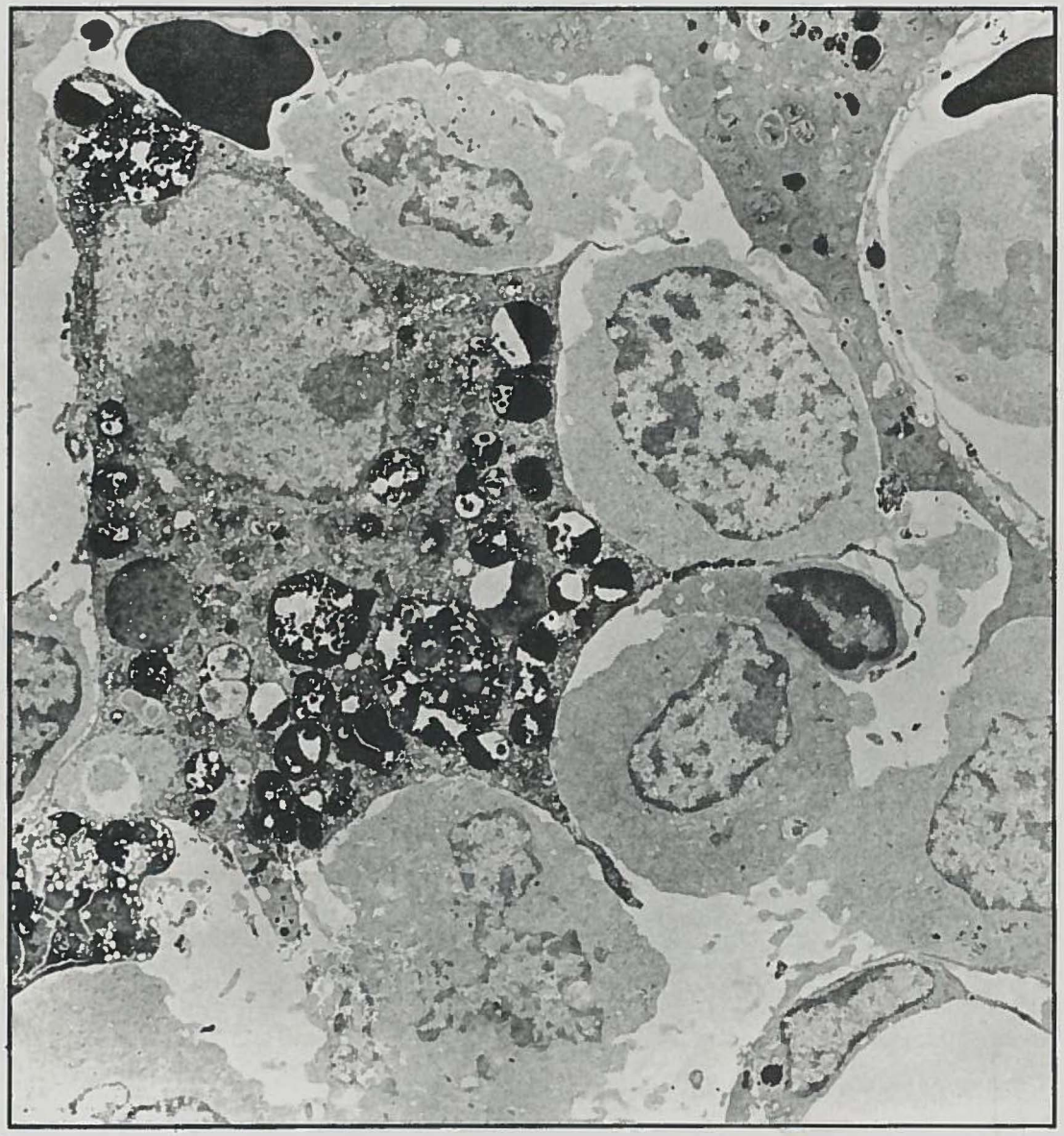
Liposomes as drug carriers in cancer chemotherapy

Ellens, Harmke

LIPOSOMES AS DRUG CARRIERS IN CANCER CHEMOTHERAPY



harma ellens

RIJKSUNIVERSITEIT TE GRONINGEN

LIPOSOMES AS DRUG CARRIERS IN CANCER CHEMOTHERAPY

PROEFSCHRIFT

ter verkrijging van het doctoraat in de
geneeskunde aan de Rijksuniversiteit
te Groningen op gezag van de
Rector Magnificus Dr. L.J. Engels
in het openbaar te verdedigen
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des namiddags te 4 uur

door

Harmke Ellens
geboren te Dokkum

PROMOTOR : Dr. G.L. Scherphof
REFERENT : Dr. A.W.T. Konings
COREFERENT: Prof.Dr. M.J. Hardonk

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S T E L L I N G E N

I

Davis *et al.* trekken ten onrechte uit hun resultaten de conclusie dat tetracyclines geen therapeutische waarde hebben in de chemotherapie van kanker.

Davis, R.C. et al. *(1977) Cancer Research 37, 4539-4545.*

II

Indien Abra en Hunt behalve multilamellaire vesicles tevens grote unilamellaire vesicles gebruikt hadden bij hun onderzoek naar de weefselverdeling van liposomen, zou dat de interpretatie van hun resultaten aanzienlijk vergemakkelijkt hebben.

Abra, R.M. and Hunt, C.A. (1981) Biochim. Biophys. Acta 669, 493-503.

III

Nicola *et al.* suggereren ten onrechte dat zij erythroid progenitor cells morfologisch gekarakteriseerd hebben.

Nicola, N.A. et al. *(1981) Blood 58, 376-385.*

IV

De mogelijke invloed van de kweektemperatuur op het phenotype van mutanten van *Neurospora crassa* is door Stade en Brambl over het hoofd gezien.

Stade, S. and Brambl, R. (1981) J. Biol. Chem. 256, 10235-10238.

V

Struck *et al.* houden bij de interpretatie van hun experimenten betreffende vesicle-cel fusie onvoldoende rekening met de mogelijkheid, dat onder hun incubatie condities vesicle-vesicle fusie zou kunnen optreden.

Struck, D.K. et al. *(1981) Biochemistry 20, 4093-4099.*

VI

De conclusie van Esparza *et al.*, dat tot de subeenheden van de mitochondriale ATPase een eiwit behoort met een molekuulgewicht van 10.000, berust op onvoldoende experimentele gegevens.

Esparza, M. et al. *(1981) FEBS Lett. 134, 63-66.*

VII

De wens als vader van de gedachte heeft een belangrijke rol gespeeld bij de interpretatie van de elektronenmicroscopische waarnemingen van Fraley *et al.*

Fraley, R. et al. *(1981) Biochemistry 20, 6978-6987.*

VIII

Volgens het nieuwe promotiereglement is de promvendus verplicht zijn/haar proefschrift uiterlijk 40 dagen voor de promotiedatum in te leveren. Menig promovendus zal zich daardoor genoodzaakt voelen vóór de promotie enige weken met vakantie te gaan.

Promotiereglement van de Rijksuniversiteit te Groningen, artikel 17, lid 5.

IX

Indien niet alleen bestemmingsplannen maar ook slechtplannen openbaar bekend gemaakt zouden worden, zou menig stadsbeeld behouden kunnen blijven als blijkt dat een slechtplan een slecht plan is.

X

IJzeren stellingen zijn de sterkste.

Stellingen behorende bij het proefschrift "Liposomes as drug carriers in cancer chemotherapy".

Harma Ellens 14 april 1982

V O O R W O O R D

Op deze plaats wil ik graag al diegenen bedanken, die mij gedurende de uitvoering van het in dit proefschrift beschreven onderzoek, wetenschappelijke of morele steun hebben gegeven. In het bijzonder geldt dit Henriëtte Morselt, die op een uiterst plezierige manier het grootste gedeelte van het experimentele werk voor haar rekening nam. Mijn promotor, Gerrit Scherphof, dank ik voor de prettige wijze waarop hij het onderzoek heeft begeleid, voor zijn bijdrage aan de totstandkoming van de tekst van dit proefschrift en, niet in het minst, voor het vertrouwen dat hij in mij gesteld heeft door mij de gelegenheid te geven een gedeelte van het onderzoek in het Roswell Park Memorial Institute in Buffalo uit te voeren. Chiel Hardonk en Ton Konings ben ik zeer erkentelijk voor de vlotte en zorgvuldige wijze waarop zij, als coreferent respektievelijk referent, de tekst van het proefschrift hebben doorgenomen en van kritisch kommentaar hebben voorzien. Ab Kroon en Jos van Renswoude dank ik voor hun aanmoediging, 4 jaar geleden, om met dit onderzoek te beginnen. Verder wil ik mijn waardering uitspreken voor de wijze waarop Bert Dontje en Jan Wybenga het vele biotechnische werk hebben verzorgd. Wim Trieling van het Laboratorium voor Radiopathologie ben ik erkentelijk voor zijn hulp bij een aantal van de experimenten. Ineke Molenaar van het Pathologisch Anatomisch Laboratorium wil ik bedanken voor de enthousiaste manier waarop zij, samen met Marijke van de Berg van de afdeling Haematologie, het karakteriseren van het lymfosarcoom op zich nam en voor haar aandeel in de formulering van het betreffende gedeelte van de tekst van dit proefschrift. De samenwerking met Caesar Hulstaert, Ruby Kalicharan en Dick Huizinga, van het Centrum voor Medische Electronenmicroscopie, in de laatste fase van het onderzoek heb ik als uiterst plezierig ervaren. I am indebted to Dr. Eric Mayhew and Dr. Joe Rustum (Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.) for their kind hospitality during my nine-months stay in their laboratory. I also want to mention Joe Bentz, who became a dear "buffalonian" friend. Karin van Wijk en Rinske Kuperus wil ik bedanken voor de vlotte en vakkundige wijze waarop ze de vormgeving van dit proefschrift hebben verzorgd. Ook dank ik Harm Schokkenbroek die de tekeningen maakte en Bert Tebbes die de fotografie van de tekeningen op zich nam.

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Voor mijn moeder

cover : the liposome
a true Trojan horse

cover design: Dick Huizinga,
Ruby Kalicharan.

C O N T E N T S

CHAPTER I

INTRODUCTION

Since cancer cells and normal cells are similar in many respects, cancer chemotherapeutic agents are often effective only at near toxic doses. The aim of all cancer chemotherapy therefore is to achieve selectivity against tumor cells, *i.e.* to deliver the cytostatic agent to the tumor cells and to protect normal cells from its effects. Selectivity in chemotherapy can be achieved in two different ways. First of all by the use of a drug that distributes throughout the body but that acts predominantly on the target cells, as is the case in the treatment of bacterial infections. With current knowledge this is not yet possible in cancer chemotherapy. Secondly, selectivity can be achieved by the use of a drug that acts on normal as well as on target cells, but that is preferentially taken up by the target cells.

Success or failure in chemotherapy is related to the pharmacological disposition of the drug. Successful therapy requires a sufficiently high drug concentration at the target site for a sufficiently long period of time. At drug-sensitive, non-target sites the presence of the drug may lead to undesired side effects. In cancer chemotherapy the doses used in treatment are often limited by toxicity of the drug to normal tissues. An intensive search for new antitumor drugs, along with an increased knowledge of tumor-cell growth kinetics have led to improvements in the therapeutic effectiveness of antitumor drugs (*e.g.* ref. 1).

The pharmacological behaviour of a drug can be influenced by the use of a drug-carrier. The *in vivo* fate and pharmacokinetics of a drug coupled to or entrapped in a carrier will be determined to an important extent by the characteristics of the carrier. An altered tissue distribution and a protection against excretion and/or untimely metabolic breakdown will conceivably result in an enhanced therapeutic index of the drug in the carrier complex over the free drug.

The capacity of liposomes to entrap lipid- and water-soluble substances led a number of investigators to promote the liposome as an attractive can-

didate for a drug delivery system (2-12, for reviews see 13-18 and books 19-23). The advantages of using liposomes as a drug carrier are, first of all, the fact that liposomes can be made of naturally occurring body-constituents and consequently are biodegradable. They are relatively non-immunogenic and almost without intrinsic toxicity. Moreover, liposomes are easily prepared and can encapsulate a wide variety of hydrophilic and hydrophobic compounds without the necessity of establishing a chemical bond between drug and carrier, so that the drug will be released from the carrier in the form in which it was originally encapsulated. In the following it will become clear that there also are disadvantages involved in the use of liposomes.

Since cells display their individuality on the cell surface, drug selectivity, at least in theory, can be achieved by using a drug-carrier that recognizes specific sites on the target cells. The idea of a "magic bullet" was first introduced by Paul Ehrlich, but only recently attempts have been made to put this concept into practice in cancer chemotherapy. Since liposomes can accomodate antibodies at their surface, they may be employed in a targetting approach. However many as yet unresolved problems, such as the production of tumor specific antibodies and the fact that most tumors are separated from the bloodstream by an endothelial barrier, await elucidation before this approach can be expected to be successful.

