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NOTES

Pharmacological Disposition of Negatively Charged Phospholipid Vesicles in Rats

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Abstract □ The pharmacological disposition of four negatively charged phospholipid vesicles with radioactive cholesteryl oleate as a tracer was investigated in rats. The acidic phospholipids included phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and diphosphatidylglycerol (cardiolipin). The blood half-life of phosphatidylserine vesicles was the shortest (<2 min), while that of phosphatidylinositol was the longest (90 min). Cardiolipin and phosphatidylglycerol vesicles had intermediate blood half-lives (7 and 18 min, respectively). The distribution of these vesicles in six major organs (liver, spleen, heart, kidneys, lungs, and brain) varied greatly. Contrary to the long-held belief, it was possible to prepare negatively charged phospholipid vesicles with long blood half-lives. Furthermore, tissue disposition of these negatively charged vesicles could be manipulated partially by judicious selection of their components.

Keyphrases □ Phospholipid vesicles, negatively charged—tissue distribution in rats □ Drug delivery systems—negatively charged phospholipid vesicles, tissue distribution in rats

Phospholipid vesicles, or liposomes when in combination with several similar preparations, are small (20–50 nm in diameter), single-bilayered, aqueous dispersions of phospholipids (1). The solutes entrapped in the closed interior aqueous compartment of phospholipid vesicles are protected from reacting with exterior chemicals. This property makes phospholipid vesicles an ideal pharmaceutical system for the *in vivo* delivery of biologically active material to target cells (2–5).

BACKGROUND

The interaction between cells and phospholipid vesicles consists of two known mechanisms (6), endocytosis and membrane fusion. Cells can take up phospholipid vesicles *via* endocytosis, producing intracellular vesicles that subsequently are degraded through fusion with lysosomes. Alternatively, phospholipid vesicles can fuse directly with the cell membrane, and the contents become incorporated into the cytoplasm. In terms of therapeutic efficacy, membrane fusion offers certain pharmacological advantages. For example, in enzyme replacement therapy in the treatment of storage disease, vesicle-entrapped enzymes retain their activity if they do not encounter the catalytic proteases in lysosomes (4).

Negatively charged phospholipids are involved in cell membrane fusion (7, 8). Vesicles prepared with negatively charged phospholipids have been used successfully to deliver biologically active chemicals to cells in culture (9). However, difficulties frequently are encountered in the *in vivo* application of negatively charged phospholipid vesicles. In plasma (or any calcium-containing medium), negatively charged liposomes sometimes aggregate to form larger particles, which are removed rapidly from circulation by the reticuloendothelial system (10). In addition, various blood proteins interact with phospholipid vesicles, and phospholipid molecules from blood lipoproteins are apt to exchange with those of the phospholipid vesicles (11, 12). These complicated interactions are expected to influence the pharmacological fate of circulating phospholipid vesicles.

These considerations prompted an investigation of the pharmacokinetics of four negatively charged phospholipid vesicles in rats, particularly since negatively charged liposomes with long blood half-lives may have enhanced bioavailability to the target tissue.

EXPERIMENTAL

Chemicals—Phosphatidylserine[1] (bovine brain), phosphatidylglycerol[1], phosphatidylinositol[1], cardiolipin[1], cholesterol[2] (chromatographically pure), cholesteryl [1-^{14}C]oleate[3] (20 mCi/mmole), and ethylenediaminetetraacetic acid disodium salt[4] were used as received. All solvents were glass distilled[5].

Liposome Preparation—Well-characterized single-bilayer vesicles were prepared by the simple method of Batzri and Korn (13). A solution of 3.75 mg of the phospholipid, 1 mg of cholesterol, and 0.05 μmole of cholesteryl [1-^{14}C]oleate (1 μCi) in 0.5 ml of chloroform was dried first under a nitrogen stream at room temperature and then in a vacuum desiccator for 2 hr at 10^{-5} torr. This procedure was essential to obtain a consistent liposome preparation.

