THE EFFECT OF PARTICLE SIZE AND CHARGE ON THE CLEARANCE RATES OF LIPOSOMES AND LIPOSOME ENCAPSULATED DRUGS.

R. L. Juliano[1,2]* and D. Stamp[1]

[1] Research Institute, The Hospital for Sick Children
and
[2] Department of Medical Biophysics, University of Toronto
Toronto, Canada

Received February 14,1975

Summary

Heterogeneous populations of liposomes were cleared from the rat bloodstream in a complex manner, with a rapid phase and a slow phase of removal. Small unilamellar vesicles were cleared less rapidly than were large multilamellar ones. Particle charge was also an important determinant of clearance since neutral and positively charged unilamellar liposomes were cleared less rapidly than were unilamellar negatively charged ones. Liposome samples homogeneous in size exhibited simple exponential clearance kinetics rather than the complex kinetics exhibited by heterogeneous samples. A liposome encapsulated drug (^{3}H-colchicine) was cleared from the circulation at a rate corresponding to the removal of the liposomal vesicle, and different from the rate of removal of free drug.

Introduction

Liposomes have been suggested as vehicles for the delivery of drugs or enzymes to afflicted tissues in cases of neoplastic and genetic deficiency diseases (1). Recent work of Gregoriadis and colleagues has shown that drugs and enzymes may be encapsulated within liposomes (2,3) and that the clearance of entrapped substances largely parallels that of the liposomes, resulting in accumulation in the liver and spleen (4); these workers have also shown that the adverse immunological effects of injected proteins may be minimized by encapsulation (5). Another recent study has analyzed the tissue distribution at two time intervals, of liposomes marked with $^{99m}TcO_4^-$ and a spin probe (6).

Most of these analyses have employed liposome populations which were physically heterogeneous, especially with respect to size. The importance of the physical characteristics of the liposomal vehicle with

respect to the biological fate of the encapsulated substance has been demonstrated by Papahadjopoulos and co-workers. These investigators have shown that cultured cells in vitro can take up liposomes possessing a variety of physical properties (7,8). However, liposome encapsulated cyclic AMP manifested one of its biological properties (growth inhibition) only when unilammelar fluid liposomes were used. Non-fluid liposomes containing cyclic AMP were also taken up by cells, but the drug exerted no biological effect, suggesting different routes of uptake for liposomes with different physical properties. In the light of these findings, we have decided to investigate the biological disposition of liposome populations which are relatively homogeneous in physical characteristics. In this communication we report on the role of particle size and charge on the rate of clearance of liposomes from the rat bloodstream.

Experimental

Preparation of liposomes. Liposomes were prepared by evaporating lipids onto the wall of a tube, followed by the addition of buffer (isotonic phosphate or Tris buffered saline pH 7.2) to the dry lipid; the mixture was then dispersed by vortex agitation and sonication. The sonic dispersion was carried out in a bath type instrument (Heat Systems, 125 watts) under a nitrogen atmosphere and continuous temperature maintenance (20°C)(9). Marker substances ({^{3}H}-DPPC, {^{3}H}-cholesterol and {^{14}C}-DOPC) to be incorporated in the lipid membrane of the liposomes were dissolved in organic solvents and added to the lipid prior to drying. Substances to be trapped in the liposomal aqueous compartment ({^{125}I}-albumin, {^{3}H}-sucrose) were added to the buffer prior to vortex agitation. Liposomes were prepared with three different chemical compositions: a) neutral liposomes containing phosphatidyl choline and cholesterol in a 2/1 molar ratio; b) negatively charged liposomes containing phosphatidyl choline, phosphatidyl serine and cholesterol in a 1/1/1 ratio; c) positively charged liposomes containing phosphatidyl choline, stearylamine and cholesterol in an 18/2/10 molar ratio. Two size categories of neutral, negative and positive vesicles were prepared by varying the time of sonication. Prolonged sonication (12 hrs) followed by centrifugation at 1.5 x 10^{6}g - min yielded an optically clear supernatant containing very small liposomes which are probably mainly unilamellar vesicles (9). Brief sonication (1 - 5 min) gave rise to a turbid sample containing large multilamellar liposomes. Separation of liposomes according to size was accomplished by gel filtration on Sepharose 4-B (10). Untrapped aqueous markers were sometimes separated from the liposomes by Sephadex column chromatography.