Liposomes

Liposomes or phospholipid vesicles are microscopic structures, consisting of one or more lipid bilayers enclosing a similar number of aqueous compartments. The lipid forms a permeability barrier for water-soluble (hydrophilic) compounds and it is therefore possible to entrap these compounds in the aqueous compartments. Hydrophobic compounds can be incorporated into the lipid bilayer. Multilamellar vesicles (MLV) are spontaneaously formed when phospholipids are dispersed in water (24,25). They are quite heterogeneous in size, with an average diameter in the order of several microns. Their size and be reduced by ultrasonic irradiation (sonication) (26,27) or extrusion (28). The size reduction produced by sonication will depend on the time and the power level of sonication and is difficult to standardize. Extrusion of MLV through polycarbonate membranes results in a reproducible size dis-

tribution of MLV and also produces vesicles of a relatively uniform size (28). Extensive sonication of MLV ultimately results in the formation of small unilamellar vesicles (SUV) consisting of one aqueous compartment surrounded by a single phospholipid bilayer. SUV preparations are relatively homogeneous in size with a minimum vesicle diameter of approximately 25 nm. Both MLV and SUV have a small aqueous volume per mol of lipid. A method of liposome preparation that produces liposomes with a large aqueous volume to lipid ratio and by which a high percentage of the aqueous material can be encapsulated was developed by Szoka and Papahadjopoulos: the reverse phase evaporation method (29). Reverse phase evaporation vesicles (REV) are formed when the organic solvent is evaporated from a suspension of lipid in a water in ether emulsion. The resulting vesicle preparation consists mainly of uni- or oligolamellar vesicles, with a large aqueous compartment. Extrusion of REV also results in the formation of vesicles relatively homogeneous in size (30). MLV, SUV and REV are the types of liposome most often used in studies on the *in vivo* fate of liposomes and on their application as a drug-carrier system.

Liposomes can be prepared from a variety of phospholipids. The incorporation of charged molecules like the amphipatic compounds stearylamine and dicetylphosphate or the natural phospholipids phosphatidylserine, phosphatidylglycerol and phosphatidic acid will increase the captured volume of MLV and SUV due to electrostatic repulsion forces. The phospholipids in the liposomal membrane can be arranged in a gel-phase (solid phase) or in a liquid-crystalline phase (fluid phase), dependent on the temperature and on the lipid composition of the membrane (for reviews see ref. 31,32). The gel phase represents a state in which the fatty acyl chains are tightly packed in an ordered structure, whereas in the liquid-crystalline phase the fatty acyl chains have a higher degree of disorder and a greater mobility. The temperature at which the liposomal membrane converts from one phase to the other is called the (gel-to-liquid-crystalline) phase transition temperature, which may vary from $-20^{o}C$ to $+60^{o}C$, depending on the phospholipid species. The phase behaviour of the liposomal bilayer changes when non-phospholipid molecules are incorporated in the membrane. Incorporation of increasing amounts of cholesterol causes a gradual disappearance of the phase transition and at

30 mol % it has disappeared completely (for a review on cholesterol effects see ref. 33). The presence of cholesterol in liposomal membranes has important consequences for their physical and chemical characteristics. Permeability for a variety of hydrophilic compounds is reduced (34,35) and also plasma-induced leakage of liposomal contents is minimized (for review see ref. 36). The liposomal membrane can accomodate molecules involved in biological recognition processes such as glycolipids (37-39), and antibodies (40-47) (for a recent review see ref. 48), thereby opening the possibility for targetting liposomes to specific cells or tissues.

Thus, liposomes characterized by size, charge, number of lamellae, encapsulation efficiency and stability can easily be prepared by varying the lipid composition and using different methods of liposome preparation. Liposomes of a defined size distribution can be prepared by extrusion of the vesicles through polycarbonate membranes. In order to make a rational use of liposomes as carriers in chemotherapy it is first of all necessary to understand the mechanisms involved in liposome-cell interactions and the processes governing the *in vivo* fate of liposomes and liposome-encapsulated drugs.

In vitro *interactions of liposomes with cells*

Liposomes can interact with cells in various ways. The most important mechanisms of liposome-cell interactions, schematically represented in Fig. 1 are: (1) exchange of lipid molecules between the liposomal membrane and the cell membrane (49,50), (2) adsorption of liposomes to the cell surface (51-54), (3) endocytosis (17,18), (4) fusion of the liposomal membrane with the cell membrane (51,55,56)(for a detailed description of these mechanisms see ref. 17,18). The exchange of lipid molecules may take place via specialized exchange proteins (57), and without cellular internalization of liposomal contents. Adsorption may be mediated by non-specific forces (electrostatic, hydrophobic) or by specific components of the vesicle or cell surface. Adsorption may result in leakage of vesicle contents into the external environment (54). The released material will either be taken up by the cell via the normal uptake mechanism, if available, or will be lost in the environment. After initial adsorption liposomes can be internalized via endocytosis. If the endocytic vacuole fuses with a lysosome it can be expected that the liposomes will be degraded by lipolytic enzymes (58) and that the

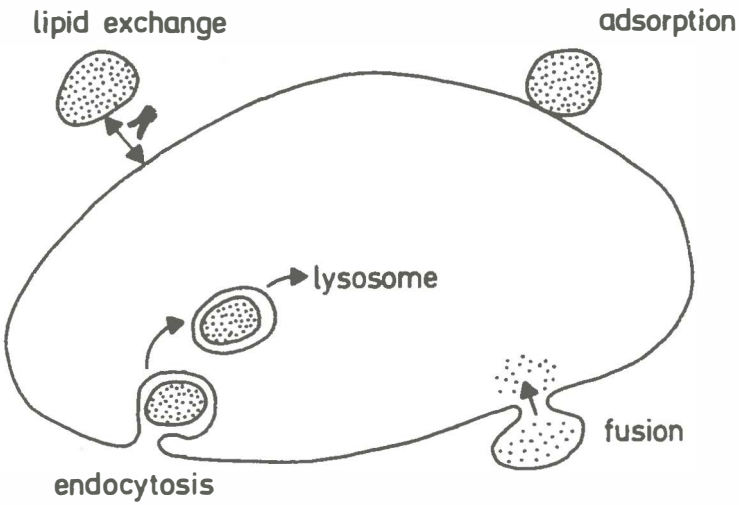


Fig. 1. Schematic representation of mechanisms involved in liposome-cell interaction.

liposomal contents will be released. Compounds which are stable in a lysosomal milieu may then act intralysosomally and compounds capable of crossing the lysosomal membrane may also act in other cell compartments. If the endocytic vacuole does not fuse with a lysosome, the liposomal contents may be slowly released into the cytoplasm. Fusion of liposomes with cells involves merging of the liposomal bilayer with the plasma-membrane bilayer, followed by a release of liposome contents directly into the cytoplasm (non leaky fusion) or in the external environment (leaky fusion). The different mechanisms of interaction are not mutually exclusive and it is very well possible that to some degree lipid exchange, adsorption, endocytosis and fusion all take place at the same time. The relative extent to which each of these mechanisms is active, will depend on the cell type, metabolic state of the cell and composition of the extracellular environment, along with liposomal lipid composition and liposome concentration.

Several investigators (17,59,60) have suggested that endocytosis of liposomes by, or fusion of liposomes with, tumor cells could be a way to overcome resistance to certain drugs due to deficiency in uptake. This concept has been an important stimulus for research into the potential applications of liposomes as drug carriers in chancer chemotherapy. However, phagocytosis is largely restricted to cells like monocytes and macrophages of the reticuloendothelial system that perform phagocytosis professionally. Only some types of tumor cells have phagocytic potential. Furthermore, with the development of more reliable methods to follow the fate of liposomes, evidence has accumulated that fusion does not play a quantitatively important role in the interactions between liposomes and cells (61,62). The possibility of adsorption is not likely to depend strongly on cell type, although some cells may adsorb liposomes more efficiently than others. It must be noted that even if the liposomal contents are not taken up directly into cells, such as when stable adsorption or leaky fusion take place, it is conceivable that, compared to the free drug, a higher amount of the entrapped material is taken up by the cell due to the generation of locally high concentrations at the cell surface.

The binding of liposomes to cells can be enhanced by the incorporation of recognition molecules in the liposomal membrane. This approach has involved adsorption or covalent binding of antibodies to the liposome (40-47) and lectin mediated binding of liposomes to cells (37-39). For instance, Leserman *et al.* (43) showed that liposomes bearing covalently coupled mouse monoclonal antibody against human β_2 microglobulin bound specifically to human, but not to mouse cells. Weissmann and coworkers (40-42) demonstrated that non-specific binding of heat-aggregated IgG resulted in an enhanced uptake of liposomes, via a binding to the F_c receptors of human polymorphonuclear cells. Szoka *et al.* (37) attached vesicles containing the glycolipids lactosylcerebroside or tetradecylmaltobionamide to BC-9 human fibroblasts, NIL 8 M_2 hamster cells or L929 mouse cells with the lectins Ricinis communis agglutinin I and concanavalin A respectively. If the cells are not professionally phagocytosing, however, increased attachment is not accompanied by a concomitant transfer of liposome contents into the cells (63).

In vivo *fate of liposomes*

After *in vivo* administration liposomes are subject to interaction with a variety of proteins and cells. Liposomes are able to bind plasma proteins such as α- and β-globulins (64,65), immunoglobulins (65,66), lipoproteins (67-70), albumin (65-71) and also some components of the clotting system (72). Interaction with plasma proteins may increase the permeability of the liposomal bilayer or, following interaction with HDL, may even result in complete destruction of the liposome (65,73). The extent of damage is largely dependent on the type of liposome [number of lamellae, curvature of the bilayer (65)]. When antigenic structures [*e.g.* glycolipids (76)] are present at the liposome surface, liposomes are susceptible to complement damage (77). In most *in vivo* studies so far the liposomes were constituted of a lipid mixture with very low antigenicity : phosphatidylcholine, cholesterol and small amounts of charged lipids.

Relatively little is known about the interactions between liposomes and blood cells. It would seem likely that circulating macrophages will take up a fraction of an injected dose of liposomes. However, since these cells constitute only a small proportion of the total number of blood cells, such uptake is not expected to be quantatively important. Little evidence is available until now of a substantial interaction between liposomes and red blood cells. Liposomes presumably cannot pass the endothelial lining of the blood vessels except in the liver where they can come in contact with parenchymal cells, thanks to the presence of numerous relatively large fenestrations in the endothelial sieve plates (78). After intravenous injection liposomes are gradually cleared from the blood. The clearance rate is dependent on size and lipid composition of the liposomes. Large liposomes are cleared more rapidly than small liposomes (79,80) and, possibly due to differences in adsorbed protein, negatively charged liposomes are cleared more quickly than neutral or positively charged vesicles (79). The rate of liposome clearance is dose dependent (81-84). At present only limited information is available about the relevance of other parameters such as total number of vesicles and total surface area (84), that may be greatly different for different liposome-types and liposome-sizes despite similarity in lipid doses (85).

Liposomes become associated with cells of the reticuloendothelial system, which are found in high concentrations in liver and spleen (for diagrams of blood circulation through liver and spleen see Fig. 2). The distribution between the cell types in the liver has long been a matter of debate and is still not entirely clear. Different methods of vesicle preparation between different laboratories and the use of inadequate markers of liposomal lipid and liposomal contents, along with the use of vesicle preparations of undefined size-frequency distributions have resulted in conflicting reports on this subject. Recently Rahman *et al.* (86) presented *indirect* evidence that small unilamellar liposomes (diameter less than 70 nm) preferentially associate with hepatocytes, while large liposomes are mainly taken up by Kupffer cells. Roerdink (87) *et al.* presented *direct* evidence that large liposomes were taken up preferentially by Kupffer cells, while only a small proportion was associated with hepatocytes and virtually none with endothelial cells. Recent experiments from our laboratory directly showed uptake of lipid and entrapped solute of SUV in hepatocytes and low uptake in non-parenchymal cells (88).

Intraperitoneally injected cholesterol-rich liposomes appear in the blood in intact form and are subsequently distributed throughout the body in a manner qualitatively similar to the distribution after intravenous injection. Transport from the peritoneal cavity to the blood presumably takes place via thoracic and right lymphatic ducts (89,90). Parker *et al.* have shown that intraperitoneal administration of liposome entrapped adriamycin results in an enhanced uptake in renal and thoracic lymph nodes (89).

After interstitial injection the general tendency is for large liposomes to be retained and desintegrated at the site of injection and for small liposomes to accumulate in regional lymph nodes and to be transported to the blood (91-93). Segal *et al.* suggested that size precluded unsonicated multilamellar vesicles from entering the lymphatics (94). Examples of the administration of liposomes to a particular organ or tissue include the injection of methotrexate containing liposomes into the brain (95), the injection of hydrocortisol containing liposomes into the joints of experimental arthritic rabbits (96) and the administration of Ara-C containing liposomes, as a liposome aerosol via the respiratory system, to the lungs (96). In all these

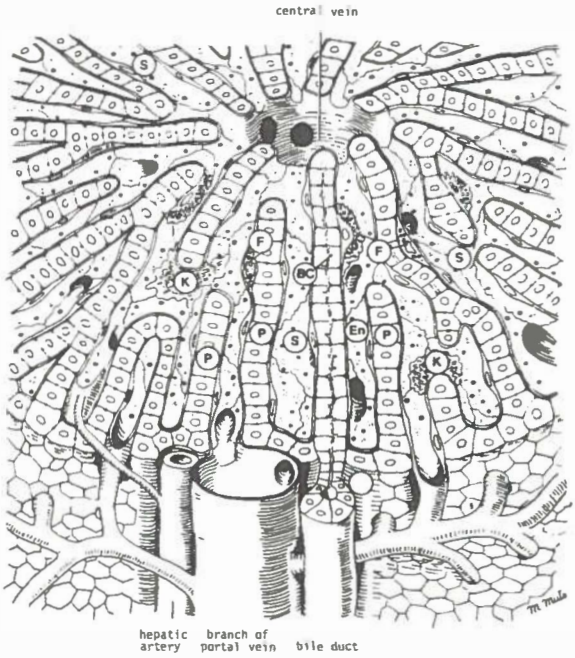


Fig. 2.A. Circulation of blood through the liver. Blood from portal vein and hepatic artery enters sinusoids where it flows towards the central vein. Kupffer cells are located in the sinusoidal lumen. BC = bile canaliculus; P = parenchymal cell; K = Kupffer cell; S = sinusoid; F = fat storing cell; En = sinusoidal endothelial cell.