The lipid mixtures were dissolved in 100 μl of ethanol at 37°. The ethanolic lipid solution was added as a fine stream with a 100-μl gastight

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Applied Science Laboratories, State College, Pa.
[3] New England Nuclear, Boston, Mass.
[4] Matheson, Coleman and Bell, Norwood, Ohio.
[5] Burdick & Jackson Laboratories, Muskegon, Mich.

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Table I—Normalized Tissue Distribution of Negatively Charged Phospholipid Liposomes in Rats 90 min after Intravenous Administration [a]

Organ	Phosphatidylserine	Cardiolipin	Phosphatidylglycerol	Phosphatidylinositol
Heart	1.01 ± 0.07	1.14 ± 0.16	1.39 ± 0.10	2.09 ± 0.11[b]
Lungs	1.49 ± 0.17	2.26 ± 0.68	1.79 ± 0.35	4.81 ± 0.54[b]
Liver	94.42 ± 0.16	92.27 ± 0.41	94.51 ± 0.76	87.40 ± 0.85[b]
Spleen	1.74 ± 0.08	3.43 ± 0.39[b]	0.89 ± 0.09[b]	2.96 ± 0.56[b]
Kidneys	1.11 ± 0.06	0.74 ± 0.09	1.15 ± 0.24	1.99 ± 0.23
Brain	0.24 ± 0.05	0.15 ± 0.02	0.27 ± 0.04	0.53 ± 0.05[b]
Percent of dose recovered	45	70	40	20

[a] Each value is the mean ± *SE* of six determinations (two groups of three rats). [b] $p < 0.05$ compared with corresponding phosphatidylserine values.

microsyringe[6] to 2 ml of isotonic saline containing 10 m*M* ethylenediaminetetraacetic acid (pH 7.4) while being vortexed at 37°. Vesicles thus prepared were transparent and remained so for at least 10 hr at room temperature. However, in these experiments, liposomes always were prepared fresh on the day of use.

Pharmacokinetic Studies—Male Sprague–Dawley rats, 200–300 g, were used. Laboratory rat chow and water were provided freely. While the rat was under pentobarbital anesthesia, a 0.5-ml vesicle preparation was administered intravenously *via* the lateral tail vein. Blood samples of 100 μl were collected by clipping the tail at various intervals and then were combusted in a sample oxidizer[7]. The radioactive carbon dioxide was absorbed in 6 ml of a high-capacity carbon dioxide-absorbing solution[8], and this solution was diluted with an equal volume of xylene-based counting fluid[9]. The radioactivity of the samples was determined with a scintillation spectrometer[10]. Quenching was determined and corrected automatically with an external standard; the counting efficiency for carbon 14 was 95%.

After 90 min, the rat was killed by decapitation. The blood was drained, and the internal organs were removed, rinsed with deionized water, blotted with tissue paper, and weighed. Tissue samples were processed in the same manner as the blood except that 11 ml each of the carbon dioxide-absorbing solution and the counting fluid was used. In the phosphatidylserine study, three rats were killed at 20 min because of the short plasma half-life of the vesicles. The tissue disposition patterns were similar to those from rats killed at 90 min.

RESULTS AND DISCUSSION

The disappearance of the four negatively charged liposomes from the blood after a single intravenous administration is depicted in Fig. 1. Phosphatidylserine liposomes were eliminated quickly from the circulation; 4 min after liposome administration, the blood radioactivity decreased almost 10-fold, with the half-life estimated to be 1.2 min by nonlinear regression analysis. In contrast, phosphatidylinositol liposomes persisted much longer (half-life of 90 min); phosphatidylglycerol and cardiolipin liposomes showed half-lives of 18 and 7 min, respectively.

Table I summarizes the normalized percent distribution of radioactivity in six major organs 90 min after intravenous administration of the four liposomes. These results must be considered in light of the fraction of the administered dose recovered in these organs: 45% of phosphatidylserine, 70% of cardiolipin, 40% of phosphatidylglycerol, and 20% of phosphatidylinositol. Clearly, in all of the studies, the liver took up almost all of the radioactivity. However, although it was still high, the hepatic radioactivity of phosphatidylinositol was the lowest (87.4%) of the four liposomes. Since the recovery of the administered phosphatidylinositol also was the lowest (20%), the smallest amount of this liposome persisted in the liver 90 min after its administration.

