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Lymphatic Transport of Liposome-Encapsulated Drugs Following Intraperitoneal Administration – Effect of Lipid Composition

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Received: March 30, 1985; accepted: June 11, 1985.

Abstract: Tumor cells often metastasize through lymphatic channels. It follows that localization of antitumor agents in the lymphatics may be therapeutically beneficial. This study determines the extent to which lipid composition controls lymphatic transport of a model compound (^{14}C-sucrose) in liposomes following intraperitoneal administration in rats. All liposomes tested had mean diameters of approximately 0.2 μm. Liposomes were administerd to thoracic duct cannulated rats, and ^{14}C was quantified in thoracic lymph, several lymph nodes, blood, urine, and peritoneal wash. Changing liposome composition altered the rate of absorption of ^{14}C from the peritoneal cavity, stability in biological fluids, and the relative ability of liposomes to be retained by lymph nodes. Stability in biological fluids (plasma and lymph) appeared to be a reasonable predictor of observed lymph node recovery. Direct measures of lymph node level alone were poor measures of the ability of liposomes to function as prototypal lymphatic drug carriers. Neutral liposomes were better at reaching the general ciruclation following absorption from the peritoneal cavity.

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Controlled release of drugs in the vicinity of target tissues may lead to improved therapeutic availability. A theoretical analysis (1) of the pharmacology and pharmacokinetics of intravenous versus intraperitoneal drug therapy concluded that the latter route can have major advantages in chemotherapy of cancers confined to the peritoneal cavity. Experimental results support these predictions (2, 3). Such improved therapeutic availability need not be limited to treatment of peritoneal tumors if a drug carrier can gain access to more distant tumors.

The findings of Parker et al. (4–7) and ourselves (8) suggest that liposomes can function both as prototypal lymphatic drug carriers and as vehicles for localized sustained drug release. Subcutaneous or intramuscular administration of anticancer agents entrapped in liposomes can result in both improved delivery to lymph nodes (9–12) and suppression of tumor metastases within the lymphatics (13, 14). The governing mechanisms and the important variables that may control drug availability are not yet known. However, it has been predicted that only drugs with specific physicochemical, pharmacokinetic and mechanistic properties will be good candidates for use with *in vivo* drug carriers such as liposomes (15).

It may become possible to engineer liposomes or other carriers to have the desired attributes by changing their composition, size, surface properties, or the dose administered. Here we focus on composition. Answers to three questions are sought. Can changes in liposome composition significantly alter the fraction of lymphatically absorbed drug that is retained in lymph nodes? Is liposome absorption from the peritoneal cavity a function of liposome composition? Is passage of liposomes through the lymphatics following absorption a function of composition?

Materials and Methods

Materials

Purified egg yolk phosphatidylcholine (PC), sodium salt of dipalmitoyl phosphatidic acid (DPPA), and bovine sphingomyelin (SM) (Avanti Polar-Lipids, Inc., Birmingham, AL 35216), along with cholesterol (Ch), stearylamine (SA) and α-tocopherol (T) (Sigma Chemical Co., St. Louis, MO) were used for liposome preparations. Sucrose (Sigma Chemical Co., St. Louis, MO) combined with (U-^{14}C)-sucrose (Amersham Corp., Arlington Heights, IL) (1.57 mCi/mg) was used as an aqueous space marker for the liposomes. PCS scintillation reagents (Amersham Corp., Arlington Heights, IL) or Liquiscint (National Diagnostics, Somerville, NJ), *tert*-butyl hydroperoxide (Mallinkrodt, Inc., St. Louis, MO), Protosol tissue solubilizer (New England Nuclear, Boston, MA) and Solusol tissue solubilizer (National Diagnostics, Somerville, NJ) were used for radioactivity quantitation. All other chemicals were of analytical reagent grade or better. Phosphate-buffered saline (pH 7.4) contained 92 mM sodium chloride, 23 mM dibasic sodium phosphate, and 11 mM monobasic sodium phosphate. Prior to use, DPPA was prepared as described before (16).

Experimental Design

There are several identifiable major independent, functional liposome variables: composition, including charge, size, stability, and surface properties. Of these, composition and surface properties are most diverse with potentially an infinite number of subsets. Our operating hypothesis has been that charge, size, and stability would impact similarly on a given dependent variable for any group of closely related liposomes. We therefore designed experiments to explore the effects of charge and size on lymphatic absorption and disposition while limiting the number of variable combinations. We selected an incomplete block design with four levels of both charge and size. Results of experiments evaluating size effects will be discussed in a separate report.