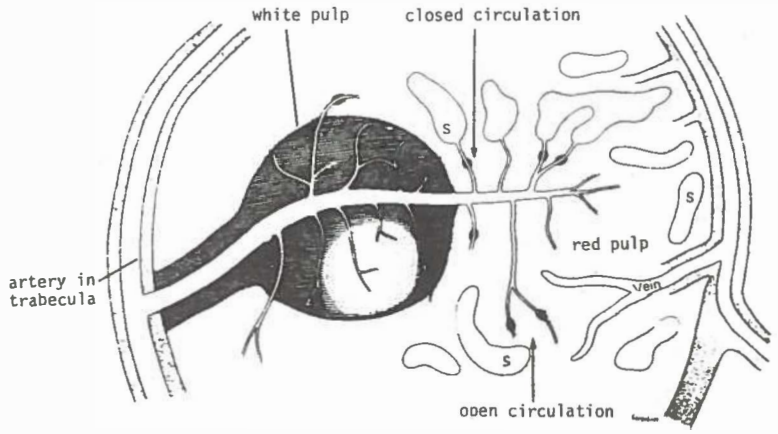


Fig. 2.B. Circulation of blood through the spleen. The central artery, surrounded by a lymphocyte sheath, gives off branches to the follicles of the white pulp, which consist of dense accumulations of lymphocytes, and continues on to enter the red pulp. Macrophages often protrude into the sinusoidal lumen between endothelial cells. Sinusoids empty into veins of the red pulp. S = sinusoid (Functional Histology, L.C. Junqueira and J. Carneiro; Ned. bew. J. James and C.J.H. van den Broek, (1981), Wet. Uitg. Bunge, Utrecht).

studies, a higher concentration and a prolonged availability (of the encapsulated drug versus the free drug) at the site of administration was demonstrated.

Conflicting results have been reported on the fate of orally administered liposomes (90,98-100). Several investigators have claimed that orally administered liposome-encapsulated insulin causes a decrease in blood glucose levels of diabetic rats (98-100). Hemker *et al.* (101) administered liposome-encapsulated clotting factor VIII to a patient with Haemophilia A and their results suggest that the efficacy of orally administered liposome-encapsulated factor VIII may by as high as the same dose when given intravenously. However from these studies it is not clear whether insulin or factor VIII were adsorbed from the intestinal contents while still encapsulated. It is difficult to envisage how orally administered liposomes could escape the action of pancreatic phospholipases or bile salts. As a possible explanation of the results with orally administered liposomes, Patel and Ryman (100) suggested that liposome-encapsulation protected the entrapped proteins from attack by digestive enzymes during passage through the stomach. The surviving liposomes would then release their contents in the ileum, where they are adsorbed as the free substances. An alternative possibility is that insulin and especially a hydrophobic protein as factor VIII are absorbed as a lipoprotein complex (102).

Thus it is possible to influence the *in vivo* fate of liposomes to a certain degree by varying size, charge, lipid composition and route of administration. The involvement of the reticuloendothelial system in the blood clearance of liposomes has been recognized for some time, but still relatively little is known about the details of the interactions between liposomes and the RES. The rate of disappearance of liposomes from the blood decreases with increasing dose (81-84), indicating that the clearance mechanism is saturable. In this respect it resembles the reticuloendothelial clearance of other particles or cells such as sheep red blood cells (SRBC). Therefore several investigators have suggested saturation of the hepatic uptake mechanism with a high dose or with a preload of liposomes as a way to reduce the blood clearance rate of liposomes (81,82,103). In two recent studies (82,83) it was reported that, compared to the effect of reticuloendothelial

blockade on hepatic uptake and clearance of SRBC, the effect of blockade on clearance and hepatic uptake of liposomes was small. Souhami *et al.* (83) suggested that in contrast to SRBC only part of the hepatic liposome uptake involved Kupffer cell phagocytosis.

A few attempts have been made to target liposomes to specific tissues *in vivo*, using immunoglobulins (104), glycoproteins and glycolipids (105, 106). Gregoriadis *et al.* incorporated rabbit IgG against the Meth A tumor in ^{111}In-Bleomycin containing liposomes. A tissue distribution study revealed that the accumulation in tumor, liver and spleen were enhanced to the same extent. This was explained by the possible presence of antibodies to mouse liver and spleen antigens in the immunoglobulin preparation. Several investigators have successfully employed molecules carrying terminal galactose residues, to target liposomes to the liver. Involvement of the galactose receptor was demonstrated in these studies (105,106). Mauk *et al.* (107,108) and Wu *et al.* (109) reported that incorporation of the 6-aminomannose derivative of cholesterol into the lipid bilayer of the liposomes resulted in an altered clearance and tissue distribution after intravenous injection and, after subcutaneous injection, in a very effective retention at the injection site and a greatly increased liposomal stability.

Fate of the liposome-encapsulated drug

The pharmacological behaviour of a liposome-encapsulated drug is determined by the behaviour of the liposome. In general the circulation time of encapsulated drugs is prolonged and a larger proportion is taken up by liver and spleen. Encapsulated drugs are protected from metabolic breakdown and excretion. In all probability, the rate at which the drug is released from the vesicles is the slow step regulating the *in vivo* metabolism and pharmacokinetics. It is only after the drug is released that it becomes available for interaction with cells. The rate at which an encapsulated drug is released will depend on the type of drug (110), the liposomal lipid composition, the type of liposome (SUV, REV, MLV) and the localization of the liposome *e.g.* as extracellular or intracellular depot. For any tumor, the successful use of liposome encapsulated drugs will greatly depend on a careful choice of therapeutic conditions as the route of liposome administration and the type of antitumor agent.

Liposome applications

Antitumor studies with liposomes have involved various drugs. Arabinofuranosylcytosine (Ara-C), (111-116) methotrexate (117-122) and actinomycin-D (118,123-125) have received most of the attention so far, but also adriamycin (162-128), daunomycin (129), cisdiaminedichloroplatinum (130) and other drugs have been employed. Most of the positive results relate to Ara-C. A single dose of liposome-encapsulated Ara-C was successfully used in the treatment of L1210 bearing mice. The observed beneficial effect of entrapment was attributed to sustained release and not to a specific delivery of the drug to the tumor cells (131). As was outlined in the previous sections, liposomes may be able to directly deliver their contents into tumor cells by fusion or endocytosis and/or may act as a depot for slow release. Since most types of tumor cells do not display phagocytic capacity, sustained release can be expected to be an important determinant of the liposomal carrier system in cancer chemotherapy. In some instances encapsulation enhances the antitumor activity whereas in others it does not, or even decreases the effect. This must be attributed to differences in tissue distribution, toxicity, pharmacokinetics and tumor sensitivity in different systems.

In an attempt to increase the uptake of encapsulated methotrexate by the tumor, liposomes with a phase transition temperature slightly higher than body temperature have been used in combination with local hyperthermia (119-122). The heating of the tumor resulted in a preferential release of the drug in the target area and in an increased antitumor effect.

One problem inherent to the use of liposomes as carriers for antitumor agents is the fact that intravenously injected liposomes do not pass the endothelial lining of the vascular system, while most tumor cells are located behind this barrier. However, when tumor cells spread throughout the body by lymphatic pathways and metastasize in lymph nodes, a direct contact between tumor cells and liposomes could be established if the liposomes were administered to afferent lymph vessels. This approach was used by Kaledin *et al.* who found that the frequency and growth rate of metastases in regional lymph nodes were decreased more efficiently with liposome-encapsulated cisdiaminedichloroplatinum (II) and hydrocortison than with the free drugs (130).

Chemotherapy of cancer is probably the only application of liposomes as a drug carrier system that largely depends on the sustained release mechanism. Most other medical applications are based on the ability of macrophages to phagocytose liposomes. Thus liposome-entrapped macrophage activating factor (MAF, a lymphokine) was demonstrated to render mouse macrophages cytotoxic to tumor cells *in vitro* (132-134) at a much lower dose than that required for free MAF. In addition, liposomes with entrapped MAF were therapeutically effective against spontaneous lung metastases of mouse melanoma, after excision of the primary tumor, indicating that liposome-entrapped MAF can be used to stimulate the host-mediated tumoricidal response (135,136).

Other applications of liposomes in medicine include their use as immunological adjuvants (137), their use in metal storage diseases (6,86), in antimicrobial therapy (7,11), and in enzymereplacement therapy (3). For a variety of antigens it has been shown that the immunological response is enhanced when the antigen is given in liposome-associated form. Van Rooyen *et al.* showed that the immune response is caused by antigens adsorbed at the liposome surface (71). The immunoadjuvant activity may present a serious problem when antibody-coated liposomes are to be used for targetting of liposomes to tumor cells *in vivo*. In several metal storage diseases metals accumulate in the lysosomes of cells. Liposomes have been used to introduce metal chelating agents into cells. For instance liposome-encapsulated desferrioxamine was directed to Kupffer cells or hepatocytes (dependent on the diameter of the liposomes) and was claimed to remove iron from intracellular sites more efficiently than the free drug (86). Alving *et al.* (7) made use of the tendency of liposomes to accumulate in Kupffer cells, for the treatment (with antimonial compounds) of experimentally induced leishmaniasis, a disease caused by a parasite that invades Kupffer cells. The objective of enzyme replacement therapy in lysosomal storage diseases is to introduce biologically active enzyme molecules into the lysosomes of cells deficient in this enzyme. It was argued that liposome-encapsulation might protect the enzyme from degradation in the circulation and might prevent the development of an immune response against the entrapped enzyme. This approach can only be expected to be useful in those storage diseases that involve macrophages of the reticuloendothelial system. Since a small fraction of the liposome-asso-

ciated enzyme is likely to be adsorbed to the liposomal surface, a problem is presented by the fact that liposomes were found to act as adjuvants in the immune reaction to adsorbed antigens.

Until now liposomes have only been used in a few clinical studies, mainly concerning patients with Gaucher's disease, but are about to be tested on a much larger scale in the treatment of leishmaniasis.

Characteristics and dissemination pattern of the murine lymphosarcoma used in this study

The lymphosarcoma which originated spontaneously in a C57 Bl mouse was maintained by weekly passage of spleen suspensions in C57 Bl or in (C57 Bl x Gr)F_1 mice, for a period of more than 20 years (138). The tumor as used in the present study was characterized morphologically, immunologically and enzyme-histochemically. A preliminary report has been published (139).

Intraperitoneal injection of 10^6 tumor cells resulted in a diffuse, massive enlargement of the spleen and, to a lesser extent, of the liver (Fig. 3). After seven days the splenic tissue appeared to be replaced entirely by large immunoblast-like cells, showing many mitosis. These tumor cells are characterized by centrally located irregular round nuclei. On the surface and in the cytoplasm of virtually every cell immunoglobulins appeared to be present (indirect immunofluorescence and immunoperoxidase techniques), consisting of kappa light chains and IgG and to a lesser exent IgM heavy chains. Acid phosphatase and non-specific esterase reactions on frozen sections showed many positive cells throughout the lymphosarcomatous spleen, presumably representing macrophages. Acid phosphatase reaction on frozen sections of the liver showed positive Kupffer cells, and no staining of the tumor cells. In frozen sections erythrocyte-antibody-complement rosettes were formed over some tumor cells, while no erythrocyte-antibody rosettes were formed, suggesting a limited number of complement receptors and the absence of F_c receptors.