As a major component of the reticuloendothelial system, the liver undoubtedly plays a critical role in the elimination of liposomes from the blood. Compared with the other three liposomes, phosphatidylinositol liposomes were taken up to the least extent by the liver. Consequently, their elimination from the blood is expected to be much slower than the elimination of the other liposomes. This finding is consistent with the observation that phosphatidylinositol showed the longest half-life in the blood *in vivo*.

The comparative distribution of liposomal radioactivity in the six organs is shown in Fig. 2, with the hepatic radioactivities normalized to 100%. Of the total administered radioactivity recovered, more phosphatidylinositol liposomes than any other liposomes were found in all organs except the spleen. This finding is attributed to the more protracted exposure of the organs to phosphatidylinositol liposomes due to the relatively long blood half-life of these liposomes.

The optimal application of liposomes to drug delivery depends on the mechanism of liposomal internalization. For encapsulated, biologically active chemicals that are resistant to liposomal enzyme catalysis, endocytosis may not be detrimental. However, for other drugs, particularly enzymes for replacement therapy, endocytosis may lead to their degradation by lysosomes. In contrast, fusion of the plasma membrane with liposomes can result in the release of encapsulated materials directly into the cytoplasm. Accordingly, membrane fusion would be preferred for liposomal delivery of drugs.

Negatively charged phospholipids are believed to be essential in the fusion process (8, 14). Tissue membranes that actively participate in fusion are high in negatively charged phospholipids, particularly in neural tissues in which exocytosis contributes significantly in neurotransmission (15). Liposomes recently were used as a model biomembrane system (16–18). The fusion of liposomes under defined conditions provides information on the nature of this process. Generally, the chemical properties of the phospholipid and the cation are critical to fusion. Of the

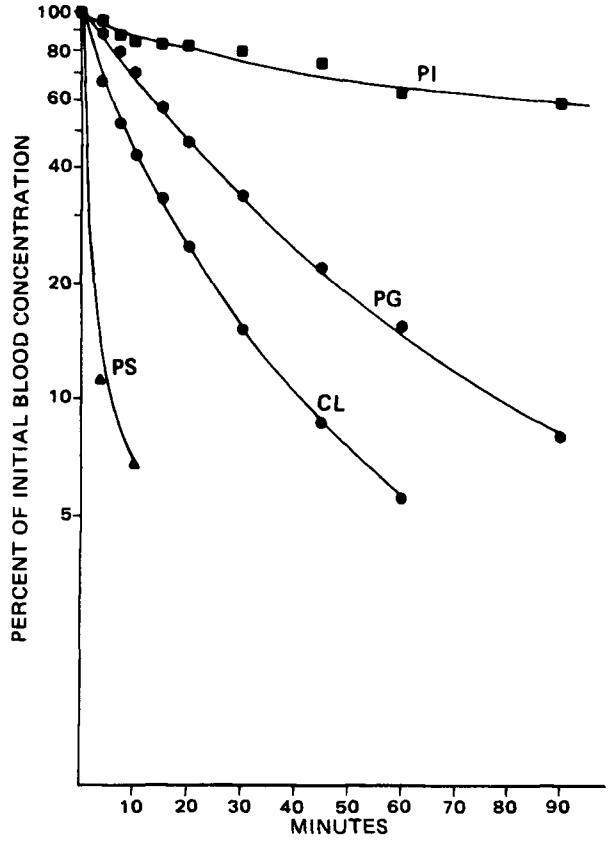

Figure 1—*Plasma disappearance of negatively charged phospholipid vesicles. Key: PS, phosphatidylserine; PG, phosphatidylglycerol; PI, phosphatidylinositol; and CL, cardiolipin. Each point represents the average value from six animals.*

[6] Hamilton.
[7] Model B306, Packard Instrument Co., Downers Grove, Ill.
[8] Carbo-sorb, Packard Instrument Co., Downers Grove, Ill.
[9] Permafluor V, Packard Instrument Co., Downers Grove, Ill.
[10] Tri-carb model 2650, Packard Instrument Co., Downers Grove, Ill.

