Biochimica et Biophysica Acta 812 (1985) 42–48
Elsevier

BBA 72354

Interactions of polymerized phospholipid vesicles with cells. Uptake, processing and toxicity in macrophages

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(Received May 24th, 1984)

Key words: Liposome-cell interaction; Multilamellar vesicle; Phospholipid polymerization; Photopolymerization; (Mouse macrophage)

We have studied the uptake of photopolymerized multilamellar vesicles composed of bis(1,2(methacryloyloxy)dodecanoyl)-L-α-phosphatidylcholine (DPL) by mouse peritoneal macrophages in vitro. Vesicles composed of polymerized DPL are taken up more rapidly and extensively than vesicles composed of conventional phosphatidylcholine. The uptake of radioactive DPL vesicles was not blocked by incubation with unlabelled phosphatidylcholine vesicles in either the fluid or gel state. Likewise, fluid-phase negatively charged vesicles failed to block uptake of DPL vesicles, whereas solid-phase negatively charged vesicles did have a blocking effect. A radioactive lipophilic marker (dipalmitoylphosphatidyl[*N-methyl*-^{3}H]choline) incorporated into DPL vesicles was metabolized at essentially the same rate whether the vesicles were polymerized or not. Nonpolymerized DPL vesicles were quite toxic to macrophages, whereas polymerized DPL vesicles or vesicles composed of conventional phosphatidylcholines were not toxic.

Introduction

Recently, a number of investigators have synthesized phospholipid analogs which contain diacetylenic, methacryloyl or sulfhydryl groups in the fatty acyl chains. These derivatives can, by chemical- or photon-catalyzed processes, be induced to undergo polymerization reactions while in the lipid bilayer configuration [1–5]. Phospholipid vesicles (liposomes) containing such polymeric derivatives share many properties with conventional liposomes, but in addition have some rather unusual characteristics due to their polymer-like structure. For example, we have recently reported that liposomes composed of photopolymerized bis(12-(methacryloyloxy)dodecanoyl)-L-α-phosphatidylcholine (termed dipolymerizable lipid or DPL) were remarkably resistant to the destabilizing effects of organic solvents or of hydrodynamic shear forces (ultrasonication); in addition, when lipophilic solutes were incorporated into DPL vesicles, the solutes were resistant to extraction with detergents [6].

The unusual characteristics of polymeric lipid membranes suggest their eventual application in a number of areas, including photochemical energy conversions [7], heterogeneous catalysis [8], modification of the surface properties of biomaterials [9,10] and development of controlled drug-delivery systems [11]. Utilization of polymerized vesicles as

Abbreviations: DPL, bis(1,2(methacryloyloxy)dodecanoyl)-L-α-phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol.

drug carriers in vivo will be predicated upon a detailed understanding of the biological fate and actions of these unusual lipid entities. As a preliminary step in that direction, we have investigated the interactions of multilamellar lipid vesicles composed of DPL, with mouse peritoneal macrophages in tissue culture.

Methods

Preparation of liposomes. The photopolymerizable phosphatidylcholine analogue DPL was synthesized as described by Regen et al. [4]. Multilamellar liposomes were formed and polymerized as described previously [6], using intense ultraviolet illumination in a Rayonet Photochemical Reactor. Complete polymerization required 30–45 min of exposure; the degree of polymerization was evaluated by thin-layer chromatography as described [6]. In some cases, [^{3}H]DPPC (dipalmitoylphosphatidyl[*N-methyl*-^{3}H]choline) (Amersham, 35 Ci/mmol) was included in the preparation as a marker for the lipid. We have previously shown that the [^{3}H]DPPC does not itself become covalently attached to the polymeric lipid [6]. Conventional liposomes formed from a variety of nonpolymerizable lipids were prepared by standard means, largely as described by Kao and Juliano [12].

Macrophage uptake studies. Cultures of thioglycollate-stimulated mouse peritoneal macrophages were prepared as described by Hsu and Juliano [13], except that in these studies the macrophage cultures were set up in 24-well Costar tissue culture trays with each well containing $1.5 \cdot 10^6$ cells. All liposome uptake and processing experiments were conducted in alpha minimal essential medium + 1 mg/cc bovine albumin (medium A); most experiments were done at 37°C in a 5% CO_2 incubator, while some control experiments were done at ice temperature. The macrophage cultures were incubated with [^{3}H]DPPC-labelled DPL vesicles at various doses and for various times as described in the figure legends; in some cases other types of vesicles or various metabolic inhibitors were included in the medium.