Pattern of tumor distribution after intraperitoneal or intravenous injection

Macroscopically no peritoneal tumor growth or ascites production was observed at any day after intraperitoneal injection of 10^6 tumor cells. Microscopic examination of peritoneal fat showed an increasing number of tumor

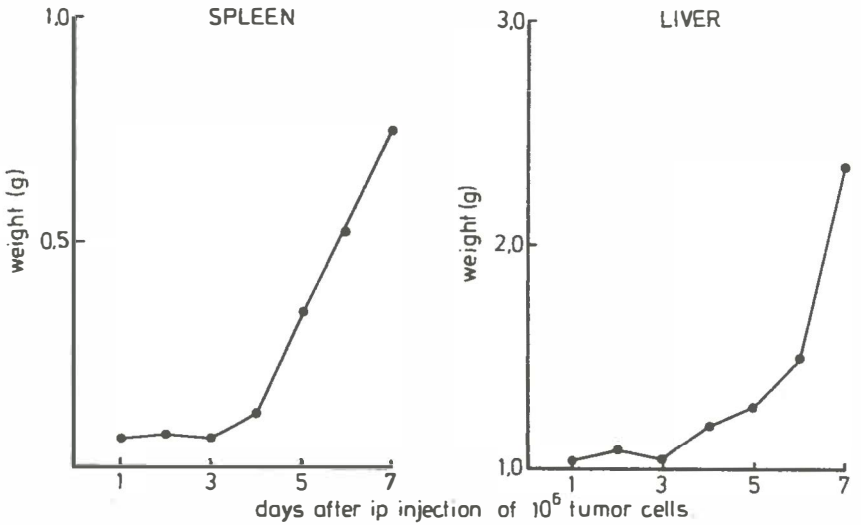
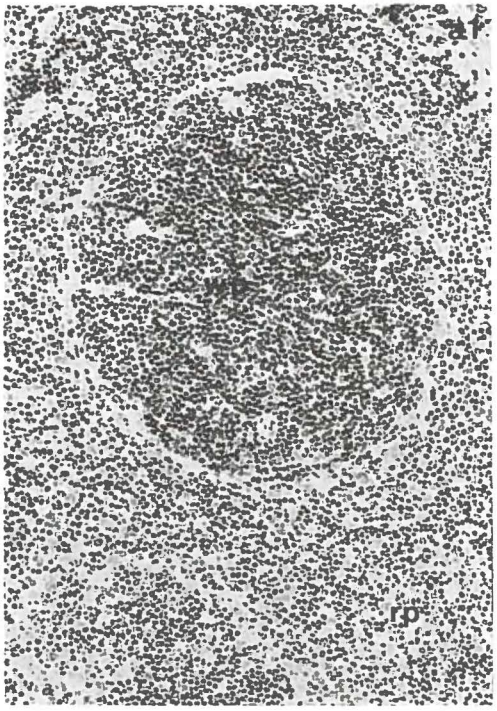


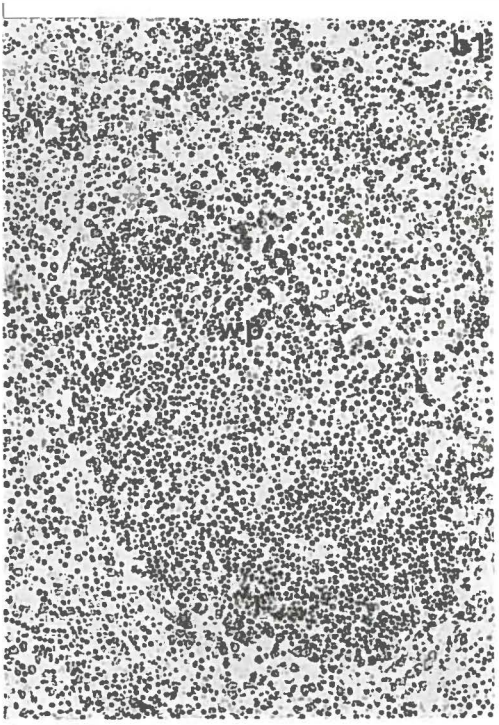
Fig. 3. Increase in weight of spleen and liver after intraperitoneal injection of 10^6 lymphosarcoma cells.

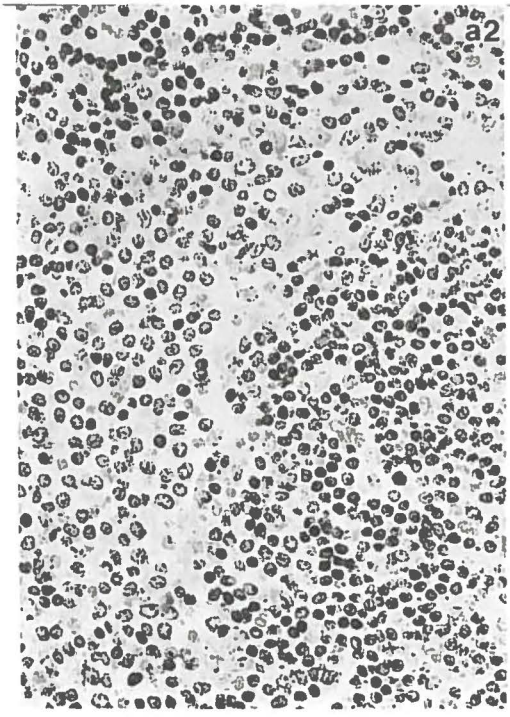
cells from the first day on. Direct infiltration from perisplenic fat into the spleen was not observed. During tumor development the spleens showed no gross changes apart from an increase in size (Fig. 3) and fragility. No tumor cells could be detected in the spleen by microscopic observation one or two days after injection. At the third day tumor cells were detected in the marginal zone of the follicles of the white pulp. In the following days tumor cells concentrically invaded the white pulp, obliterating the red pulp, leaving only follicular remnants at day five. At day six the spleen consisted mainly of tumor cells showing a somewhat nodular pattern and after seven days a diffuse tumor proliferation was found. In Fig. 4 sections of the spleen are shown one and three days after intravenous injection of 10^6 cells. A rim of tumor cells is clearly visible in the marginal zone of the follicles. The distribution of tumor cells in the spleen after intraperitoneal injection is identical to the pattern after intravenous injection (see below).

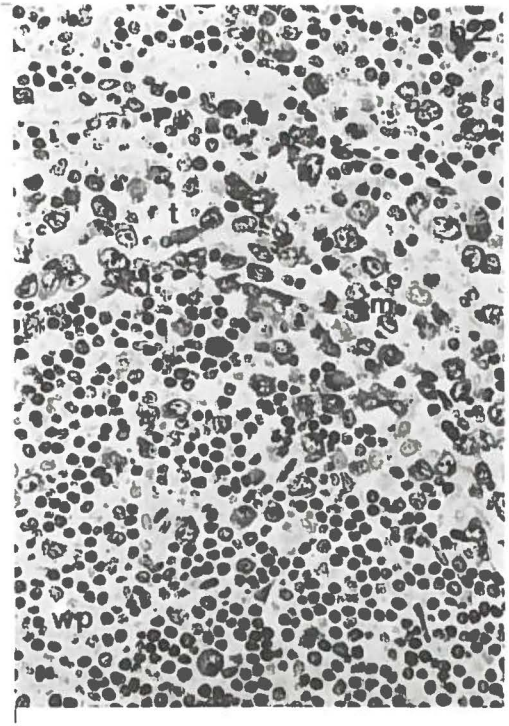
The livers started to increase in weight from the fourth day on (Fig. 3). The tumor cells in the liver were located predominantly in the sinusoids,

Fig. 4. Sections of spleen, 1 day (A1 and A2) and 3 days (B1 and B2) after intravenous injection of 10^6 tumor cells. Giemsa stain. A1 and B1 140x, A2 and B2 350x. WP, Follicle of the white pulp; RP, red pulp; T, rim of tumor cells; M, mitosis. (Courtesy of Dr. W.M. Molenaar).


a1
wp
rp


b1
wp


a2


b2
t
m
wp

but also in branches of hepatic and portal veins. In lymph nodes, which were regularly seen upon examination of the organs (*e.g.* kidney, pancreas, lungs), tumor cells were present from the third day on. Tumor cells in the lymph nodes were distributed initially in the sinusoids while ultimately they almost entirely replaced preexistent tissue. Bone marrow, lungs and kidneys showed tumor infiltration from the fourth day onwards. The tumor cells were present in small vessels, progressively infiltrating the interstitial space in later stages of tumor growth. In heart and thymus tumor cells were present in surrounding fat and some within these organ at the fifth through seventh day after injection. In the brain tumor involvement remained restricted to the meninges. In the blood increasing numbers of tumor cells were observed from the fourth day on. Tumor bearing mice invariably die eight or nine days after intraperitoneal inoculation of 10^6 tumor cells.

After intravenous injection of 10^6 tumor cells the macroscopic findings (hepatic and splenic enlargement) were identical to those after intraperitoneal injection. Tumor growth in peritoneal fat appeared to be limited on microscopic examination and did not occur before the fifth day after injection. The pattern of tumor distribution was similar to that after intraperitoneal injection, but the various stages of tumor development were found one to two days earlier.

These results characterize the tumor as a high-grade malignant B-lymphosarcoma. This tumor model has been used in several other studies (140-142).

Scope of the thesis

In this study various aspects of liposomes as drug carriers in cancer chemotherapy were investigated. From work of other investigators it became increasingly clear, during earlier stages of this project, that effects of liposome-encapsulated cytotoxic drugs were mainly due to sustained release of the cytotoxic agent and not to a specific interaction of the drug with the tumor cells. Therefore, in the present study, much attention was given to this sustained release aspect. In particular we investigated:

(1) the effect of the route of administration (intraperitoneal versus intravenous) and dose of liposomal lipid on the rate of elimination of liposomes from the blood and on the uptake liposomes by liver and spleen (Chapter II and III).

(2) the effect of liposomes on the activity of the reticuloendothelial system (Chapter IV).
(3) the possibility of local sustained release of liposome-encapsulated Ara-C from liposomes associated with liver and spleen (Chapter V).
(4) the effect of liposome-encapsulation on the therapeutic activity of Adriamycin and Ara-C (Chapter VI).
(5) the capacity of lymphosarcoma cells to take up liposomes *in vivo* (Chapter VI).

The experiments in Chapters II, III and IV were undertaken in relation to the use of liposomes as a circulating depot for sustained release. The experiments in Chapters V and VI were undertaken to investigate the occurrence and usefulness of local sustained release of encapsulated Ara-C and Adriamycin in liver and spleen.

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CHAPTER II

IN VIVO FATE OF LARGE UNILAMELLAR SPHINGOMYELIN-CHOLESTEROL LIPOSOMES AFTER INTRAPERITONEAL AND INTRAVENOUS INJECTION INTO RATS

SUMMARY

We investigated the fate of intraperitoneally and intravenously injected reverse phase evaporation vesicles of fairly uniform size (100-200 nm) with respect to blood clearance, tissue distribution and integrity *in vivo*. The vesicles are composed of sphingomyelin and cholesterol in a molar ratio 3 : 2 and contain ^{125}I-labeled poly(vinyl pyrrolidone) in the aqueous compartment. It is shown that following an intraperitoneal injection the vesicles are transported intact, and not associated with cells, from the peritoneal cavity to the blood and are subsequently taken up mainly by liver and spleen, where, particularly in liver, the phospholipid is partially metabolized. After an intraperitoneal injection the rate of vesicle-uptake by liver and spleen is reduced by a factor of 2-3 compared to the rate of vesicle-uptake by liver and spleen following an intravenous injection. The peritoneal cavity functions as a reservoir of vesicles for some hours.

INTRODUCTION

Understanding and control of the pharmacokinetic properties of cytostatic agents is an important aspect of the rational design of cancer chemotherapy. Encapsulation of cytostatic agents in the aqueous compartment of liposomes modifies the pharmacokinetic properties of these drugs. Liposome-encapsulation has been shown to change the tissue distribution (1,2) and to increase the pharmacological efficacy of various encapsulated agents (3-8). Among the various ways in which liposomes can conceivably be applied they offer the possibility of being used as carriers gradually releasing an entrapped cytostatic agent. In this way one avoids initially high, and potentially toxic, peak concentrations in the plasma and at the same time a sustained availability of the drug in therapeutically effective concentration ranges is generated. Before liposomes can be used as vehicles for such sustained release, a

number of criteria must be met: once administered the liposomes must not be cleared from the body too rapidly by cells of the monocytic phagocytosing system localized mainly in liver and spleen and they must possess a certain degree of stability in a physiological environment. We investigated the usefulness, for this purpose, of large unilamellar vesicles prepared by the reverse-phase evaporation method (9). Such vesicles are known to entrap water-soluble substances with high efficiency. To obtain a high degree of stability we composed the vesicles of sphingomyelin and cholesterol. This lipid combination was expected to produce vesicles which are highly resistent to plasma HDL, because sphingomyelin interacts with cholesterol even more strongly than phosphatidylcholine (10). Furthermore, incorporation of 40 mol % cholesterol would greatly reduce spontaneous diffusion of entrapped material from the vesicles (11,12). ^{125}I-labeled poly(vinyl pyrrolidone) was chosen as a marker of the aqueous compartment of the vesicles because it is metabolically inert, in a free form it is taken up by liver and spleen only to a minor extent and once taken up it is removed from these tissues very slowly (13). Thus, ^{125}I-label found associated with liver and spleen, following injection in liposome-entrapped form, can reliably be taken to reflect uptake of whole vesicles. Blood clearance of intravenously injected vesicles is dependent on vesicle size: large vesicles are cleared more rapidly than small vesicles (14,15). Therefore, we decided to use vesicles of intermediate size, prepared by extrusion through a polycarbonate filter (16), thus combining the advantage of a large internal aqueous volume with a relatively slow blood clearance. In addition, we compared the intravenous and intraperitoneal routes of vesicle - administration in order to see how clearance and tissue uptake might be influenced by varying the way of administration.