Preparation of Liposomes

Liposomes of PC/DPPA/CH/T (molar ratio, 4:1:5:0.1), PC/CH/T (5:5:0.1), and PC/CH/SA/T (4:5:1:0.1) were prepared using the procedure for medium sized liposomes described previously (17). The lipids were dissolved in chloroform, mixed in a round-bottom flask, and subsequently evaporated to dryness under vacuum. An aliquot of buffer was added to the dried lipids. The mixture was then agitated (at 20°C after nitrogen purging) until all lipids were suspended, giving 20–25 μmol lipid/ml. Then the lipid suspension was extruded sequentially with 1.0, 0.8, 0.6, 0.4 μm and then 0.3 μm Unipore polycarbonate membranes (Bio-Rad Labs., Richmond, CA) at a flow rate of about 5 ml/min controlled by nitrogen pressure (17). Liposomes of SM/CH/T (6:4:0.1) were prepared using another method (18). The lipid mixture (total 120–150 μmol) was dissolved in 14.4 ml diethyl ether, mixed with 2.40 ml of phosphate-buffered saline containing 5 mM sucrose with tracer amount of ^{14}C-sucrose, and sonicated at 20°C under a N_2 atmosphere in a bath type sonicator until an emulsion was obtained. The organic phase was then removed at 25–30°C in a rotary evaporator. The resulting suspension was extruded twice through a 0.4 μm pore size polycarbonate membrane and then twice more through a 0.3 μm membrane. All liposomes were dialyzed at 4°C in the dark, using a 1-ml dialysis cell fitted with a 0.2 μm polycarbonate membrane, against frequent changes of phosphate-buffered saline for 2–3 days to both remove unencapsulated sucrose and narrow the liposomal size distribution. The final concentration of lipids was adjusted to 20 μmol/ml. The free sucrose content was below 0.5 % of total sucrose after dialysis in all cases. All liposomes were stored at 4°C in the dark and usually used within 24 h of preparation.

Animal Experiments

Male SD rats (from Simonsen) weighing 250–300 g were used. Each rat was given an oral dose of soybean oil (Crisco®, Proctor and Gamble, Cincinnati, OH) (4.0 ml/kg) and 1 h later anesthetized with an injection of urethane (Sigma Chemical Co., St. Louis, MO) (1.2 g/kg, s.c.). A PE-10 polyethylene cannula (Clay Adams, Parsippany, NJ) was inserted into the thoracic duct proximal to the juglosubclavian junction according to the method of Saldeen and Linder (19). A PE-50 cannula was inserted into the left femoral artery, and another was used to cannulate the urinary bladder. The anesthesia was maintained during the duration of the study. Each rat was placed on a heated plate and kept at 37°C in a supine position. Fluid balance was maintained with an infusion (4 ml/h/kg) of saline containing 2.5 units/ml heparin via the arterial cannula. Animals with lymph flow rates of 0.5 ml/h or less were ineligible for the study. The test liposome (40 μmol lipid/2 ml; 2 ml/kg) or free sucrose (5 mM in phosphate-buffered saline, 2 ml/kg) was administered intraperitoneally 30 min after surgery. Lymph and urine were collected continuously. Blood was sampled periodically over the 5-h study period, and then rats were killed. The peritoneal cavity was rinsed with over 60 ml of saline followed by 20 ml of 1 % Triton X-100 (Amersham Corp., Arlington Heights, IL) to recover unabsorbed marker. Several lymph nodes adjacent to the peritoneal cavity (iliac, renal, cisternal, splenic, posterior gastric, portal, and superior mesenteric) and those around the thymus (left and right mediastinal, and parathymic) were excised for assay.

For each liposome composition aliquots of both the final lymph collection and the first peritoneal wash were centrifuged at 3,000 xg for 12 min to sediment any monocytes and macrophages. The sediment and aliquots of the supernatent were analyzed for total ^{14}C to estimate the fraction of collected material associated with sedimented cells.

Results are referenced to administered dose, absorbed dose and corrected dose. The absorbed dose is defined as the administered dose minus the amount recovered from the peritoneal cavity after 5 h. Corrected dose is defined as the administered dose minus the sum of the amounts recovered from the peritoneal cavity and collected in thoracic lymph.

Liposome Size and Stability

The size-frequency distribution of each liposome preparation was estimated by negative staining and subsequent electron

microscopy (20). Liposome stability was examined by incubation at 37°C in phosphate-buffered saline (pH 7.4), plasma, and lymph to which 10 units each of penicillin G and streptomycin sulfate were added per ml. A volume of liposomes (20 μmol lipid/ml) was mixed with an equal volume of medium in a length of dialysis tubing (mol. wt. cut-off, 12,000–15,000; Spectrum Medical Industries, Inc., Los Angeles, CA), and dialyzed against excess buffer to remove unencapsulated sucrose. The fraction remaining encapsulated was then calculated.

