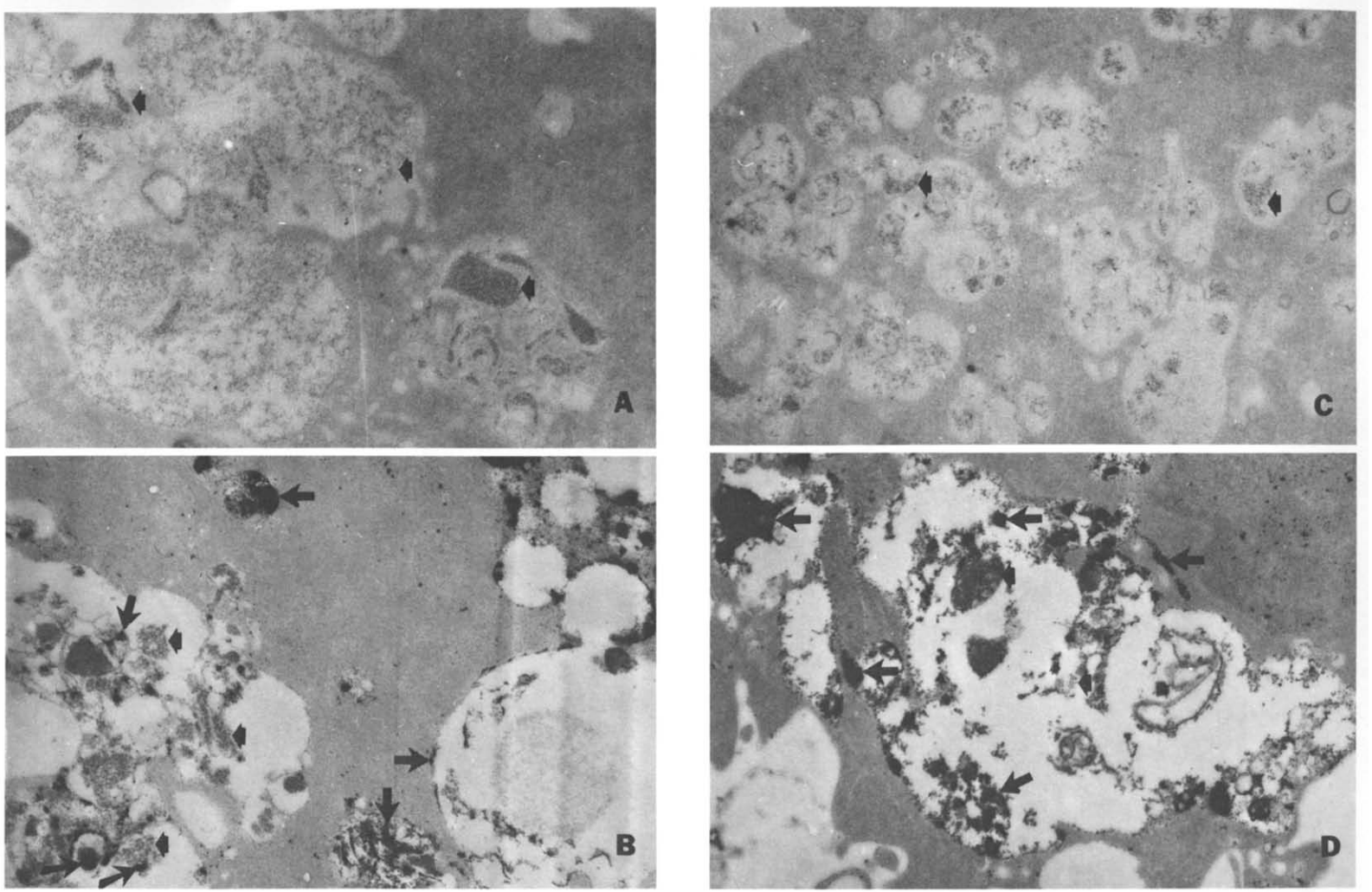
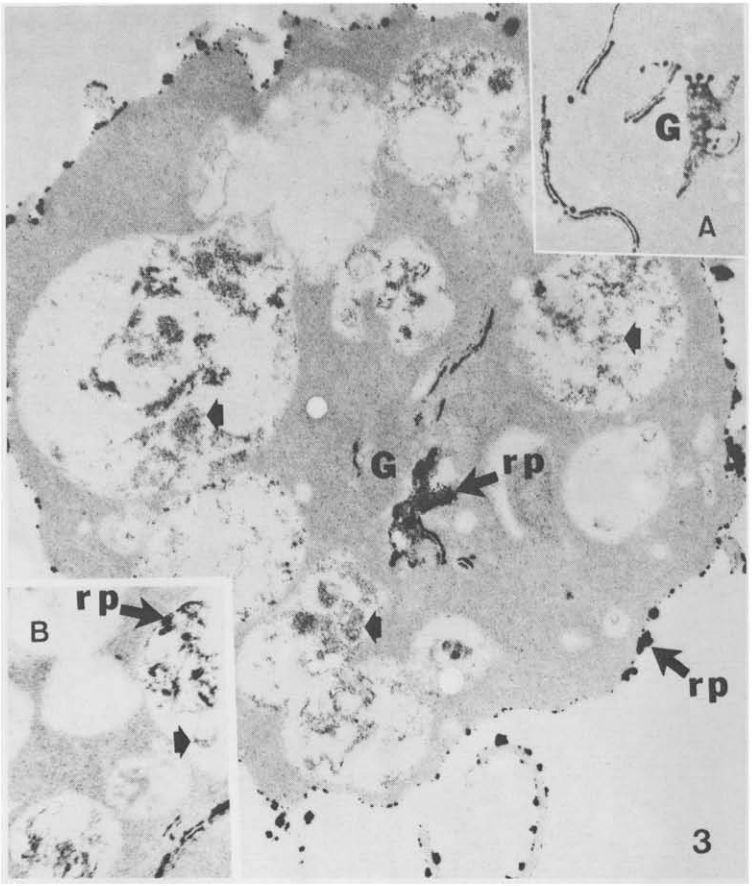