MATERIALS AND METHODS

Materials

Sphingomyelin (from bovine brain), cholesterol and fluorescein isothiocyanate-labeled Dextran (average molecular weight 67 600; 0.007 mol fluorophore/mol glucose residue) were obtained from Sigma. Sphingomyelin was labeled in the choline-group by transfer of a methyl group from [^{14}C]methyl-iodide to demethylated sphingomyelin, essentially as was described for label-

ing of phosphatidylcholine by Stoffel (17). The lipids were chromatographically pure, as was demonstrated by TLC on silica gel with $CHCl_3/CH_3OH/NH_3/H_2O$ (65 : 35 : 4 : 1, v/v) as an eluant. ^{125}I-labeled poly(vinyl pyrrolidone) (spec. act. 46.3 μCi/mg; average molecular weight 35 000) and [1-^{14}C]cholesteryl oleate (34 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, U.K. Unipore polycarbonate filters were from Biorad. Ficoll® (molecular weight 70 000) and Sepharose CL-2B were purchased from Pharmacia. Soluene was obtained from Packard. All organic solvents used were reagent grade.

Methods

Vesicle preparation. Reverse-phase evaporation vesicles were prepared essentially as described by Szoka and Papahadjopoulos (9). A chloroform solution of 30 μmol sphingomyelin and 20 μmol cholesterol was evaporated to dryness in a round-bottom flask on a rotary evaporator. When lipid-labeled vesicles were prepared approx. 15.10^6 dpm [^{14}C]sphingomyelin were added to the lipid mixture. The dry lipid film was dissolved in 6 ml of a 1 : 1 mixture of chloroform/diethyl ether and 1 ml of the aqueous phase (either Tris-buffered saline with 0.5 mg unlabeled poly(vinyl pyrrolidone), or a succinate-buffered solution, containing approx. 25 μCi ^{125}I-labeled polymer was added. This mixture was sonicated under a N_2 atmosphere in a bath type sonicator (Branson) until a homogeneous suspension of inverted micelles was obtained. The organic phase was then removed at 30°C in a rotary evaporator. The aqueous suspension of vesicles thus obtained was extruded through a Unipore polycarbonate filter with a pore size of 0.4 μm (16). Non-encapsulated poly-(vinyl pyrrolidone) was removed by gel chromatography on Sepharose CL-2B. The void volume fractions were pooled and used in further experiments. Vesicle-lipid concentrations were determined by lipid phosphorus assay (18). The unilamellar character of the vesicles as well as the lipid composition may contribute to the discrepancy between pore size and vesicle size. Such discrepancy was not observed by Olson *et al.* (16) using multilamellar vesicles of different lipid composition. The diameter of the vesicles varied between 100 and 200 nm as was shown by electron microscopic examination of negatively stained preparations.

In vivo experiments. Male Wistar rats, weighing about 200 g were starved overnight and injected intraperitoneally or intravenously with approx. 2 μmol vesicle lipid. For the determination of tissue distributions of the injected sphingomyelin the animals were anesthesized with Nembutal^{R} (intraperitoneally, 6 mg/100 g body weight). At the time chosen a blood sample was taken from the portal vein and the liver was briefly perfused with saline to remove blood. Liver and spleen were homogenized in cold 0.25 M sucrose and the homogenate was used for determination of total uptake of radioactive label by the organ. Samples were bleached with hydrogen peroxide and the radioactivity of the sample was determined by liquid scintillation counting in a xylene-based scintillator. For characterization of labeled lipids a sample of the homogenate was extracted according to Bligh and Dyer (19). The amounts of radioactivity associated with the methanol/water- and the chloroform-phase were determined by liquid scintillation counting in xylene-based scintillator and chloroform-soluble material was submitted to TLC on silica gel, with $CHCl_3/CH_3OH/NH_3/H_2O$ (65 : 35 : 4 : 1) as a solvent system. The various lipid spots, visualized by ultraviolet light, were assayed for radioactivity. Occasionally a number of organs and tissues other than liver and spleen were removed as well. They were rinsed in buffered saline and blotted on filter paper. Tissue samples of 10-100 mg were digested in 1 ml soluene. The digests were bleached with hydrogen peroxide and isopropanol, mixed with 10 ml toluene-based scintillator and radioactivity was determined. When vesicles containing ^{125}I-label were injected, 3-4 ml blood were obtained from the inferior caval vein, the liver was briefly perfused and the amounts of radioactivity in the peritoneal cavity and associated with blood, liver, spleen and several other organs were determined. Blood volume was taken as 5.8 ml/100 g body-weight.

Vesicle-associated radioactivity in blood and peritoneal cavity. 2 ml heparinized blood or peritoneal wash fluid from animals injected with ^{125}I-labeled poly(vinyl-pyrrolidone) containing vesicles were layered on a 20% solution of Ficoll^{R} in saline and centrifuged for 10 min at 1400 x *g*. Vesicles and plasma remain on top of the Ficoll layer, while blood cells with any adsorbed vesicles sediment at the bottom of the tube. Free ^{125}I-labeled polymer is distributed between upper layer and Ficoll, the major fraction

(approx. 70%) remaining in the upper layer.

In vitro incubation of fluorescent dextran-containing vesicles in plasma. Vesicles were prepared as described above. They were labeled with 0.75 mol % [1-^{14}C] cholesteryl oleate and contained fluorescent Dextran (see Materials) in the aqueous compartment. Fluorescence measurements were performed on a Perkin Elmer MPF 43 spectrofluorimeter with excitation and emission wavelenghts at 490 and 520 nm, respectively. Plasma was obtained from starved rats and dialyzed in tubing with molecular weight cut-off 3500 (Thomas Laboratory specialities), for 2 h at 37^{o}C against 100 mM Tris/50 mM NaCl, pH 7.6. This high buffer concentration was essential to keep pH at 7.6 during the incubations. 0.1 ml of the vesicle suspension was added to 0.9 ml dialyzed plasma (37^{o}C) and the mixture was chromatographed again on Sepharose CL-2B either immediately after mixing or 2 or 4 h later. Both Dextran fluorescence and the amount of vesicle-lipid radioactivity in the void volume fractions were measured after addition of Triton X-100 (1% final concentration) in order to dissolve the vesicles.

RESULTS AND DISCUSSION

Intraperitoneal administration of the vesicles

Table I shows the association of the ^{14}C- and ^{125}I-labels with blood, liver and spleen following intraperitoneal administration of vesicles containing [^{14}C]sphingomyelin or ^{125}I-labeled poly(vinyl pyrrolidone). In blood as well as in liver and spleen the two labels run closely parallel following their injection during the entire 12-h period. Organ uptake of non-encapsulated ^{125}I-poly(vinyl pyrrolidone) is much lower than that of the encapsulated marker. Less than 2% of the injected dose is associated with the liver at 2 h, increasing to not more than 6% after 12 h. At that time less than 1% of the injected dose of free ^{125}I-labeled polymer is recovered in the spleen. The parallelism between ^{14}C- and ^{125}I-labels suggests that liver and spleen take up intact vesicles. It appears, however, that the two organs handle the vesicle-lipid differently, once taken up. Although both in liver and spleen 80-90% of the radioactivity is chloroform-soluble, TLC showed considerable differences between the organs in the distribution of radioactivity among the phospholipids. At 2 h after injection, 80% of the chloro-

TABLE I

UPTAKE OF [^{14}C]SPHINGOMYELIN AND ^{125}I-LABELED POLY(VINYL PYRROLIDONE) BY BLOOD, LIVER AND SPLEEN AFTER INTRAPERITONEAL INJECTION OF LABELED VESICLES

Wistar rats were injected intraperitoneally with 2 ml of a vesicles suspension containing 2 μmol vesicle-lipid. The vesicles were composed of sphingomyelin and cholesterol (molar ratio 3 : 2), contained poly(vinyl pyrrolidone) (PVP) and were labeled with either [^{14}C]sphingomyelin or ^{125}I-labeled poly(vinyl pyrrolidone). The uptake of [^{14}C]sphingomyelin and ^{125}I-labeled poly(vinyl pyrrolidone) was determined 2 and 12 h after injection. The data given are means ± S.D. of 3-4 animals. When [^{14}C]spingomyelin-labeled vesicles were injected, liver and spleen homogenates were extracted according to Bligh and Dyer (19) and the chloroform-soluble material was analyzed by TLC.

	Time after injection (h)	Uptake ^{125}I-labeled PVP (% of injected dose ± S.D.)	Uptake [^{14}C]-sphingomyelin (% of injected dose ± S.D.)	% of chloroform-soluble material associated with sphingomyelin
Blood	2	19 ± 9	25 ± 9	
	12	6 ± 5	9 ± 6	
Liver	2	7 ± 4	11 ± 3	80
	12	32 ± 14	29 ± 6	44
Spleen	2	4 ± 2	8 ± 4	80
	12	23 ± 16	19 ± 4	80

form-soluble label in the liver was associated with sphingomyelin; 12 h after injection not more than 44% of the lipid label remained as sphingomyelin, 56% having been converted to phosphatidylcholine. By contrast, 12 h after injection as much as 80% of the chloroform-soluble label in the spleen was still associated with sphingomyelin. Apparently the sphingomyelin in the liver is subject to considerable degradation, whereas, at least during the first 12 h, there is only limited metabolization in the spleen. This might be due either to different activities in phospholipid metabolism in liver and spleen expressed, for example, in the levels of sphingomyelinase activity (20) or to different mechanisms by which the organs accomodate the liposomes. As was demonstrated by Roerdink *et al.* (21), in the liver vesicles are taken up by Kupffer cells, where they are most probably degraded in the

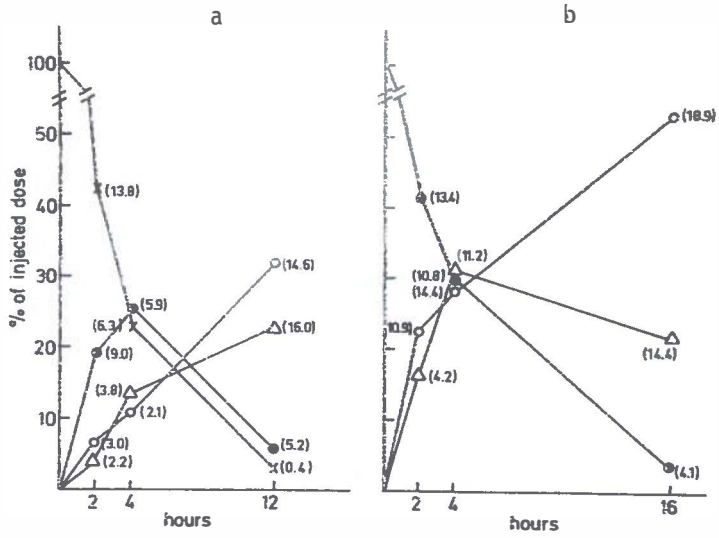

Fig. 1. Time course of clearance of ^{125}I-labeled poly(vinyl pyrrolidone) from the peritoneal cavity and uptake of the label in blood, liver and spleen after an intraperitoneal (a) or intravenous (b) injection of vesicle-encapsulated ^{125}I-labeled poly(vinyl pyrrolidone). 2 µmol vesicle lipid, containing approx. 5 . 10^5 dpm of ^{125}I-labeled poly(vinyl pyrrolidone) were injected into Wistar rats. At various times after injection the amounts of radioactivity associated with blood, liver and spleen were determined. Mean values of four animals are given. Numbers in parentheses denote the standard deviations. X—X, peritoneal cavity; ●—●, blood; O—O, liver; Δ—Δ, spleen.

lysosomes, whereas in the spleen they may, for the greater part, be simply adsorbed without significant intracellular uptake, as was suggested by Van Rooyen and van Nieuwmegen (22). Furthermore, in the liver the metabolically very active hepatocytes may contribute substantially to metabolization of the lipids following primary uptake by Kupffer cells as was suggested by Scherphof *et al.* (23).

Fig. 1 (a) shows that 12 h after intraperitoneal injection of vesicle-entrapped ^{125}I-labeled poly(vinyl pyrrolidone) the label is almost completely removed from the peritoneal cavity and has gradually become associated with liver and spleen with a transient rise in activity in the blood. Only small amounts of radioactivity were recovered in other tissues. This time course is indicative of a transport of vesicle-associated ^{125}I-label from the

peritoneal cavity to the blood. For comparison, the same vesicles were also injected intravenously (Fig. 1b). It is obvious that the average rate of uptake by liver and spleen, during the first few hours is faster by a factor of 2-3 in this case. The proportion of spleen-associated label tends to level off after 4 h, in particular after intravenous administration. This might be indicative of a lack of real intracellular uptake of vesicles by this organ. Other investigators recently reported that small unilamellar vesicles composed of sphingomyelin as the main phospholipid constituent, were removed from the circulation more slowly than small unilamellar vesicles with phosphatidylcholine as the major phospholipid component (24,25). Possibly sphingomyelin or its mixture with cholesterol constitutes a lipid composition which is less favorable for interaction with cells, *e.g.* as a result of significantly smaller size which conceivably would result in relatively long survival times in the cirulation. Further investigation is required before definite conclusions on these potentially important observations can be drawn.