Assay Method

^{14}C Radioactivity in lymph, blood, washings of the peritoneal cavity, lymph nodes, and urine was quantified with the use of a scintillation counter equipped with automatic quench correction (LS 7800, Beckman Instrument, Inc., Mountainview, CA). Blood (100 μl) was digested with 200 μl 0.1 N KOH and bleached with 200 μl tert-butyl hydroperoxide, and then mixed with 8 to 50 ml scintillant. Lymph node tissue was mixed with 100 μl water and digested with 1 ml tissue solubilizer at 37°C, and then mixed with 15 ml of scintillant (PCS) for counting. Phosphorous content in each liposome preparation was determined by the modified Bartlett method (21).

Results

Size and Stability of Liposome

To evaluate the effect of lipid composition on the ability of liposomes to carry drugs to and through the lymphatics it is desirable to control size. Table I lists the liposomes used, their compositions and their characteristics. All four liposome preparations had a number-average diameter of approximately 0.2 μm.

Although liposomes were typically used within 24 h of preparation, no significant ^{14}C-sucrose release occurred for aliquots stored for four weeks at 4°C in the dark.

Results of stability studies are listed in Table II. The percent release of ^{14}C-sucrose from liposomes *in vitro* was determined after 4-h incubations with either phosphate-buffered saline (pH 7.4), lymph rich in chylomicrons (Lymph A), lymph poor in chylomicrons (Lymph B) or plasma. Liposomes incubated with either lymph (A and B) or plasma lost more of the entrapped sucrose than those incubated with buffer. The ability of lymph A, lymph B and plasma to destabilize liposomes was similar within each composition tested. There were modest differences in stability between compositions, with composition III being the most stable and the negative composition (II) being the least stable. Overall, *in vitro* stability was adequate. All liposomes retained at least 87% of the encapsulated marker after 4 h of incubation in biological fluids.

Disposition and Lymphatic Transport of Liposomes

Figure 1 shows the time-course of ^{14}C in both blood and thoracic lymph after administration of free ^{14}C-sucrose and two of the four liposome compositions (I and III). Free sucrose was rapidly absorbed following injection with peak blood levels occurring at or before 0.5 h. A fraction of the injected sucrose remained in lymph until collected. The mean thoracic lymph-blood concentration ratios ranged from 4.6 to 1.7 during the 5 h experiment. The time-averaged thoracic lymph/blood ratio for positive liposomes (III) was 100, whereas that for composition I it was only 6.1. The rising values for thoracic lymph between 1.5 and 4.5 h suggested continued absorption of encapsulated ^{14}C. Similar results (not shown) for larger (x3) and smaller (x0.1) doses of both free and encapsulated sucrose indicated the absence of a dose effect within this dose range.

Table II. Per cent Release of ^{14}C-Sucrose from Liposomes Incubated With Buffer, Plasma and Lymph for 4 Hours at 37°C

Lipsome[a] Designation	Mole Per cent Charged Lipid	Percent Release[b] Medium[b] Buffer[c]	Plasma[d]	Lymph-A[e]	Lymph-B[f]
I	0	0.36	11.0	8.5	7.3
II	10% Neg.	0.51	13.1	9.5	9.4
III	10% Pos.	0.42	2.3	3.7	2.1
IV	0	0.21	8.3	4.0	4.4

[a] See Table I.
[b] One volume of liposomes (20 μmol lipid/ml) was incubated with an equal volume of medium.
[c] pH 7.4, isoosmotic, phosphate buffered saline.
[d] Pooled from three rats.
[e] Lymph rich in chylomicrons collected from three rats predosed orally with 4 ml/kg of soybean oil.
[f] Lymph from normal rats. The clear lymph collected between 2.5 and 5 h after thoracic duct cannulation was used.

Figure 2 summarizes recovery of ^{14}C in thoracic duct lymph, urine, and from the peritoneal cavity 5 h after administration. Negligible amounts of ^{14}C were found associated with the cellular pellet of both thoracic lymph and peritoneal wash. Free sucrose was completely absorbed from the peritoneal

Table I. Liposomes Tested

Designation	Composition (molar ratio)	Method[a]	Characteristics[b]	Diameter (μm)[c]
I	PC/Ch/T (5:5:0.1)	Extr. (0.3 μm) Dial. (0.2 μm)	MLV Neutral	0.22
II	PC/Ch/PA/T (4:5:1:0.1)	Extr. (0.3 μm) Dial. (0.2 μm)	MLV Negative	0.17
III	PC/CH/SA/T (4:5:1:0.1)	Extr. (0.3 μm) Dial. (0.2 μm)	MLV Positive	0.20
IV	SPM/Ch/T (6:4:0.1)	Extr. (0.3 μm) Dial. (0.2 μm)	REV Neutral	0.23

[a]Liposomes were first extruded (Extr.) and then dialyzed (Dial.) with the membrane pore-size indicated as described in the text.
[b]MLV, multilamellar vesicle; REV, reversed phase evaporation vesicle.
[c]Measured by electron microscopy. Values are number-average diameters.

cavity (99.7 ± 0.2%) and excreted in urine (90.8 ± 0.1%) within 5 h. Liposome-encapsulated sucrose showed a delayed absorption and an enhanced transport in thoracic lymph. Among the different compositions, the negative composition (II) gave the most rapid apparent absorption of ^{14}C from the peritoneal cavity and the highest urinary recovery. The positive composition (III) showed the smallest degree of apparent absorption.