Macrophage processing studies. The ability of macrophages to metabolically process lipophilic markers ([^{3}H]DPPC) deriving from internalized polymerized or nonpolymerized vesicles was evaluated as follows. After a period of incubation with radiolabelled vesicles in medium A, the cell monolayer was rinsed several times in medium and the culture wells were filled with fresh medium A. The cultures were then returned to the 37°C incubator and sets of wells were harvested (both medium and cells) at various time intervals. The medium and the cells were each subject to a Folch extraction [14] to separate lipid from non-lipid constituents, and both the aqueous and organic phases were analysed for total radioactivity and fractionated by thin-layer chromatography on Analtech silica plates using chloroform/methanol/water (65 : 25 : 4, v/v) as a solvent system.

Macrophage toxicity studies. The short-term toxicity of polymerized and non-polymerized vesicles to macrophages was evaluated by measuring effects of preincubation with the vesicles on the ability of the cells to incorporate [^{3}H]leucine or [^{3}H]uridine into macromolecules; incorporation of leucine and uridine are presumptive measures of protein synthesis and RNA synthesis, respectively [15]. Cells previously incubated with various vesicles for 90 min at 37°C were then incubated in medium A at 37°C which contained [^{3}H]leucine (1 μCi) or [^{3}H]uridine (1 μCi) for 90 min. The cell layer was throughly rinsed and then removed by scraping, extracted with ice-cold 10% trichloroacetic acid and the insoluble material was collected by centrifugation, washed, dissolved in 1% SDS and counted in a beta counter. Results were expressed initially as cpm incorporated per μg cell protein (by the assay of Lowry et al. [16]) and then as percentage of control (cells not exposed to vesicles).

Results

Uptake of polymerized and nonpolymerized vesicles by macrophages

The kinetics of uptake of polymerized and nonpolymerized multilamellar vesicles composed of DPL are shown in Fig. 1a. At 37°C, polymerized vesicles were taken up about 3-times as rapidly as the nonpolymerized version (67 ng/μg per h vs. 21 ng/μg per h). At this temperature, vesicle uptake represents a combination of vesicle adsorption to

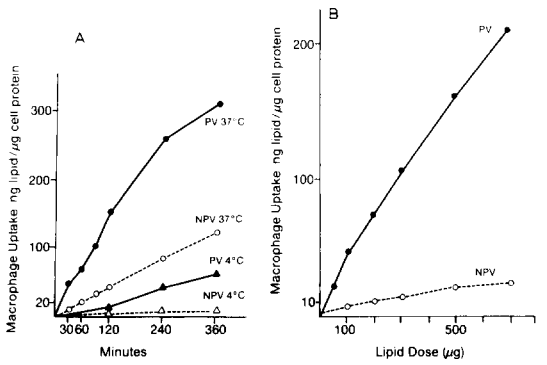

Fig. 1. Uptake of DPL vesicles by macrophages. (A) Kinetics. Macrophage monolayers were incubated at 37 and 4°C with 200 μg polymerized (PV) or nonpolymerized (NPV) DPL vesicles labelled with [^{3}H]DPPC. Uptake of vesicles by the cells was measured as described in Methods. (B) Uptake versus dose. Macrophage monolayers were incubated at 37°C for 90 min with various doses of PV or NPV vesicles labelled with [^{3}H]DPPC. Vesicle uptake was measured as described in Methods. Points shown in A, B are the means of triplicate determinations differing by less than 10%.

the cell surface and active cellular endocytosis [6]. At 4°C, the polymeric vesicles were also taken up more rapidly than the nonpolymerized ones; at this temperature, active uptake processes are inhibited [17] and the cell-associated liposomes are primarily bound to the cell surface. Thus, the polymeric vesicles do tend to adsorb more rapidly to cells even when active cellular processes are blocked.

In Fig. 1b, the uptake by macrophages versus dose of liposomes is compared in experiments at 37°C. Thus, polymerized vesicles are taken up to a substantially greater degree than nonpolymerized vesicles at every dose; no evidence of saturation of uptake was seen with either vesicle type at the doses used in this study. The amount of lipid taken up in the case of the polymerized vesicles seems to be considerably greater than the uptake of uncharged DPPC/cholesterol vesicles by macrophages described previously [13]. Thus, while macrophages took up, on the average, 5 ng lipid/μg cell protein per h for DPPC/cholesterol vesicles, they took up approx. 50 ng/lipid/μg cell protein per h of the polymerized vesicles at comparable (200 μg) input doses of lipid. Higher levels of uptake with conventional liposomes were previously observed when the vesicles were negatively charged [13] or if they were coated with IgG and thus capable of interacting with macrophage Fc receptors [13]. Thus, the cell-uptake behavior of polymerized DPL vesicles seems quite different from the behavior of vesicles composed of conventional (i.e., nonpolymerizable) phosphatidylcholines.