Animal Experiments. Liposome samples (1-2 mg in 0.5 ml buffer) were injected into the tail veins of male rats. A small and constant amount of lipid was used in order to avoid saturating the system responsible for clearance (4). Blood samples were obtained by retro-orbital puncture from anaesthetized animals. The plasma and cells were separated by centrifugation and a sample

(0.1 cc) of the plasma was taken for radiation counting. Tritium containing samples were dissolved in scintillation solvent and analyzed in a Packard Tri-Carb liquid scintillation counter. Samples containing {^{125}I} were analyzed on a nuclear Chicago gamma counter, while samples labelled with both isotopes were analyzed by counting the {^{125}I} on the gamma counter and then correcting the counts derived from liquid scintillation using an appropriate standard curve.

Other. The chemical stability of {^{3}H}-dipalmitoyl phosphatidyl choline and {^{14}C}-dioleyl phosphatidyl choline was evaluated by thin-layer chromatography and autoradiography (11). The permeation of trapped markers from liposomes was measured using previously described techniques (9). Data analysis, and graphic curve fitting of clearance data to a sum of exponentials function were performed on a Hewlett Packard model 7810A desk top computer equipped with a model 9862A plotter.

Materials. Bovine phosphatidyl choline (PC), cholesterol (Chol), bovine phosphatidyl serine (PS) and {^{14}C}-dioleyl phosphatidyl choline (DOPC) were obtained from Applied Science Labs. Stearylamine was from K and K Laboratories. {^{3}H}-dipalmitoyl phosphatidyl choline ({^{3}H}-DPPC) was the generous gift of Dr. D. Papahadjopoulos. Radioalbumin ({^{125}I}) was obtained from Charles Frosst & Co., Toronto, while tritiated cholesterol, sucrose and colchicine, as well as scintillation solvents, were obtained from New England Nuclear. Sephadex and Sepharose products were from Pharmacia Canada Ltd. Other chemicals were of reagent grade and were generally obtained from Fisher Scientific Ltd.

Results

Characterization of Liposome Properties

a) Size. Brief sonication gave rise to a population of large multilamellar liposomes which were almost entirely excluded by a Sepharose 4-B column and which migrated in the void volume. By contrast, prolonged sonication followed by centrifugation gave rise to a population of small unilamellar vesicles which were almost entirely retarded by the column.

Thus, one may readily prepare populations whose size distribution falls entirely above or below the exclusion limit for the column packing. By collecting a few column fractions, one may obtain liposomal samples with a relatively narrow size range (10).

b) Stability. Cholesterol containing liposomes (10) are suitable vesicles for drug encapsulation, since they retain their impermeability to polar solutes, even in the presence of blood components. Thus, PC/PS/Chol liposomes containing entrapped {^{3}H}-sucrose released (9) less than 2% of their contents per hour during incubation in 50% serum at 37°C. Similarly, PC/Chol vesicles containing {^{3}H}-colchicine released less than 1% of the trapped drug

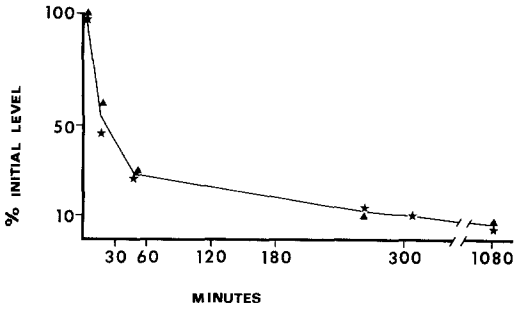

Fig 1. Clearance of labelled PC/PS/Chol liposomes: Liposomes labelled in the aqueous compartment with {^{125}I}-albumin and in the lipid membrane with {^{3}H}-DPPC were injected into rats. The plasma levels of the individual isotopes were measured ▲{^{3}H}★{^{125}I} . ordinate: percent of initial plasma level. abscissa: time in minutes.

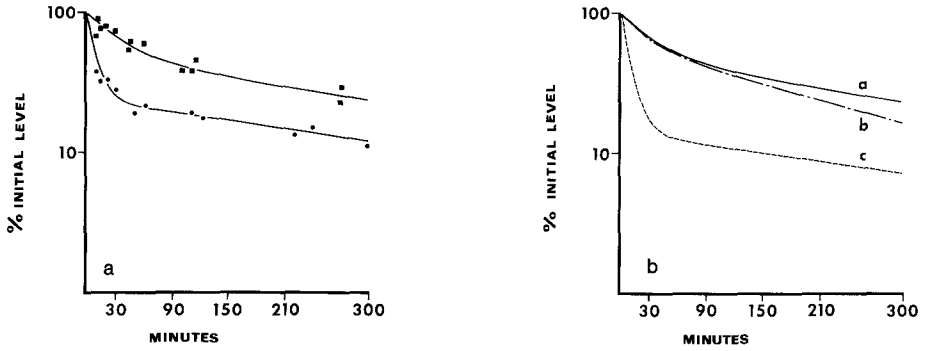

Fig 2a. Clearance of liposomes as a function of particle size: Unilamellar ■ or multilamellar ● PC/Chol liposomes labelled with {^{3}H}-DPPC were injected into rats and the plasma levels of {^{3}H} were measured. The data represents pooled results from 3 - 4 animals.