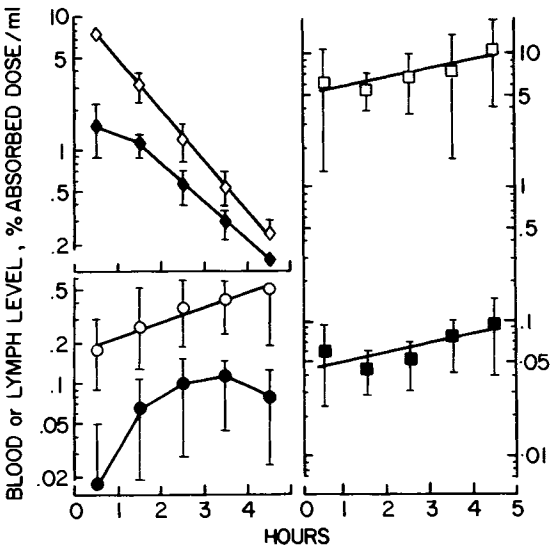

Fig. 1 Semilogarithmic plots of the time-course of ^{14}C levels in blood (closed symbols) and lymph (open symbols) following intraperitoneal administration of free ^{14}C-sucrose (◆, ◇), ^{14}C-sucrose encapsulated in composition I liposomes (○, ●), or ^{14}C-sucrose encapsulated in composition III liposomes (■, □). See Table I for compositions. Values are means for 3 to 5 rats. Vertical bars are ± one standard deviation. Lymph levels are plotted at the mid-point of the designated collection interval.

An analysis of variance for each of the four data sets in Fig. 2 resulted in rejection ($\alpha = 0.1$) of the null hypothesis that absorption of the four liposomes would be essentially the same. The degree of apparent absorption from the peritoneal cavity (Fig. 2A) averaged 49% for compositions II and IV which was 61% better than the combined results for I and III (30.4%). There was no statistical difference in the urinary recovery of ^{14}C (Fig. 2C) following administration of compositions I, III and IV (range of mean recoveries: 3.4–6.5%). However, urinary recovery following administration of composition II was significantly (α=0.1) higher, by a factor of 4. For these liposomes measure of *in vitro* stability (Table II) were poor measures of relative *in vivo* stability as measured by 0–5 h recovery of ^{14}C in urine.

Lymph Node Level

In order to evaluate uptake by lymph nodes, several were removed and analyzed. Results are shown in Table III. The approximate anatomical location of these lymph nodes is depicted in Fig. 3. Iliac, renal, splenic, posterior gastric, portal, superior mesenteric, and cisternal lymph nodes (hereafter referred to as Group 1) are located in the peritoneal region. The lymph fluid passing through these nodes eventually enters the cannulated thoracic ducts (22, 23). Several nodes around the thymus, such as the parathymic and left and

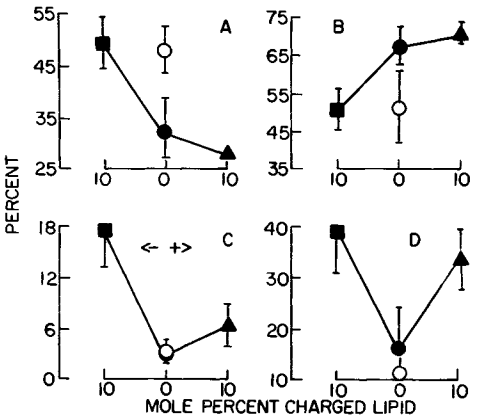

Fig. 2 A: Per cent of administered dose absorbed from the peritoneal cavity 5 h after dosing is shown as a function of the mole per cent charged lipid comprising the liposomes, as listed in Table II: composition I (●), II (■), III (▲) and IV (○). B: Per cent of administered dose recovered from the peritoneal cavity 5 h after dosing. C: Per cent of administered dose recovered in urine 0–5 h after dosing. D: Per cent of corrected dose recovered in urine 0–5 h after dosing, where corrected dose is defined as the fraction of administered dose reaching the circulation; and is approximately equal to the administered dose minus both that fraction recovered from the peritoneal cavity and that fraction collected in thoracic duct lymph after 5 h. All values are means (± SE) for 3 to 5 rats.

right posterior mediastinal nodes were also examined. It has been shown (22, 23) that some part of the lymph fluid draining the peritoneal cavity flows into lymph ducts other than the thoracic duct and then passes through this node group (hereafter referred to as Group 2).