Experimental proof that the ^{125}I-label found in the blood and peritoneal cavity is indeed vesicle encapsulated is given in Table II. Blood and peritoneal fluid were sampled 4 h after injection of the vesicles and the supernatant fraction, obtained after Ficoll centrifugation, was chromatographed on Sepharose CL-2B. A representative pattern of the distribution of ^{125}I-labeled poly(vinyl pyrrolidone) between supernatant, Ficoll layer and the sediment is given in Table II. Nearly all of the label, present in the upper layer eluted in the void volume of the column (Table II and Fig. 2). When either free ^{125}I-labeled polymer or free ^{125}I-labeled polymer mixed with empty vesicles was injected no radioactivity was recovered in the void volume of Sepharose CL-2B, following the Ficoll-centrifugation treatment (Table II and Fig. 2). This demonstrates that the ^{125}I-label eluting in the void volume is encapsulated in and not adsorbed to the vesicles. Considerable amounts of radioactivity were found associated with the blood cells collected at the bottom of the centrifuge tube. However, two repeated washings sufficed to remove virtually all of this radioactivity, indicating that the blood cells do not take up significant amounts of liposomes. For this reason, and also because of our finding that only very small amounts of radioactivity in the peritoneal cavity were cell-associated, we consider it unlikely that circu-

TABLE II

VESICLE-ASSOCIATED RADIOACTIVITY IN BLOOD AND PERITONEAL CAVITY

4 h after intraperitoneal and intravenous injection of vesicle-entrapped ^{125}I-labeled poly(vinyl pyrrolidone) (PVP) blood and peritoneal fluid were sampled (see Materials and Methods) and centrifuged on a Ficoll layer. Blood and peritoneal fluid were resolved in three fractions: a supernatant fraction (A) containing the plasma with the bulk of the liposomes and free poly(vinyl pyrrolidone); a Ficoll fraction (B) containing a minor fraction of the liposomes plus free poly(vinyl pyrrolidone) and a sediment (C) containing cells and adsorbed vesicles. The supernatant fraction was chromatographed on Sepharose CL-2B; the percentage of the radioactivity eluting in the void volume is given (D). The numbers given are percentages of the total amount of radioactivity applied on the Ficoll layer. Mean values of two separate experiments are shown.

	Vesicle entrapped ^{125}I-PVP intraperitoneal		Vesicle entrapped ^{125}I-PVP intravenous	Empty vesicles plus free [^{125}I]PVP intraperitoneal	Control vesicles
	Blood	Peritoneal fluid	Blood	Blood	
A (plasma)	65.2	96.0	69.7	74.5	97.6
B (Ficoll)	9.1	2.5	8.6	21.7	2.4
C (sediment)	25.6	2.1	21.6	3.8	-
C' (washed sediment)	3.0	0.8	1.0	0.1	-
D (% of radioactivity in void volume)	78.0	87.0	87.0	0	-

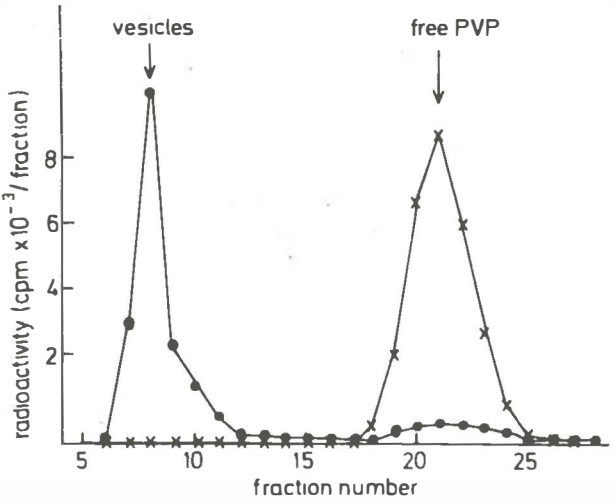

Fig. 2. Elution profile, on Sepharose CL-2B, of ^{125}I in plasma, following intraperitoneal injection of free or vesicle-entrapped ^{125}I-labeled poly-(vinyl pyrrolidone). Plasma was obtained by centrifugation of blood on Ficoll, following intraperitoneal injection of encapsulated ^{125}I-labeled poly(vinyl pyrrolidone) (●——●), or free ^{125}I-labeled poly(vinyl pyrrolidone) mixed with empty vesicles (X——X).

lating macrophages play an important role in the transport of vesicles from the peritoneal cavity to the blood and subsequently to liver and spleen.

Integrity of the vesicles

Our finding that a large fraction of the ^{125}I-label in the blood is still encapsulated does not exclude, that the vesicles lost part of their contents followed by rapid clearance of the released solute from the blood compartment. Therefore, we measured the ratio of the labels of lipid and aqueous phase before and after injection.

We prepared vesicles labeled in the lipid phase with a trace amount of [1-^{14}C]cholesteryl oleate (0.75 mol %), which at least in rats, does not interact with lipoproteins (26), and in the aqueous phase with fluorescently-labeled Dextran.

Vesicles recovered from the blood 4 h after intraperitoneal injection

TABLE III

IN VIVO AND *IN VITRO* INTEGRITY OF THE VESICLES

Vesicles were labeled with a trace amount of [1 ^{14}C]cholesteryl oleate (0.75 mol %) in the lipid phase and with fluorescein isothiocyanate-labeled Dextran in the aqueous compartment. The specific activity, defined as the amount of fluorescein isothiocyanate-labeled Dextran per μmol vesicle-lipid was taken as a measure of integrity. The integrity of the vesicles was measured *in vivo* (in the blood) 4 h after an intraperitoneal injection and *in vitro* during a 4-h incubation in plasma, followed by reisolation on Sepharose.

		Specific activity of the vesicles
Vesicle preparation		100%
In vivo 4 h after injection		77%
In vitro incubation in plasma	0 h	75%
	2 h	70%
	4 h	66%

contained 76.8 ± 7.7% of the amount of Dextran, which was present in the original vesicle-preparation. Most of the approx. 25% of Dextran which is lost, is probably released immediately after injection. *In vitro* incubations in rat plasma showed that a similar extent of release takes place immediately after mixing the vesicles with plasma (Table III).

After this sudden loss of Dextran the further release takes place at a moderate rate of a few percent/h. Probably the first loss represents a fraction of Dextran which was not really entrapped in the aqueous phase but rather became associated with the lipid bilayer during formation of the veiscles (27). This would mean that the entrapped aqueous space of the vesicles remains well-separated from the environment for at least a few hours after intraperitoneal injection. Similar results were obtained by Kirby *et al.* (28) and Sieber *et al.* (29) with small unilamellar vesicles.

Our observations on the fate of the liposomal sphingomyelin are not obscured by an intervention of lipoproteins. Scherphof *et al.* showed before (30) that phosphatidylcholine is readily transferred from small unilamellar vesicles to high density lipoprotein and, thus may be donated to liver

parenchymal cells by way of this plasma constituent (23). Incorporation of cholesterol into the liposomal membrane abolishes the net transfer of phosphatidylcholine to the lipoprotein but can not prevent exchange of phospholipid between liposomes and lipoprotein (Damen, J., Regts, J. and Scherphof, G., unpublished data). The latter will thus take up liposomal lipid label which then no longer represents the fate of the liposome as such. We found no transfer of sphingomyelin label from the vesicles we used in the present study to high density lipoprotein or to other lipoproteins during an incubation of such vesicles in 90% rat plasma for 1 h, thus excluding the possibility that liver and/or spleen uptake of sphingomyelin label are mediated by such plasma constituents. Similar results were found by Damen *et al.* (unpublished data) using sonicated unilamellar vesicles composed of sphingomyelin and cholesterol.

In conclusion, our results are compatible with the view that liposomes administered *in vivo* may serve as a depot from which sustained release of entrapped drugs may take place. The type of vesicle we used seems particularly useful for this purpose because of its high trapping efficiency and its relative stability.

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C H A P T E R III

THE PRESENCE OF TUMOR CELLS INFLUENCES BLOOD CLEARANCE AND TISSUE DISTRIBUTION OF DIFFERENT DOSES OF LARGE UNILAMELLAR LIPOSOMES

SUMMARY

In the present study it is shown that high liposome doses cause a dose dependent saturation of the phagocytosing capacity of reticuloendothelial cells in the liver and presumably also in the spleen of the mouse. The capacity to eliminate liposomes from the blood is inhibited one day after injection of 10^6 lymphosarcoma cells. This is probably due to a decreased activity of the reticuloendothelial system caused by the tumor cells, as was indicated by the simultaneously occurring inhibition of carbon clearance. Six days after injection of the tumor cells the elimination of liposomes from the blood is similar to the elimination in normal mice, but the accumulation per g tissue in liver and spleen of the tumor mice is decreased significantly. This indicates that although tumor cells *per se* are not directly involved in the accumulation of liposomes in these tissues, their mere presence may have significant effects on blood clearance and tissue distribution.

INTRODUCTION

The potential clinical usefulness of liposomes as a system for the controlled delivery of therapeutic agents has been discussed in a number of recent reviews (1-3). After intravenous or intraperitoneal injection a significant proportion of the liposomes accumulates in liver and spleen. Liposomes can be taken up by tissue macrophages (mainly located in liver and spleen) as has been demonstrated for Kupffer cells in the liver (4,5), but relatively little is known about the factors which govern such uptake. If liposomes contain 40-50 mol % cholesterol they do not release substantial proportions of their contents and therefore the distribution of liposome-encapsulated therapeutic agents will initially follow the distribution of the liposomes. Consequently, the pharmacokinetic properties of the encap-

sulated agents will be largely different from those of the free drugs (7,8, 9).

Alving *et al.* (10) made use of the ability of liposomes to localize in Kupffer cells, for the treatment of experimentally induced *Leishmaniasis*, a disease caused by a parasite that invades Kupffer cells. In cancer chemotherapy only few successful applications of liposomes as drug carriers have been reported (11-16) and the presently available evidence supports the hypothesis that the therapeutic effects from a liposome-encapsulated cytotoxic drug are due to a delayed elimination from the blood and a sustained release of the drug from the liposomes, rather than to a selective uptake of the encapsulated drug by the tumor cells (17,18). For such a mechanism to be optimally effective, uptake of liposomes by reticuloendothelial cells may be a serious drawback.

Depression of reticuloendothelial activity has been suggested to serve as a means of reducing liver and spleen uptake of liposomes and prolonging the circulation time of the vesicles (19-22). The rate of disappearance of liposomes from the blood decreases with increasing dose (20,22,23), indicating that the clearance mechanism is a saturable process. Concomitantly one would expect the proportional uptake of liposomes in the liver to decrease with increasing dose, although conflicting results have been reported on this subject (20,23). Tumor cells may influence reticuloendothelial activity (24) but thusfar little attention has been paid to the possible effects of the presence of tumor cells on the elimination of liposomes from the blood.

In this study we investigated the dose dependency of the circulation time and tissue distribution of large unilamellar vesicles and we investigated the influence of the presence of lymphosarcoma cells on these processes.

MATERIALS AND METHODS

Liposome preparation

Sphingomyelin (from bovine brain) and cholesterol were obtained from Sigma. Phosphatidylserine, purified from bovine brain as was described by Papahadjopoulos and Miller (25), was a gift from Dr. J.C. Wilschut of our laboratory. ^{125}I-labeled poly (vinylpyrrolidone) (spec. act. 38 μCi/mg; molecular weight 30 000 - 40 000) was purchased from the Radiochemical Centre

Amersham. Unipore polycarbonate filters were from Biorad. Sepharose CL-2B and Ficoll(R) (molecular weight 70 000) were obtained from Pharmacia.

Reverse phase evaporation vesicles (REV) were prepared essentially as described by Szoka and Papahadjopoulos (26). Sphingomyelin, cholesterol and phosphatidylserine (molar ratio 4:5:1) were dispersed in diethyl ether to give a concentration of 20 μmol of total lipid per ml (the diethyl ether was distilled in the presence of sodiumbisulfite immediately before use). The aqueous phase (Tris-buffered saline; 150 mM NaCl/5 mM Tris-HCl, pH 7.4) contained ^{125}I-labeled poly (vinylpyrrolidone) (2.6 mg/ml with varying specific activity). Per ml of the organic phase 0.3 ml of the aqueous phase was added and the mixture was sonicated under nitrogen in a bath type sonicator (Branson). After formation of a homogeneous suspension of inverted micelles the organic phase was removed on a rotary evaporator and the vesicl s thus formed were extruded through a polycarbonate membrane with 0.4 μm pores (27). Non-encapsulated ^{125}I-poly (vinylpyrrolidone) was separated from encapsulated material by gel filtration on Sepharose CL-2B. Void volume fractions, containing the liposomes, were pooled and concentrated in an Amicon concentration cell (XM-300 filter). Vesicle lipid concentrations were determined by lipid phosphorus assay according to Bartlett *et al.* (28) as modified by Böttcher *et al.* (29).

Animals

3 months old female (C57BlxGr) F_1 mice were obtained locally. A B-lymphosarcoma, which originated spontaneously in a C57Bl mouse (30), was transplanted weekly by intraperitoneal injection of 10^6 tumor cells. Seven days after inoculation the spleen was significantly enlarged (from approx. 100 mg to approx. 800 mg) and consisted almost entirely of tumor cells. The lymphosarcomatous spleen was excised and squeezed through nylon gauze in ten parts of ice cold saline. The single-cell suspension thus obtained was used for tumor transplantation.