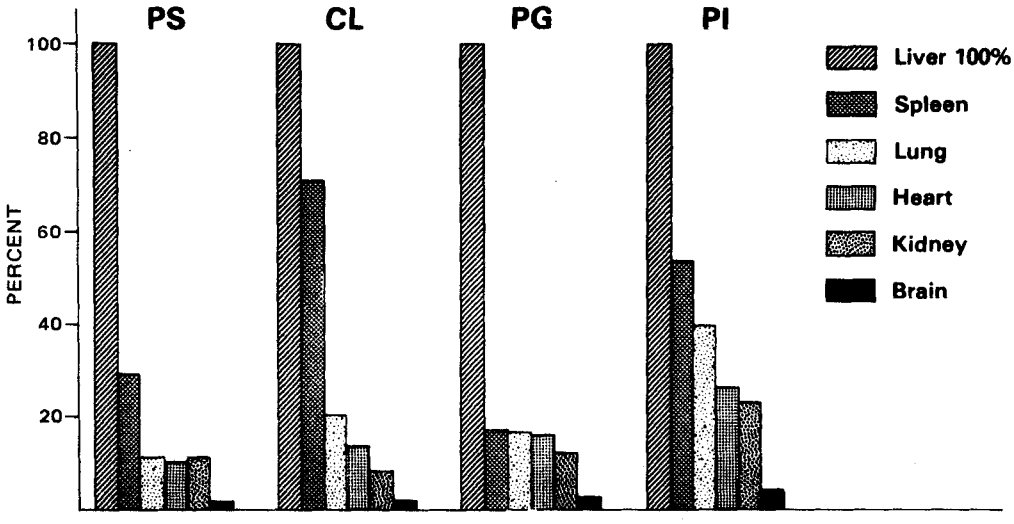

Figure 2—*Relative organ distribution of vesicle-associated radioactivities in the rat. Key: PS, phosphatidylserine; CL, cardiolipin; PG, phosphatidylglycerol; and PI, phosphatidylinositol. The liver-specific uptake was normalized to 100%. For each phospholipid, the data represent the average values from six rats (two experiments with three animals each).*

negatively charged phospholipids, phosphatidylserine fuses at the lowest cation concentration. Furthermore, among divalent cations, calcium is the most effective in inducing fusion. The free calcium concentration in plasma is ~1 m*M*. At this calcium concentration, phosphatidylserine vesicles alone, but not other negatively charged phospholipid vesicles, precipitate and cause disorganization of the membrane and leakage of the contents. This finding is consistent with the observation that the phosphatidylserine liposome blood half-life is <2 min, indicating that phosphatidylserine vesicles are not useful for drug delivery.

The various components in plasma may interact with liposomes. Some phospholipids exchange with blood lipoproteins and vesicles, and various apolipoproteins may bind to liposomes. These interactions must be quantified before a serious application of negatively charged liposomes to drug delivery can be attempted. The interactions between blood components and phosphatidylglycerol and cardiolipin liposomes probably account for their relatively short blood half-life. Although the pharmacological experiments were not designed to identify these interactions, the results suggested their existence.

Phosphatidylinositol liposomes were comparatively stable in the bloodstream since their half-life *in vivo* was the longest. Dapergolas *et al.* (19) showed that insulin encapsulated with phosphatidylinositol liposomes had hypoglycemic activity in rats when given orally. It is inferred that phosphatidylinositol liposomes can maintain their integrity in an extreme chemical environment. Because inositol, a carbohydrate, is highly hydrated in aqueous media, the bonded water molecules surrounding a phosphatidylinositol liposome may contribute to its low reactivity and thus sustain its long blood half-life.

In conclusion, based on pharmacokinetic observations of four negatively charged phospholipid vesicles in the rat, phosphatidylinositol vesicles clearly deserve further studies; they have a very long circulation half-life and a relative high uptake by organs in which the reticuloendothelial system plays a minor role.

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