The data in Table 3 indicated enhanced uptake of liposome-encapsulated ^{14}C-sucrose by most lymph nodes relative to corresponding uptake values for free sucrose. The resulting lymph node levels covered a 1000-fold range. The highest were for cisternal, parathymic, left mediastinal and renal lymph nodes, with the lowest levels found in superior mesenteric, iliac and portal lymph nodes. Lymph node levels of ^{14}C following administration of either composition I or II liposomes were higher than those obtained with the other two liposome compositions. This trend is clearly shown in Fig. 4, where nodal uptake of ^{14}C is represented as both per cent absorbed and per cent administered dose. An analysis of variance allowed use to reject the null hypothesis that composition had no effect on 5 h lymph node levels (α=0.1). Compositions I and II gave similar values, as did compositions III and IV. However, the mean lymph node values for these two pairs of compositions consistently differed by a factor of 2.5, and they were 3-times larger than that associated with Group 1 lymph nodes.

Lymph Node Retention

A potentially better measure of the degree of retention or clearance of liposomes by a group of lymph nodes is the ratio of the amount of ^{14}C recovered in nodes to the sum of both node levels and the amount in the lymph passing through these nodes (Fig. 5A) after some fixed interval (e.g. 5 h). This measure of lymph node retention is shown in Fig. 5C. Most significantly, the pattern seen for recovery of ^{14}C in thoracic lymph a percentage of the absorbed dose (Fig. 5B) is reversed. The pattern in Fig. 5C is similar to lymph node recovery as a per cent of the absorbed dose (Fig. 4C). Neutral composition I was apparently retained best by sampled nodes, whereas

Table III. Concentration of ^{14}C-Sucrose in Each Lymph Node at 5 h after Intraperitoneal Administration

	Lymph Node	Concentration in Lymph Node [a]				
		I[b] PC/Ch	II[b] PC/Ch/PA	III[b] PC/Ch/SA	IV[b] SPM/Ch	Free
Group 1	Iliac	0.05 (0.01)	0.10 (0.02)	0.06 (0.01)	0.05 (0.02)	0.06 (0.03)
	Renal	37.72 (37.33)	1.19 (0.56)	2.04 (1.81)	10.85 (9.95)	0.45 (0.04)
	Splenic	6.99 (6.83)	1.03 (0.37)	1.57 (0.92)	0.17 (0.10)	1.85 (1.56)
	Posterior Gastric	12.69 (6.66)	6.91 (5.24)	4.73 (2.28)	1.48 (1.32)	0.51 (0.36)
	Portal	0.32 (0.11)	0.70 (0.39)	0.17 (0.04)	0.14 (0.04)	0.06 (0.01)
	Superior Mesenteric	0.03 (0.01)	0.03 (0.01)	0.02 (0.01)	0.03 (0.01)	0.03 (0.01)
	Cisternal	69.27 (20.42)	61.23 (24.14)	9.81 (3.87)	12.46 (6.64)	4.73 (1.40)
Group 2	Parathymic	45.91 (12.50)	47.66 (25.04)	9.98 (2.41)	21.99 (16.39)	0.29 (0.03)
	Mediastinal (left)	31.42 (9.81)	63.88 (22.43)	17.85 (1.67)	13.88 (4.55)	1.02 (0.80)
	Mediastinal (right)	0.19 (0.13)	0.13 (0.04)	0.10 (0.04)	0.20 (0.06)	0.65 (0.24)
Blood		0.085 (0.061)	0.029 (0.006)	0.034 (0.002)	0.502 (0.164)	0.015 (0.002)

[a] Per cent of dose/g. Mean (S.E.) of 3 to 5 rats.
[b] See Table I for details.