Fig 2b. Clearance of liposomes as a function of particle charge: The clearance of unilamellar {^{3}H}-DPPC labelled liposomes of the following compositions were measured: a) PC/Chol molar ratio (2/1). b) PC/SA/Chol molar ratio (18/2/10). c) PC/PS/Chol molar ratio (1/1/1). The curves were fitted by computer to pooled results from 3 - 4 animals. ordinate: percent of initial (2 min post injection) plasma level. abscissa: time in minutes.

---

per hour under the same conditions. Double label experiments indicate that the clearance of intact liposomes from the bloodstream may be measured without interference from marker loss due to exchange or vesicle breakdown. Thus fig 1 displays the clearance rate of PC/PS/Chol liposomes marked in the aqueous

compartment with {^{125}I}-albumin and in the lipid compartment with {^{3}H}-DPPC. It can be seen that the two isotopes were cleared at identical rates. Therefore, the disappearance of labelled PC from the bloodstream in all probability measures the loss of intact liposomes and not exchange or breakdown phenomena. The marker {^{3}H}-DPPC has also been shown to be stable during the preparative procedures, since only 5-10% radioactive breakdown products were visualized by thin layer chromatography and autoradiography after 18 hours of sonication.

Clearance of liposomes from the rat bloodstream. Injected liposomes typically are cleared from the bloodstream in two phases; there is an initial rapid loss, followed by a slower period of decline. These observations may readily be fitted to a function of the form $^{*}P = Ae^{-.693\ t/T_1} + Be^{-.693\ t/T_2}$: that is, a sum of exponentials. However, we must regard this as only an approximation of what may be a very complex kinetic pattern.

Effect of size. Particle size is an important determinant of the clearance rate as is illustrated by fig 2a. Here one sees that neutral unilamellar PC/Chol liposomes were maintained in the plasma substantially longer than multilamellar ones. The half times (T_1 and T_2) were approximately 22 and 200+ minutes in the first case and 6 and 200+ minutes in the second case. A similar size dependence of the initial rapid clearance was seen in the case of positive PC/SA/Chol liposomes where the unilamellar type was retained in the plasma longer (T_1= 30 minutes) than the multilamellar one (T_1= 10 minutes). In the case of negative PC/PS/Chol liposomes, both unilamellar (T_1= 8 minutes) and multilamellar (T_1= 8 minutes), vesicles were cleared very rapidly and no differences could be discerned.

Effect of charge. As implied in the previous paragraph, particle charge has important effects on clearance rates. Thus, samples comprised of unilamellar neutral or positively charged liposomes were retained in the circulation longer than were samples composed of unilamellar negatively charged liposomes (fig 2b).

* P equals the % of the initial plasma level Po (sampled at 2 min post injection), A + B = Po; T_1 and T_2 are half times for the rapid and slow phases of clearance.

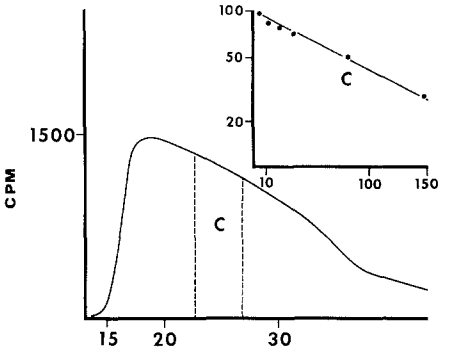

Fig 3. Clearance of a homogeneous sample: Neutral unilamellar liposomes (PC/Chol/{^{14}C}-DOPC) were fractionated on a Sepharose 4-B column. ordinate: cpm {^{14}C}-DOPC. abscissa: fraction number (void volume at #10).

Fig 3 inset. Pooled sample "C" was injected into a rat and the rate of clearance was determined. ordinate: percent of initial plasma level. abscissa: time in minutes.

In contrast to previous investigators who used multilamellar populations, we have not discerned any appreciable differences between clearance rates of neutral and positively charged liposomes (4). We concur in the observation that negative liposomes are cleared most rapidly.