Fig. 2. Several experiments utilizing ferritin-containing model membranes are shown. In all cases thin-sections of RAW264 macrophages are given. The type of target is listed, followed by its particular lipid composition. (A) Phagocytosed ferritin liposomes (DMPC). (B) Phagocytosed ferritin liposomes (egg PC + PS) stained for the lysosomal enzyme trimetaphosphatase. The reaction product (long arrows) can be seen near ferritin (short arrows), the internal volume marker of the liposomes. (C) RAW264 macrophages after phagocytosis of ferritin vesicles (DMPC). (D) A similar experiment using ferritin vesicles (DPPC), including reaction for the lysosomal enzyme acid phosphatase. As in the previous example, reaction product is in close proximity to the ferritin marker. Only representative data are shown because of space limitations. Lamellar structures are difficult to observe because of extraction during dehydration; this is particularly important for synthetic lipids.



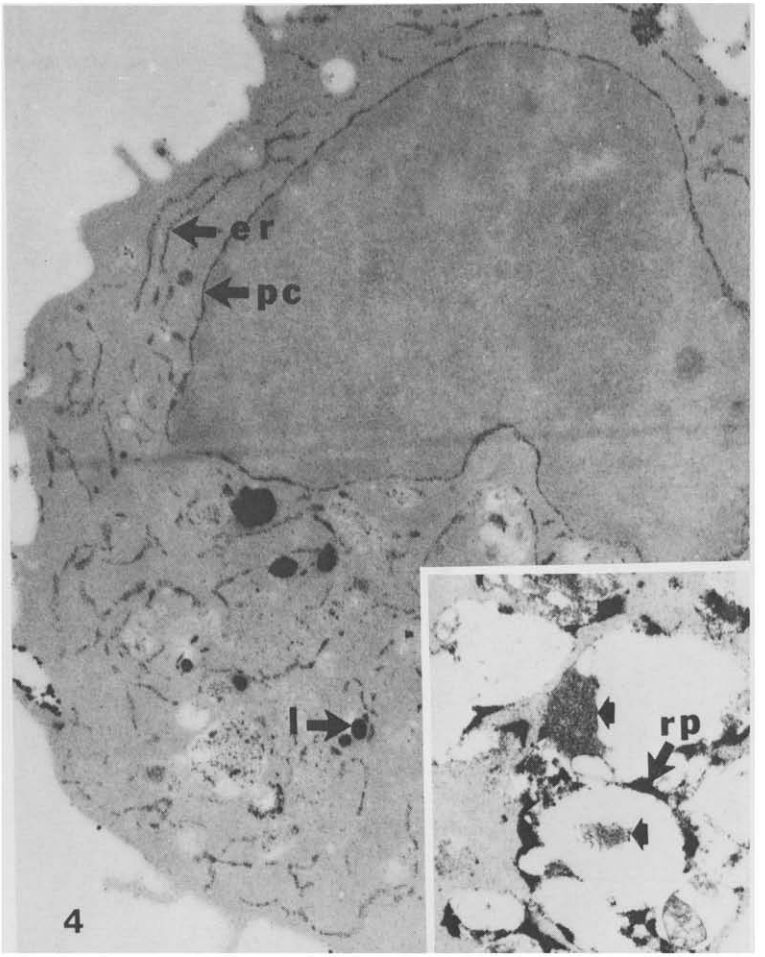


Fig. 3. RAW264 macrophage after antibody-dependent phagocytosis of ferritin liposomes (egg PC) reactd for the enzyme ATPase. Ferritin liposomes can be seen in the cytoplasm. Reaction product (indicated by rp) is associated with the plasma membrane and Golgi apparatus (indicated by G). Another example of the Golgi is shown in insert A. Budding vesicles can be easily seen. In most cases, it is difficult to associate reaction product and ferritin, although some reaction product can be found. Reaction product can be easily seen in insert B.

Fig. 4. An RAW264 macrophage stained for the enzyme glucose-6-phosphatase. Reaction product is seen in the rough endoplasmic reticulum (indicated by er) and perinuclear cisternae (pc). Some lysosomes (l) are also labeled by this procedure. The insert shows phagocytosed ferritin liposomes (egg PC) which are associated with this enzymatic activity (reaction product = rp).

The present studies indicate that target membrane fluidity does not influence phagolysosome formation. In addition, the inclusion of a negatively charged phospholipid, phosphatidylserine, at 4 mol% (in addition to the 1–2 mol% charged hapten headgroup) to increase the surface charge density of the targets did not influence the formation of phagolysosomes. Our studies indicate that target membrane fluidity and surface charge (up to 4 mol%) do not influence the formation of phagolysosomes. The signal which controls the delivery of lysosome contents to phagosomes is apparently not strongly dependent upon the lateral diffusion of antibody-Fc receptor complexes in the phagosomal membrane. It should be understood that we are concerned with a three-membrane problem: the lysosomal membrane, the phagosomal membrane and the target membrane bound within the phagosome. These studies are important with regard to: (i) target membrane factors which influence phagolysosome formation and (ii) the delivery of specific molecules or drugs to cells, including macrophages.