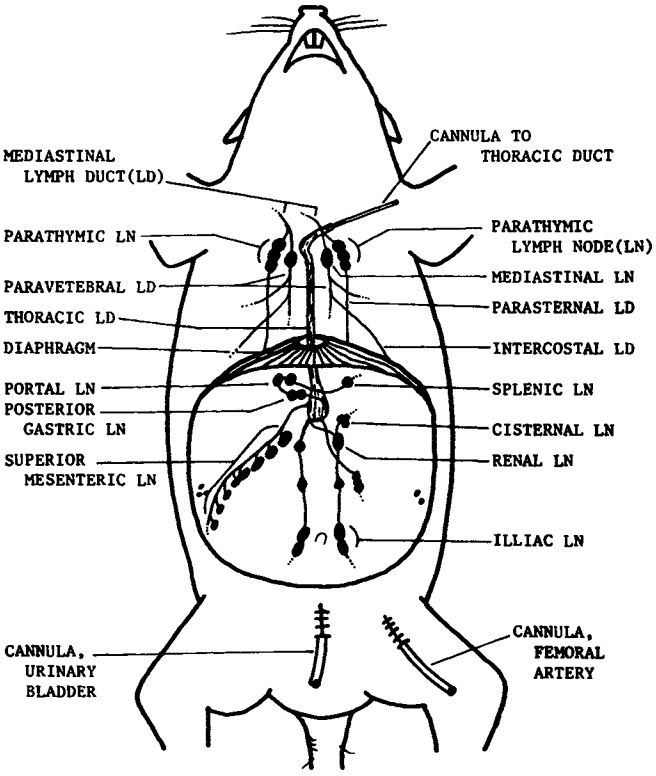

Fig. 3 Diagram of cannulated rat showing locations of different lymph nodes (LN) and lymph ducts (LD). Lymphatics drain cranially via the parasternal and paravertebral lymph ducts to the mediastinal and parathymic nodes, and caudally via the retroperitoneal lymphatics to renal and/or cisternal lymph nodes.

neutral composition IV and positive liposomes (III) were retained the least.

Discussion

Lymph Node Uptake

The dramatic range for lymph node levels may reflect differences in relative, fractional lymph flow from the peritoneal

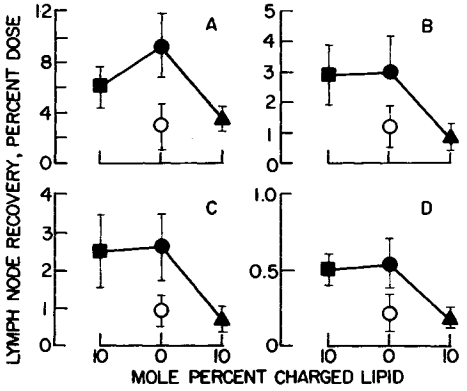

Fig. 4 Lymph node recovery, as per cent dose, is plotted as a function of the mole per cent charged lipid comprising the liposomes. Symbols are as defined in Fig. 2. A: Totals of group 1 plus group 2 nodes (see Table III) as per cent absorbed dose. B: Totals of Group 1 plus Group 2 nodes as per cent administered dose. C: Totals of Group 2 nodes as per cent administered dose. D: Totals of Group 1 nodes as per cent administered dose. Each value is the mean (± SE) for 3 to 5 rats.

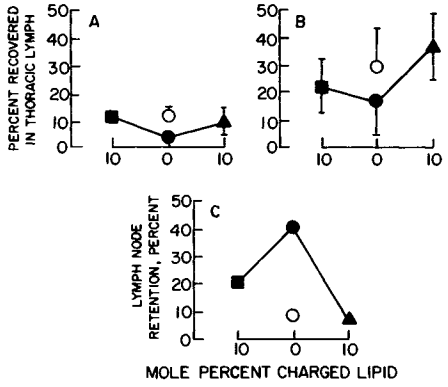

Fig. 5 A: Per cent of administered dose recovered in thoracic duct lymph after 5 h is shown as a function of the mole per cent charged lipid comprising the four liposomes. Symbols are as defined in Fig. 2. B: Per cent absorbed dose collected in thoracic lymph. C: Values for per cent lymph node retention are shown and were calculated as 100 N/(N + L), where N is the fraction of administered dose recovered from all sampled nodes after 5 h, and L is the total ^{14}C collected in thoracic lymph.

cavity or differences in a given node's ability to retard and clear liposomes. A common feature of all four compositions was that total uptake in Group 2 lymph nodes was 5-times higher than that for Group 1 lymph nodes, indicating the importance of lymph flow, especially the relative, fractional lymph flow draining the peritoneal cavity. The lymph flow from the peritoneal cavity associated with Group 2 nodes has been estimated to be 2- to 3-times larger than that associated with Group 1 lymph nodes (24).

Feasibility

Treatment of tumor metastases is a difficult problem. Because tumor metastases can travel from their site of origin to their new location via the lymphatics, the optimum therapeutic strategy may be to take advantage of a pharmaceutical system that can increase therapeutic availability by producing preferentially high lymphatic drug levels coupled with preferentially high drug levels around tumor metastases. The feasibility of such an approach has been demonstrated. For example, Osborne et al. (25, 26) injected a variety of ^{99m}Tc-labelled liposomes into the footpad of rats and examined the distribution of ^{99m}Tc by gamma-camera. They report that ^{99m}Tc became clearly associated with normal lymph nodes and lymph nodes involved in metastatic tumor spread. Kaledin et al. (13) and Khato et al. (14) showed an enhanced suppression of tumor metastases in regional lymph nodes by interstitial administration of liposome encapsulated chemotherapeutic agents. These studies, and those of Speyer et al. (3) provide further support for the feasibility of using liposomes as carriers for treatment of cancers confined to the lymphatics or to the peritoneal cavity.

Estimating Systemic Availability and Lymph Node Retention

When considering lymphatic absorption of liposome-encapsulated agents, there are three questions one would like answered. What fraction of the administered dose reaches the circulation, and what fraction is retained by lymph nodes? Finally, how do changes in liposome properties alter the kinetics and magnitude of these fractions? Direct quantitative answers are not possible for the experimental system used here, but estimates are possible, given several mechanistic assumptions.