During the course of these experiments, it was noted that the macrophages treated with nonpolymerized DPL vesicles tended to round up and detach from the substratum; some of the cells also became stainable with Trypan blue dye. Thus, treatment with the nonpolymerized form of DPL seemed to result in some degree of toxicity to the cells.

Metabolic processing of a lipophilic marker

In order to study the intracellular metabolism of polymerized or nonpolymerized DPL vesicles, one should ideally have a radiolabelled derivative [18]. At this time, however, no such derivative is available and thus we have chosen a less direct way of making a preliminary evaluation of whether polymerized and nonpolymerized vesicles are metabolized differently by macrophages. Radiolabelled DPPC is an excellent marker for the lipid phase of liposomes and has been used to trace their uptake and cellular processing [19]. In particular, we have shown that radiolabelled DPPC is tightly but noncovalently associated with the membranes of polymerized DPL vesicles [6]; we have also studied the metabolism of this lipid by monocytes [20]. Thus, the cellular processing of [^{3}H]DPPC, including catabolic breakdown and release into the medium, may give an indication of the breakdown of the vesicle itself. We admit that this analysis does not directly address the fate of the polymerizable lipids and, in particular, the fate of the polymethacrylate chain formed during polymerization; at minimum, however, it should give a good indication of the likely metabolic fate of lipophilic compounds trapped in polymeric liposomes.

As seen in Table Ia, macrophages retain substantial amounts of radioactivity from [^{3}H]DPPC in polymerized or nonpolymerized vesicles for more than 24 h. The TLC data (Table Ib) indicate that most of the cellular material comigrates with

TABLE Ia

MACROPHAGE PROCESSING OF [^{3}H]DPPC IN POLYMERIZED OR NONPOLYMERIZED DPL VESICLES

Macrophage monolayers were incubated with [^{3}H]DPPC-labelled polymerized (PV) or nonpolymerized (NPV) vesicles composed of DPL for 3.5 h at 37°C in medium A. The lipid dose was 300 μg/well and the amount of radioactivity $6 \cdot 10^6$ dpm/well. The cells were then rinsed in medium A and further incubated in medium A at 37°C. At intervals, cells and medium were each extracted as described in Methods and the amount of water-soluble and organic-soluble material remaining in the cells or released into the medium was determined. The organic-soluble material in the cells and in the medium at 20 h was further analysed by thin-layer chromatography for the PV case. Results represent the means of triplicate determinations and are typical of several experiments of this type.

Time (h)	% Total dpm			
	in medium		in cells	
	water	organic	water	organic
NPV 0	0.3	5.8	1.4	92.4
3	2.2	17.6	1.3	78.9
20	7.2	23.6	1.4	67.8
26	9.1	21.9	0.6	68.0
PV 0	0.3	5.1	2.0	92.5
3	2.5	24	0.8	62.6
20	5.4	20.3	1.0	73.4
26	7.1	19.0	1.2	72.0

TABLE Ib

TLC ANALYSIS OF MACROPHAGE PROCESSING OF ^{3}H-LABELLED DPL VESICLES

Polymerized DPL vesicles labelled with [^{3}H]DPPC were incubated with macrophages, as described in Table Ia. The organic-soluble fractions derived from the cells or the culture medium at 20 h were analysed on thin-layer chromatograms versus a [^{3}H]DPPC vesicle standard, as described in Methods. TLC lanes were divided into 20 fractions, the fractions scraped into scintillation vials and counted. Authentic unlabelled DPPC migrates in fractions 8–12.

Fraction No.	% total cpm		
	[^{3}H]DPPC vesicle standard	^{3}H in cells	^{3}H in medium
1– 7 (Origin)	15.42	4.12	8.38
8–12	75.63	88.70	50.7
13–15	8.6	2.06	32.2
16–20	0.5	5.2	4.8

authentic [^{3}H]DPPC and thus likely represents unmetabolized liposomes. If the vesicles and their associated [^{3}H]DPPC had been broken down and the label reutilized, one would expect to find radioactivity associated with other lipid classes such as sphingomyelin, phosphatidylethanolamine and phosphatidylserine [18], which would migrate at different positions on the chromatogram. A crude estimate of the $t_{1/2}$ for breakdown of [^{3}H]DPPC from both polymeric and nonpolymeric DPL vesicles is 50 h, a number which agrees quite well with the rate of breakdown of conventional phospholipid vesicles by monocytes previously observed by Mehta et al. [20]. The macrophages also reexport a substantial amount of label (25% of total in 26 h) into the medium. In contrast to the cellular material, about 30% of the radioactivity found in the medium represents breakdown products of the [^{3}H]DPPC; thus, a substantial fraction of the counts are water-soluble and the material in the organic-soluble fraction includes both DPPC and a faster-migrating (less polar) product. Thus, the cells seem to reexport the breakdown products of the vesicles rather than incorporating them into cell lipids; the mechanisms involved are unknown at this time. Our results seem to suggest that there are no major differences in the cellular processing of a lipophilic marker incorporated in either polymerized or nonpolymerized liposomes composed of DPL.