The enzyme ATPase has been associated with the plasma membranes of many cell types. Ultrastructural cytochemistry has demonstrated that this enzyme is present in the Golgi complex and plasma membrane of macrophages [22]. This study has confirmed these findings. In addition, we have shown that ATPase can be found in approx. 15% of the phagolysosomes. A previous study did not find ATPase associated with phagosomes following the phagocytosis of sheep erythrocytes. However, the antibody-dependent phagocytosis of the smaller lipid vesicles and liposomes results in a greater loss and turnover of plasma membrane area. This greater stimulus might account for the minor difference between these studies. Since it is known that plasma membrane encompasses the target, it is somewhat surprising that only approx. 15% of the phagosomes contained a small amount of the reaction product. This cannot be accounted for by exclusion of cytochemical reactants from the phagosomes since the heavily-labeled Golgi complex was, in nearly all cases, farther from the plasmalemma (in the planes-of-section examined). Since we have shown that these stuctures contained lysosomal enzymes, a possible explanation is that the plasma-membrane ATPase was destroyed by lysosomal enzymes. Two additional hyptheses must be entertained. In light of recent work, these enzymes could be recycled to the plasma membrane before lysosomal destruction [27]. Alternatively, these components could be excluded from the phagocytosed membrane at the level of the cell surface [28]. Since transport sites have been shown to possess ATPase activity, these observations would fit with Berlin's suggestion regarding the exclusion of transport sites from phagocytosed plasma membrane.

We have also examined the enzyme glucose-6-phosphatase. Our studies confirm a recent report regarding its ultrastructural localization in the rough endoplasmic reticulum and perinuclear cisternae. However, we have also found this enzyme in association with several primary and secondary lysosomal structures. This may be due to the fact that we are employing a transformed macrophage cell line. The small amount of staining associated with the ferritin-containing model membrane is probably a reflection of the limiting staining of lysosomal bodies.

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