Figure 6 is a plausible schematic of the fate of ^{14}C-sucrose in these studies. Any sucrose released from liposomes is quickly absorbed into blood and then rapidly cleared to urine (27). Much larger macromolecules, however, may be confined to the lymphatics. Encapsulated sucrose can pass freely from the peritoneal cavity (PC) into the lymphatics (L). Some of the absorbed liposomes will be retarded or trapped by nodes (N). Of those passing all nodes in their path, a fraction is collected in thoracic lymph (ThL). Liposomes following other lymph channels will reach blood (B), and either be destabilized or be transported to tissues (T) where they may be retained or taken up. Whenever an extracellular liposome becomes permeable to sucrose, the released sucrose will be excreted in urine (U). Intercellular sucrose, either free or remaining in liposomes after cellular uptake, was not expected to be recoverable in urine during the experiment.

Transport of ^{14}C through the system to collected lymph and blood by cells that previously had phagocytized liposomes is not included in this schematic, because experimental data indicated this was a negligible process. For other liposomes or experimental protocols this process may become quantitatively significant.

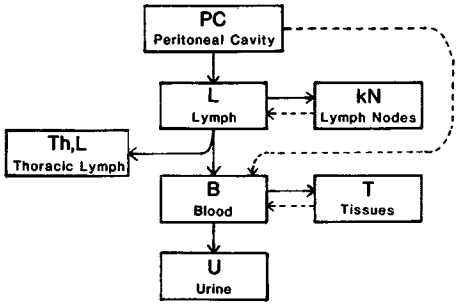

Fig. 6 Schematic diagram of drug transport from the peritoneal cavity as described in the text. The dotted line from PC to B indicates the primary route for absorption of free sucrose. Dotted lines from kN and T indicate that free sucrose can be released from liposomes in these locations. Transfers need not be apparent first order.

Equation (*1*) accounts for the location of the administered

$$\text{DOSE} = \text{PC} + \text{L} + \text{kN} + \text{ThL} + \text{T} + \text{B} + \text{U} \qquad \text{Eq. (1)}$$

dose at the end of each experiment. The amount of ^{14}C in all nodes is kN, whereas the amount in Group 1 plus Group 2 nodes is N. Because of the small volume of lymph in the system at any one time, the amount in uncollected lymph (L) is assumed to be small relative to either the amount in nodes, collected in thoracic lymph or remaining in the peritoneal cavity. The dose fraction that has been absorbed and has reached blood or tissues, although not measured directly, can be calculated from Eq. (*2*).

$$\text{B} + \text{T} = \text{Dose} - \text{PC} - \text{ThL} - \text{U} - \text{kN} \qquad \text{Eq. (2)}$$

The term B + T is a measure of the systemic availability of liposomes because it reflects the dose fraction passing through the lymphatics and reaching blood and other tissues. In general, several hours after dosing, blood levels of both liposomes and free sucrose will be small (Table III), and so the term B can be neglected, resulting in Eq. (*3*). The actual value of k is unknown, but

$$\text{T} = \text{DOSE-PC-ThL-U-kN} \qquad \text{Eq. (3)}$$

based on available literature (22, 23) is expected to be greater than one, but less than five. Values of T, as per cent dose are given in Fig. 7A, and are a measure of systemic availability of the liposomes (with cargo) following lymphatic absorption. Of the three phosphatidylcholine-containing liposomes (I, II, III) the uncharged liposome (I) was best. The difference between the two uncharged liposome compositions was more dramatic (IV > I). The trend suggested is one where inclusion of a charged lipid reduces systemic availability.

To assess the ability of liposomes to be retained (retarded) by lymph nodes one can define a lymph node retention index as the ratio, R, of the total content of all lymph nodes at some time, t, to the absorbed fraction of dose, F, that has actually passed the nodes as intact liposomes over the same time interval.

$$\text{R} = \text{kN/F} \qquad \text{Eq. (4)}$$

Neither all nodes nor F can be reliably measured. Our best estimate of R is the apparent lymph node retention index, R', given by Eq. (*5*). The fraction of dose

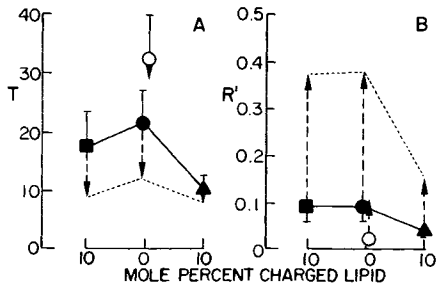

Fig. 7 A: Estimates of the per cent administered dose remaining in tissues, T, 5 h after intraperitoneal administration are plotted as a function of mole per cent charged lipid comprising the liposomes. Symbols are as defined in Fig. 2. Values of T were calculated using Eq. (*3*) with k=1, as described in the text. Vertical bars represent 1 SE for 3 to 5 rats. The arrows indicate an alternate value of T calculated from Eq. (*3*) when k=4. B: Values of the apparent lymph node clearance index, R', are shown and were calculated from Eq. (*5*) with k=q=1. Verticle bars are 1 SE for 3 to 5 rats. The arrows indicate alternate values of R' from Eq. 5 when k=4 and q=1.