Competition experiments

Since the uptake characteristics of polymerized DPL vesicles seem quite different from those of conventional phosphatidylcholine vesicles, we decided to examine the ability of different types of vesicles to compete with polymerized DPL vesicles for uptake by macrophages. There is a good deal of evidence suggesting that different types of vesicles are taken up via different mechanisms or at least via different cell-surface-binding sites. Thus, Szoka et al. [21] and Pagano et al. [19] have reported that solid liposomes bind more avidly to cells than fluid vesicles. Hsu and Juliano [13] and Schroit and Fidler [22] have shown that negatively charged vesicles are endocytosed more extensively than neutral vesicles by macrophages, while Margolis et al. [23] have shown that different types of vesicles interact differently with epithelial cells.

Fraley et al. [24] have demonstrated that neutral and negatively charged vesicles do not compete with each other for cellular uptake and thus are presumably taken up by different pathways.

In Fig. 2, we see that the uptake of radiolabelled polymerized DPL vesicles can be readily competed out by excess unlabelled polymerized DPL vesicles; the apparent competition by nonpolymerized DPL vesicles may, in part, be due to the toxic effects of these liposomes (see below). By contrast, liposomes prepared from 'conventional' phosphatidylcholines, whether in a fluid phase (dimyristoylphosphatidylcholine, DMPC) or in a solid phase (distearoylphosphatidylcholine, DSPC), were unable to compete for uptake of polymerized DPL vesicles, even at very high doses of lipid. These observations suggest that DPL vesicles and conventional phosphatidylcholine vesicles are bound at different sites on the cell surface and are taken up by different mechanisms. We also examined the effect of negatively charged vesicles on the uptake of polymerized DPL vesicles. Interestingly, negative fluid vesicles (DMPC/DMPG) failed to compete, whereas high doses of negative solid vesicles (DSPC/DPPG) did compete.

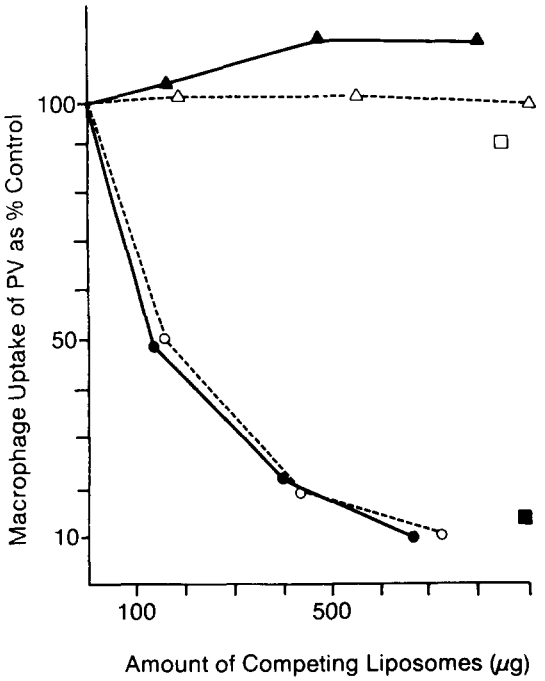

Fig. 2. Competition for macrophage uptake. Macrophage monolayers were incubated at 37°C for 90 min with [^{3}H]DPPC-labelled polymerized DPL vesicles (PV). Various doses of several types of unlabelled vesicles were coincubated with the radiolabelled vesicles. The amount of uptake of ^{3}H was determined as described in Methods; the results are expressed as percentage of a control where no unlabelled vesicles were added. Points are the means of triplicate determinations. Competing vesicles: ●, polymerized DPL; ○, nonpolymerized DPL; △, DMPC; ▲, DSPC; □, DMPC/DMPG (7:3) (negative fluid vesicles); ■, DSPC/DPPG (7:3) (negative solid vesicles).