At the third day after intraperitoneal injection, tumor cells were present in liver and spleen as observed microscopically. In the liver the tumor cells were located predominantly in the sinusoids and in the spleen in the marginal zone of the follicles of the white pulp. During the tumor development the tumor cells in the spleen concentrically invaded the white pulp, obliterating

the red pulp. At seven days after inoculation a diffuse tumor cell proliferative pattern was found in the spleen and massive infiltrations were found in liver, lungs, bone marrow and kidneys. Macroscopically, no tumor growth or ascites production was observed in the peritoneal cavity at any day. Tumor bearing mice died 8 or 9 days after intraperitoneal inoculation of 10^6 lymphosarcoma cells. The growth pattern and characteristics of this tumor are described in more detail in chapter I.

Tissue distribution experiments

Normal or tumor bearing mice (either 1 or 6 days after inoculation of 10^6 lymphosarcoma cells) were injected intraperitoneally or intravenously (lateral tail vein) with various doses of liposomes. Four hours after the injection, the animals were anesthetized with ether and the peritoneal cavity of mice that received an intraperitoneal liposome injection, was rinsed with 2 ml of Tris-buffered saline to recover liposomes. A blood sample (0.2 ml) was taken from the vena cava and the liver was briefly perfused with Tris-buffered saline to remove blood. Various organs were excised, rinsed in buffer, blotted on filter paper and weighed. Radioactivity was measured in whole organs. For calculation of the proportion of the dose present in blood the total blood volume of the mice was taken as 2 ml. In some experiments mice were treated with lanthanum by intravenous injection of 0.4 μmol $La(NO_3)_3$ (Merck) in 0.2 ml Tris-buffered saline, 24 h prior to the injection of liposomes. Control animals received Tris-buffered saline.

RES function assay

Reticuloendothelial activity was measured by determining the rate of elimination of intravenously injected carbon from the blood. Mice received 0.2 ml of India Ink 4415 (Higgins Ink Co., NJ) diluted ten-fold with Tris-buffered saline to give a 0.55% suspension. Three and eight minutes after injection of carbon, groups of mice were anesthetized with ether and blood was sampled from the vena cava. 0.1 ml of blood was diluted with 2.9 ml of distilled water and the carbon concentration was measured by determining light absorbance at 620 nm. The phagocytic index was calculated according to Donald and Tennent (31).

RESULTS

^{125}I-labeled poly (vinylpyrrolidone) was chosen as a marker of the aqueous compartment because it is metabolically inert, and in a free form is taken up by liver and spleen only at very low rates (32). Once inside cells it is also released very slowly, thus ^{125}I-label found associated with liver and spleen can be taken to reflect uptake of vesicles without taking into consideration their subsequent metabolic fate. In preliminary experiments, using a Ficoll$^{(R)}$ centrifugation method as described previously (32), we found that following an intraperitoneal injection of ^{125}I-poly (vinylpyrrolidone)-containing vesicles, a considerable proportion of the radioactivity in blood and peritoneal cavity was liposome-associated (table I). At 4 h after intraperitoneal injection of 125 μmol liposomal lipid/kg, less than 10% of the injected dose was recovered in the peritoneal wash and 61% was recovered in liver and spleen (see below, table 7). The latter value is comparable to the 75.3% recovered in liver and spleen after intravenous injection of a similar dose (see below, table 2). This indicates that ^{125}I-poly (vinylpyrrolidone) is transported from the peritoneal cavity to the blood in liposome-entrapped form. Only a small fraction of the radioactivity was associated with cells (table 1) suggesting that circulating macrophages are not extensively involved in the transport of liposomes from peritoneal cavity to blood. In further experiments ^{125}I-radioactivity in blood and peritoneal cavity was taken to represent liposomes.

Intravenously injected vesicles; dose dependent blood elimination and tissue distribution

^{125}I-poly (vinylpyrrolidone)-containing vesicles were injected intravenously into normal and tumor bearing mice in doses of 50-500 μmol of liposomal lipid/kg. The mice were sacrificed 4 h after injection and the distribution of liposomes between blood and various tissues was determined (tables 2 and 3). In all cases 70-80% of the injected dose was recovered in blood, liver and spleen. With increasing liposome dose larger proportions remained in the circulation after 4 h. Concomitantly, smaller fractions were taken up by liver and spleen.

One day after injection of tumor cells, the mice were clearly inhibited in

TABLE 1

LIPOSOME-ASSOCIATED AND CELL-ASSOCIATED RADIOACTIVITY IN BLOOD AND PERITONEAL CAVITY

REV, composed of sphingomyelin and cholesterol in a molar ratio 3:2, containing ^{125}-labeled poly (vinylpyrrolidone) in the aqueous compartment were injected intraperitoneally at a dose of 50 μmol/kg into normal and lymphosarcomatous mice (6 days after inoculation). 2 and 4 h after injection, blood and peritoneal fluid were sampled and centrifuged on a Ficoll layer as described in ref. 32. Blood and peritoneal fluid were resolved in three fractions: a supernatant fraction (A) containing plasma with the bulk of the liposomes and free poly (vinylpyrrolidone), a Ficoll fraction (B) containing a minor fraction of the liposomes plus free poly (vinylpyrrolidone) and a sediment (C) containing cells and adsorbed vesicles. The supernatant fraction was chromatographed on Sepharose Cl-2B, the percentage of the radioactivity eluting in the void volume is given (D). The numbers are proportions of the total amount of radioactivity applied on the Ficoll layer. Each value represents the mean of 2 mice.

	A (plasma)	B (Ficoll)	C (sediment)	C' (washed sediment)	D (% of radioactivity in void volume)
normal mice					
2 h blood	93.0	2.9	4.1	0.6	91.9
peritoneal cavity	97.1	1.9	1.0	0.3	93.6
4 h blood	92.7	2.4	4.9	1.6	85.5
peritoneal cavity	94.9	0.9	4.2	3.2	89.7
lymphosarcoma mice					
2 h blood	93.2	2.5	4.9	0.5	85.0
peritoneal cavity	91.4	1.9	6.9	5.3	88.0
4 h blood	88.8	4.4	6.9	2.6	84.6
peritoneal cavity	72.5	2.0	25.7	23.4	87.4

TABLE 2

INTRAVENOUSLY INJECTED VESICLES; DOSE DEPENDENT BLOOD ELIMINATION AND TISSUE DISTRIBUTION

	Tissue radioactivity (% of injected dose)			
	125 μmol/kg (n=7)		500 μmol/kg (n=3)	
	whole tissue	per g of tissue	whole tissue	per g of tissue
blood	0.8 ± 0.2	-	23.9 ± 10.1	-
liver	55.6 ± 7.7	54.8 ± 9.2	34.7 ± 5.5	33.5 ± 6.2
spleen	19.7 ± 1.9	250.0 ± 63.0	13.6 ± 1.6	166.8 ± 25.3
lungs	0.5 ± 0.1	3.6 ± 1.5	0.7 ± 0.3	5.6 ± 2.0
heart	0.0 ± 0.0	-	0.1 ± 0.1	-
kidneys	0.1 ± 0.1	-	0.8 ± 0.2	-

The vesicles were injected intravenously at 125 μmol/kg and 500 μmol/kg. 4 h after injection a blood sample (0.2 ml) was taken from the vena cava and various tissues were removed and weighed. Radioactivity was determined in the whole tissues. The results are expressed as the mean percentage of the injected dose per whole tissue and per gram wet weight (± S.D.). n = the number of mice involved.

their capacity to eliminate liposomes from the blood (compare tables 2 and 3). The increase in the level of circulating liposomes in the tumor bearing mice corresponds to a decrease in hepatic uptake. For instance, 4 h after the injection of 500 μmol of liposomal lipid per kg, 76.1% was cleared from the blood of normal mice, but only 38.7% from the blood of tumor bearing mice, while liver uptake values amounted to 34.7% and 10.3% respectively. It is interesting to note that in the tumor bearing mice the splenic component of clearance became of relatively more importance than in normal mice: in normal mice approximately half of the eliminated liposomes was recovered in the liver and one-fifth in the spleen, while in one-day tumor mice, liver uptake accounted for approx. one-third of the eliminated dose, and spleen uptake for one-third or more.

In tumor bearing mice the amount of liposomes deposited in the lung was also enlarged.

TABLE 3

INTRAVENOUSLY INJECTED VESICLES IN ONE-DAY TUMOR MICE; DOSE DEPENDENT BLOOD ELIMINATION AND TISSUE DISTRIBUTION

	Tissue radioactivity (% of injected dose)							
	50 μmol/kg (n=3)		125 μmol/kg (n=7)		250 μmol/kg (n=4)		500 μmol/kg (n=11)	
	whole tissue	per g of tissue	whole tissue	per g of tissue	whole tissue	per g of tissue	whole tissue	per g of tissue
blood	3.6±2.7	-	29.3±8.1	-	46.5±1.9	-	61.3±7.4	-
liver	32.4±6.8	32.5± 2.8	21.8±4.6	21.4± 4.2	14.1±3.0	13.6± 2.8	10.3±2.2	9.3±1.6
spleen	40.8±5.8	439.7±95.5	30.0±4.2	299.8±57.4	19.7±2.6	181.4±23.9	12.2±1.6	110.1±17.2
lungs	4.2±1.5	38.8±13.5	2.7±0.7	29.1± 5.2	2.3±0.3	22.1± 2.1	1.6±0.4	12.7± 2.4
heart	0.1±0.1	-	0.2±0.1	-	0.3±0.1	-	0.3±0.2	-
kidneys	0.5±0.3	-	1.0±0.2	-	1.3±0.3	-	1.7±0.2	

The vesicles were injected intravenously at doses varying from 50 μmol/kg - 500 μmol/kg in lymphosarcoma mice 24 h after inoculation of tumor cells. 4 h after injection a blood sample (0.2 ml) was taken from the vena cava and various tissues were removed and weighed. Radioactivity was determined in whole tissues. The results are expressed as the mean percentage of the injected dose per whole tissue and per gram wet weight (± S.D.). n = the number of mice involved.

Liposome uptake expressed in absolute amounts rather than as % of the injected dose, is plotted as a function of the injected dose in fig. 1. Uptake in both liver and spleen increased with the injected dose, with a tendency to reach a saturation level, particularly in the one-day tumor mice. Clearly, the maximal uptake capacities in these animals are several times lower than those in the controls.

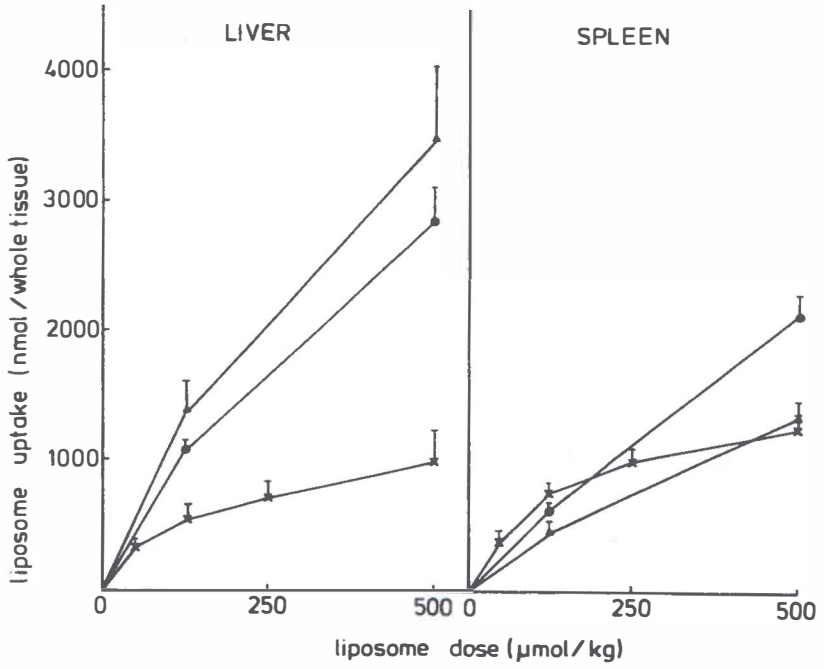

Fig. 1. Total uptake of intravenously injected liposomes in liver and spleen of normal mice and tumor-bearing mice. REV containing ^{125}I-labeled poly (vinylpyrrolidone) in the aqeous compartment were injected intravenously at 50-500 μmol/kg. 4 h after injection the tissues were removed and radioactivity was measured in the whole tissues. The results are expressed as nmol of lipid (calculated from the amount of ^{125}I-poly (vinylpyrrolidone) encapsulated per nmol of lipid) taken up per whole tissue.
▲—▲ normal mice; X—X one-day tumor mice; ●—● tumor mice, six days after inoculation of the lymphosarcoma cells.

TABLE 4

INTRAVENOUSLY INJECTED VESICLES IN SIX-DAY TUMOR MICE; DOSE DEPENDENT BLOOD ELIMINATION AND TISSUE DISTRIBUTION

	Tissue radioactivity (% of injected dose)			
	125 μmol/kg (n=3)		500 μmol/kg (n=3)	
	whole tissue	per g of tissue	whole tissue	per g of tissue
blood	1.2 ± 0.0	-	20.6 ± 2.9	-
liver	42.8 ± 3.5	29.7 ± 1.3	28.7 ± 2.6	20.7 ± 2.7
spleen	25.4 ± 1.4	36.7 ± 4.0	22.1 ± 1.2	32.3 ± 3.7
lungs	1.0 ± 0.1	3.8 ± 0.4	1.6 ± 0.1	6.6 ± 0.5
heart	0.0 ± 0.0	-	0.1 ± 0.0	-
kidneys	0.2 ± 0.1		0.6 ± 0.1	-

The vesicles were injected intravenously in lymphosarcoma mice 6 days after injection of the tumor cells at 125 and 500 μmol/kg. 4 h after injection a blood sample (0.2 ml) was taken from the vena cava and various tissues were removed and weighed. Radioactivity was determined in whole tissues. The results are expressed as the mean percentage of the injected dose per whole tissue and per gram wet weight (± S.D.). n = the number of mice involved.