$$R' = kN/(DOSE\text{-}PC\text{-}qU) \qquad \text{Eq. (5)}$$

retained by all lymph nodes can be assumed to be proportional to that amount retained by the measured nodes (Group 1 plus Group 2), when a sufficiently large sample is used. In Eq. (*5*), q is the fraction of drug release that occurred either in the peritoneal cavity or after liposomes reached the circulation. Subtracting the amount remaining in the peritoneal cavity after 5 h (PC) from the dose gives the total amount of sucrose absorbed. However, not all of this was absorbed in liposomes. Some leaked out and was excreted in urine. Undoubtedly a fraction of the ^{14}C recovered in urine was released from liposomes after beginning lymphatic absorption. However, a majority of release from liposomes generally occurs quickly following contact with biological fluids (28). Therefore, we can postulate that the urine value resulted primarily from ^{14}C released in the peritoneal cavity, i. e. q=1. So, substracting the 5 h values of both PC and U from dose gives our best estimate of F and is used in Eq. (*5*).

Values of R' are shown in Fig. 7B. Values for the three phosphatidylcholine-containing liposomes covered a 2.4-fold range with the negative (II) and neutral liposomes (I) having the highest values (R'=0.092 and 0.096, respectively for k=1). R' for composition I was 3.6-times R' for composition IV (0.026 for k=1). Because composition IV is best at reaching the circulation intact (Fig. 7A), it follows that it would be the composition that is retained least by lymph nodes.

The levels of ^{14}C from liposomes III and IV in node groups 1 and 2 were similar (Fig. 4), yet the positive liposomes, with a mean R' value of 0.039 (k=1), were retained by nodes fifty per cent better than composition IV. This apparent inconsistency may exist because compositions III and IV had different *in vivo* stabilities (Fig. 2D) and were absorbed from the peritoneal cavity at different rates (Fig. 2A). Lymph node levels alone, therefore, cannot be relied upon to give an accurate reflection of the relative lymph node retention of liposomes.

Lymphatic Variability

Among lymph nodes analyzed, cisternal, left mediastinal, renal, and parathymic lymph nodes showed some of the highest uptake values (Table III). The pattern of uptake among the various nodes was not always the same, e. g. compare results for renal and left mediastinal. The variance in node levels was also large.

Large variances and inconsistent trends for measured lymph node uptake are likely a reflection of differences in the lymphatic systems between animals combined with differences in their physiological status. Such variability may limit the number of situations where one can successfully use lymphatic drug carriers, especially when physiological factors are expected to play a major role in determining the fate of the carrier and its drug release rate.

Major Trends

Several potentially interesting trends have emerged, each deserving further investigation. Composition clearly impacts on the rate of absorption from the peritoneal cavity (Fig. 2A). In each situation there will be a maximum rate of absorption which is anatomically and physiologically determined and cannot be exceeded. However, there may be a therapeutic advantage to being able to systematically regulate and reduce the rate of absorption. The apparent half-life for absorption of positive liposomes (III) – the least absorbed — was 9.7 h (assuming a first order process), whereas that for negative liposomes (II) was 5.1 h. The retarded absorption of the positive liposomes may have been caused by interactions with negatively charged cell surfaces.

It is not clear if the actual passage of liposomes through the lymphatics was, in fact, altered by changes in composition. An unexpected observation was the apparent correlation between stability and lymph node retention. Can lymph node retention be increased further? What is the role of cellular uptake? In the absence of marker release, retardation of liposomes by nodes would be indistinguishable from cellular uptake in these studies. It remains to be determined if the observed release of sucrose from these liposomes primarily occurred either before lymphatic absorption while they were retained in nodes, or after reaching blood. Retarded liposomes that remain intact should eventually pass on to blood. More information is needed on the mechanism underlying the higher levels of lymph node retention.

One type of idealized lymphatic carrier would be completely retained extracellularly within nodes, where it would provide predictable sustained release. Compositions I and II came closest to this ideal. Another idealized carrier would provide a high degree of systemic availability with minimal lymph node retention. Neutral composition IV, one of the most stable, had the highest level of systemic availability.

Acknowledgements

The authors wish to thank Drs. R. M. Abra and F. C. Szoka, and other members of the Drug Delivery Research Group for helpful discussions, and Miss G. Kurtovich for preparing this manuscript. This work was supported by DHHS grant GM-24612.

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