Effect of size heterogeneity. In an attempt to understand the basis of the complex kinetics of clearance for liposomes, we studied the fate of more homogeneous samples. Unilamellar PC/Chol/{^{14}C}-DOPC liposomes were fractionated on a Sepharose 4-B column and a few fractions from the center of the liposomal peak were pooled for use (fig 3). These samples were injected into rats and plasma levels of radioactivity were measured. As shown (fig 3, inset) the plasma level of this material followed a simple exponential decay ($P = Be^{-.693\ t/T_2}$) with a half time of about 80 min, suggesting a single rate and mechanism of clearance. Further experiments with homogeneous samples suggest that the rate of clearance is directly related to particle size. These findings indicate that the complex kinetics of clearance mentioned above and observed by other groups (4) is due to the presence of a heterogeneous size array of particles in the samples. We might suggest (see fig 2a) that

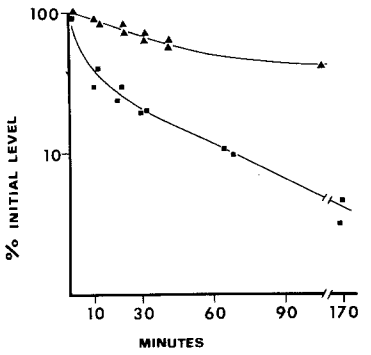

Fig 4. Clearance of free and encapsulated {^{3}H}-colchicine: {^{3}H}-colchicine was encapsulated (10% trapping efficiency) in unilamellar PC/Chol vesicles and the free colchicine was removed by filtration on a Sephadex G-50 column. The clearance rates of free ■ and encapsulated ▲ {^{3}H}-colchicine are illustrated. The rate of clearance of the encapsulated form is typical of the clearance rate of neutral unilamellar vesicles. ordinate: percent initial plasma level. abscissa: time in minutes.

the rapid phase of clearance represents the disappearance of large multilamellar forms, while the slow phase represents the loss of small unilamellar vesicles. The observation that negatively charged liposomes, even when they are initially unilamellar in form, are mainly cleared during the rapid phase may be due to the known tendency of these vesicles to coalesce in the presence of proteins and calcium ion (12) at levels similar to those found in blood plasma.

Several previous communications have evaluated the tissue distribution of injected liposomes and have noted substantial accumulation in the liver and spleen (3,4,6). However, these studies involved the use of heterogeneous liposome populations and were usually conducted during the rapid phase of clearance. Thus, the tissue distribution measured in these studies may reflect primarily the fate of large multilamellar species. In view of present results, it may be worthwhile to re-investigate the question of tissue distribution using homogeneous populations of unilamellar vesicles, to determine if these species might accumulate at sites other than tissues rich in reticuloendothelial elements. These studies, as well as studies attempting to establish the relative importance of particle size and charge, are currently under way.

Drug distribution. The rate of clearance of liposome encapsulated drugs has been studied in the case of heterogeneous (probably multilamellar) populations of vesicles. We wished to ascertain if unilamellar vesicles could also act as efficient carriers of drugs. In vitro experiments (see above) have shown that the drug {^{3}H}-colchicine remains firmly associated with liposomal carriers, even in the presence of blood elements. When unilamellar PC/Chol liposomes containing{^{3}H}-colchicine were injected into the rat bloodstream, the vesicle associated drug was cleared at a rate characteristic of unilamellar neutral liposomes, and dramatically different from that of free drug (fig 4). This demonstrated that the pharmacokinetics of chemotherapeutic agents may be modulated by encapsulation in unilamellar liposomes.

Acknowledgements

This study was supported by the National Cancer Institute of Canada. We wish to thank Mrs. Doris Wills for typing and editorial assistance.

Bibliography

1. Gregoriadis, G. (1973) New Scientist 60, 890-893.
2. Gregoriadis, G. (1973) FEBS Letters 36, 292-296.
3. Gregoriadis, G. and Ryman, B. (1971) Eur. J. Biochem. 24, 485-491.
4. Gregoriadis, G. and Neerunjun, D.E. (1974) Eur. J. Biochem. 47, 179-185.
5. Gregoriadis, G. and Allison, A.C. (1974) FEBS Letters 45, 71-74.
6. McDougall, I.R., Dunnich, J.K., McNamee, M.C. and Kriss, J.B.(1974) Proc. Natl. Acad. Sci. (US) 71, 3487-3491.
7. Papahadjopoulos, D., Mayhew, E., Poste, C. and Smith, S. (1974) Nature (in press)
8. Papahadjopoulos, D., Poste, C. and Mayhew, E. (1974) Biochim. Biophys. Acta 363, 404-418.
9. Papahadjopoulos, D. (1970) Biochim. Biophys. Acta 211, 467-477.
10. Papahadjopoulos, D., Nir, S. and Ohki, S. (1972) Biochim. Biophys. Acta 266, 561-583.
11. Randerath, K. (1970) Anal. Biochem. 34, 188-205.
12. Papahadjopoulos, D., Poste, C., Schaeffer, B.E. and Vail, W.J. (1974) Biochim. Biophys. Acta 352, 10-28.