These observations suggested that the polymerized DPL vesicle might be behaving like negatively charged solid vesicles. There is evidence, based on ESR studies [25], that polymerization markedly reduces the fluidity of DPL vesicles. However, we were unable to obtain any evidence that polymerized DPL vesicles have a negative surface charge. Thus, we used the technique of Abbracchio et al. [26], namely measurement of particle adsorption onto ion-exchange filters, and showed that polymerized DPL vesicles, nonpolymerized DPL vesicles and DMPC vesicles all adsorbed to the same small degree to anion-exchange filters (data not shown). While the technique of detecting surface charge via adsorption to ion-exchange filters has not been previously applied to liposomes, it has been used on other types of small particles and has been shown to correlate well with surface charge estimates based on zeta potentials determined by particle electrophoresis [27]. Thus, while polymeric DPL vesicles seem to be taken up by macrophages via a pathway which is distinct from that used to take up 'conventional' phosphatidylcholine vesicles, it is unclear at this time if the DPL-uptake pathway overlaps that for negatively charged vesicles.

Toxicity studies

As mentioned above, the uptake experiments suggested that nonpolymerized DPL vesicles might be somewhat toxic for macrophages. This was confirmed by measuring effects of various types of vesicles on cellular protein and RNA synthesis. As seen in Fig. 3, even very large doses of DMPC liposomes or of polymerized DPL liposomes are innocuous in terms of effects on macromolecular synthesis in macrophages. By contrast, nonpolymerized DPL vesicles markedly inhibited cellular synthesis of protein and RNA even at rather mod-

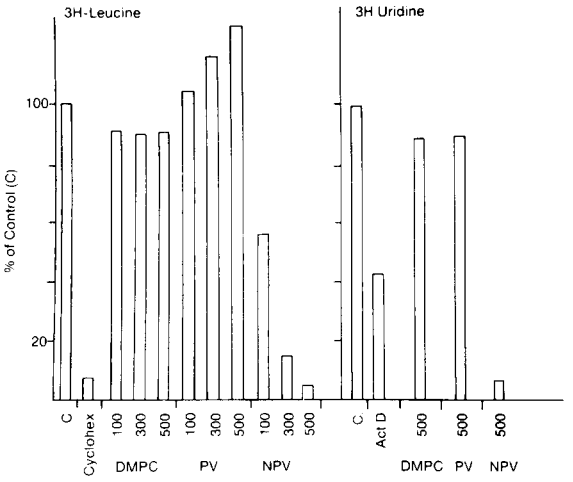

Fig. 3. Toxic effects of vesicles on cells. Macrophage monolayers were preincubated with various doses of DMPC vesicles, polymerized DPL vesicles (PV) or nonpolymerized DPL vesicles (NPV) for 90 min at 37°C. The monolayers were rinsed and then assayed for inhibition of protein synthesis ([^{3}H]leucine incorporation) or RNA synthesis ([^{3}H]uridine incorporation) as described in Methods. Results are expressed as percentage of a control which received no vesicles. Cycloheximide and actinomycin D were used as positive controls for inhibition of protein synthesis and RNA synthesis, respectively. Results are the means of triplicate determinations.

est doses. It should be noted that these studies only addressed acute toxic effects manifested over a few hours; longer-term effects manifest during many hours or days in culture have not been explored. Presumably, the toxicity of the nonpolymerized vesicles is due to the availability of methacryloyloxy residues which might react with critical cellular components. The active generation of oxidizing species, such as superoxide radical, by macrophages [28] might be suspected to be part of the toxic process.

Discussion

In many respects, vesicles composed of DPL, a photopolymerizable derivative of phosphatidylcholine, interact with macrophages in ways quite distinct from the interactions of conventional phosphatidylcholine (PC) vesicles. Thus, polymeric DPL vesicles bind at 4°C and are taken up at 37°C much more avidly than conventional PC vesicles. Competition studies (Fig. 2) also indicate that polymeric vesicles and conventional PC vesicles are taken up by different, noncompeting routes. However, once inside the cell, polymerized and nonpolymerized DPL vesicles containing a radioactive lipophilic marker are metabolically processed in similar fashion (Table I) and in a manner comparable to previous reports of intracellular processing of conventional (PC) liposomes [18,20]. An interesting aspect of these studies is the finding that DPL is quite toxic to macrophages before polymerization but is much less toxic after polymerization; we were surprised by this result, having originally anticipated the converse outcome. However, in view of the macrophages' ability to generate oxidizing species, which might then trigger free radical reaction of the methacryloyloxy groups, the toxicity of the nonpolymerized form may not be so bizarre. In summary, these studies suggest that the interactions of polymerizable and polymerized liposomes with cells present a number of novel features not readily predicted from studies of the interactions of conventional liposomes with cells.

Acknowledgement

This work was supported by NIH grant CA 28891.

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