Six days after inoculation of the tumor cells, the fraction of the administered dose eliminated in 4 h in tumor bearing mice, was very similar to that in normal mice (table 4). The total liver uptake in the tumor bearing mice was slightly decreased compared to normal mice, but the accumulation in the spleen was increased. However, as a result of a weight increase of liver (from approx. 1 g to 1.4 g) and spleen (from approx. 100 mg to 700 mg) the accumulation per g tissue was decreased significantly (table 4). This would argue against an extensive involvement of tumor cells *per se* in the accumulation of liposomes in these tissues, particularly as, morphologically, the increased weight correlates with an increase in the number of tumor cells.

The increased circulation time of the liposomes in mice with a one-day tumor and the concomitantly decreased hepatic uptake are indicative of a decreased activity of the reticulo-endothelial system in such animals. This was confirmed by a direct measurement of the phagocytic index (by

TABLE 5

EFFECT OF LYMPHOSARCOMA CELLS ON RES ACTIVITY

	OPTICAL DENSITY AT 620 nm (± S.E.) 3 min	8 min	phagocytic index
control mice	0.66 ± 0.07 (5)	0.41 ± 0.05 (4)	0.042
1-day lymphosarcoma mice	0.67 ± 0.07 (5)	0.51 ± 0.10 (3)	0.023
6-day lymphosarcoma mice	0.47 ± 0.10 (4)	0.15 ± 0.02 (4)	0.101

$$\text{phagocytic index equals: } \frac{\text{Log}_{10}\ \text{O.D.}_{3\ \text{min}} - \text{Log}_{10}\ \text{O.D.}_{8\ \text{min}}}{t_8 - t_3}$$

Lymphosarcoma cells (10^6) were injected intraperitoneally. RES activity was me sured as described in the methods section. The number of animals used at eaLn time are given in parentheses.

carbon clearance) in normal and tumor bearing mice (table 5): the phagocytic index of one-day tumor mice was decreased compared to the control mice. In six-day tumor mice the animals had recovered from the depression of reticulo-endothelial activity. The carbon clearance was even substantially increased. In additon, $La(NO_3)_3$, known to depress the phagocytic activity of Kupffer cells in mice (33), causes a change in the tissue distribution in normal mice quantatively very similar to that in the one-day tumor mice (table 6).

Intraperitoneally injected vesicles; dose dependent blood levels and tissue distribution

When the liposomes were injected intraperitoneally the results were essentially similar to those obtained with intravenously injected vesicles. Blood levels after 4 h were dose dependent and, similar to what was found after intravenous injection, were substantially increased in one-day tumor mice (tables 7 and 8). The uptake in liver and spleen on the other hand, was less clearly dose dependent than after intravenous injection. Six days after inoculation with tumor cells, blood levels and tissue distribution were almost restored to control values (table 9). As before, the increased weight of liver, and especially, spleen caused the value per gram tissue weight to be decreased significantly.

TABLE 6

EFFECT OF LANTHANUM TREATMENT ON BLOOD ELIMINATION AND TISSUE DISTRIBUTION OF I.V. INJECTED VESICLES

	control (n=2)		$La(NO_3)_3$ (n=4)	
	per whole tissue	per g of tissue	per whole tissue	per g of tissue
blood	33.6	-	56.6 ± 11.8	-
liver	34.9	40.5	17.0 ± 2.7	18.9 ± 5.0
spleen	12.7	203.4	10.9 ± 4.3	133.0 ± 22.9
lungs	0.9	7.1	1.2 ± 0.3	7.8 ± 1.4
heart	0.2	-	0.2 ± 0.1	-
kidneys	1.1	-	1.3 ± 0.3	-

24 h after i.v. injection of $La(NO_3)_3$ or Tris-buffered saline, mice were injected intravenously with vesicles (500 µmol/kg) labeled in the aqueous compartment with ^{125}I-poly (vinylpyrrolidone). 4 h after injection of the liposomes a blood sample (0.2 ml) was taken from the vena cava and various tissues were removed and weighed. Radioactivity was determined in the whole tissues. The results are expressed as the mean percentage of the injected dose per whole tissue and per gram wet weight (± S.D.). The numbers in parentheses denote the number of animals involved.

TABLE 7

INTRAPERITONEALLY INJECTED VESICLES; DOSE DEPENDENT BLOOD ELIMINATION AND TISSUE DISTRIBUTION

The vesicles were injected intraperitoneally at 125 µmol/kg and 500 µmol/kg. 4 h after injection the peritoneal cavity was rinsed with saline, a blood sample (0.2 ml) was taken from the vena cava and various tissues were removed and weighed. Radioactivity was determined in the whole tissues. The results are expressed as the mean percentage of the injected dose per whole tissue and per gram wet weight (± S.D.). n = the number of mice involved.

	125 µmol/kg (n=5)		500 µmol/kg (n=5)	
	whole tissue	per g of tissue	whole tissue	per g of tissue
blood	0.88 ± 0.22	-	11.3 ± 3.0	-
liver	46.6 ± 2.2	47.8 ± 5.8	39.9 ± 4.4	35.3 ± 6.3
spleen	14.4 ± 1.7	265.2 ± 51.2	13.7 ± 2.1	244.8 ± 19.3
lungs	1.2 ± 0.0	9.6 ± 2.9	1.0 ± 0.2	8.1 ± 1.4
heart	0.0 ± 0.0	-	0.0 ± 0.0	-
kidneys	0.1 ± 0.0	-	0.3 ± 0.2	-
peritoneal cavity	3.0 ± 0.6	-	6.0 ± 2.0	-

TABLE 8

INTRAPERITONEALLY INJECTED VESICLES IN ONE-DAY TUMOR MICE; DOSE DEPENDENT BLOOD ELIMINATION AND TISSUE DISTRIBUTION

The vesicles were injected intraperitoneally at 125 µmol/kg and 500 µmol/kg in one-day tumor mice. 4 h after injection the peritoneal cavity was rinsed with saline, a blood sample (0.2 ml) was taken from the vena cava and various tissues were removed and weighed. Radioactivity was determined in the whole tissues. The results are expressed as the mean percentage of the injected dose per whole tissue and per gram wet weight (± S.D.). n = the number of mice involved.

	125 µmol/kg (n=5)		500 µmol/kg (n=5)	
	whole tissue	per g of tissue	whole tissue	per g of tissue
blood	19.7 ± 8.2	-	51.7 ± 3.6	-
liver	16.7 ± 2.9	20.2 ± 4.8	10.6 ± 4.8	10.7 ± 5.4
spleen	22.3 ± 3.2	364.1 ± 59.9	14.3 ± 2.6	215.7 ± 20.4
lungs	2.5 ± 0.6	24.4 ± 2.3	1.9 ± 0.3	17.2 ± 2.7
heart	0.1 ± 0.1	-	0.2 ± 0.1	-
kidneys	0.8 ± 0.2	-	1.2 ± 0.5	-
peritoneal cavity	8.0 ± 2.8	-	7.1 ± 2.4	-

TABLE 9

INTRAPERITONEALLY INJECTED VESICLES IN SIX-DAY TUMOR MICE; DOSE DEPENDENT BLOOD ELIMINATION AND TISSUE DISTRIBUTION

The vesicles were injected intraperitoneally at 125 µmol/kg and 500 µmol/kg. 4 h after injection the peritoneal cavity was rinsed with saline, a blood sample (0.2 ml) was taken from the vena cava and various tissues were removed and weighed. Radioactivity was determined in the whole tissues. The results are expressed as the mean percentage of the injected dose per whole tissue and per gram wet weight (± S.D.). n = the number of mice involved.

	125 µmol/kg (n=5)		500 µmol/kg (n=5)	
	whole tissue	per g of tissue	whole tissue	per g of tissue
blood	1.1 ± 0.2	-	6.4 ± 5.7	-
liver	36.4 ± 1.9	28.8 ± 1.8	33.4 ± 12.5	29.8 ± 16.6
spleen	15.9 ± 1.7	33.3 ± 4.1	18.6 ± 3.4	36.5 ± 9.4
lungs	0.7 ± 0.1	3.6 ± 0.6	1.2 ± 0.3	6.0 ± 1.9
heart	0.0 ± 0.0	-	0.0 ± 0.0	-
kidneys	0.2 ± 0.0	-	0.3 ± 0.2	-
peritoneal cavity	5.6 ± 2.2	-	6.0 ± 2.0	-

The different doses of vesicles were injected in the same total volume (0.45 ml) and apparently were transported to the blood at a relatively high rate, since even with the high dose less than 10% was recovered from the peritoneal cavity after 4 h. The recovery of the vesicles tends to be lower after intraperitoneal than after intravenous injection. For instance, after i.v. injection of 125 μmol/kg, 70-85% was recovered in blood and various tissues (tables 2,3,4) while 60-70% was recovered in peritoneal wash, blood and various tissues after intraperitoneal injection of a similar dose (table 7,8,9). It is possible that a fraction of the vesicles in the peritoneal cavity is not easily removed by washing, and/or is taken up by lymph nodes.

DISCUSSION

The results described in this paper show that elimination from the blood of intravenously and, to a lesser extent, intraperitoneally injected vesicles is dose dependent. The fraction of liposomes cleared from the blood in a 4 h period and, concomitantly, the fraction of liposomes accumulating in liver and spleen decreases with increasing liposome dose. Since liposomes of the size we used are known to be taken up by reticuloendothelial cells in the liver (5), our results indicate that high doses of liposomes cause a saturation of the phagocytosing capacity of reticuloendothelial cells in the liver and, presumably, the spleen. Saturation of the reticuloendothelial clearance mechanism in the liver by high numbers of intravenously injected foreign particles is a well known phenomenon (34,35).

Souhami *et al.* (23) recently reported a dose dependence of the blood clearance of liposomes (at lipid doses comparable to ours), however, they did not observe a saturation of the hepatic uptake of liposomes. Apparently the slower blood elimination of the high dose of liposomes was not due to a saturation of the hepatic uptake mechanism. The actual site of saturation in these experiments remained unknown.

The vesicles used by Souhami and coworkers had a mean diameter of 58 nm and at least a fraction of the vesicles could therefore pass through the approximately 100 nm wide fenestrations in the endothelial sieve plates in the liver (36). Consequently, part of the liposomes could thus become associated with the parenchymal cells. Rahman *et al.* (37) recently presen-

ted indirect evidence that small unilamellar vesicles preferentially associate with liver-parenchymal cells. Experiments from our own lab directly showed high uptake of both lipid and entrapped solute of SUV in hepatocytes and low uptake in non-parenchymal cells (Scherphof *et al.*, in preparation).

In this study we used a different type of liposome, with a mean diameter larger than 100 nm (27), so that the liposomes are likely to be, at least partially, prevented from association with liver-parenchymal cells. Roerdink *et al.* recently demonstrated, in rats, the association of liposomes (very similar to the vesicles we used) with Kupffer cells, while only a small proportion was associated with the hepatocytes and virtually none with the endothelial cells (5). It is conceivable that quantitative differences in the distribution between the cell types in the liver are responsible for the discrepancy between the results of Souhami *et al.* and the data presented in this paper.

Clearance and tissue distribution of liposomes were very similar in one-day tumor mice and in lanthanum treated mice. Both conditions probably involve a depression of Kupffer cell activity, as measured by carbon clearance in the tumor animals. A functional change of the reticuloendothelial system following tumor cell challange has been reported previously by various investigators (38,39) and may be due to tumor components in the serum or to altered plasma opsonin levels. Roos and Dingemans have shown that intravenously injected tumor cells can interact with Kupffer cells (40). The growth pattern of the lymphosarcoma used in our experiments indicates that the tumor cells can travel from the peritoneal cavity to the blood, thus in our experiments also a direct interaction between tumor cells and Kupffer cells may be involved in the observed inhibition of Kupffer cell activity. In six-day tumor mice elimination from the blood and hepatic uptake of liposomes were comparable to those in control animals. Apparently the tumor mice had recovered from the reticuloendothelial depression. Surprisingly, in the six-day tumor mice carbon clearance was increased to even higher values than those found in control animals. At present we do not know what causes this phenomenon. Saba *et al.* (39) reported that Walker 256 tumor-cell-induced changes of reticuloendothelial carbon clearance in rats, correlated well with changes in plasma opsonin levels. Thus, increased levels of opsonic

proteins in the plasma may be involved in the increased carbon clearance, observed in our experiments. The depression of Kupffer cell activity in the one-day tumor mice causes a shift, especially at the lower lipid doses, of liposome accumulation towards the spleen. There is apparently an overflow of liposomes to the spleen, which in turn tends to become saturated at the higher lipid doses. The increase of liposome accumulation in the lungs of one-day tumor mice may be due to uptake by macrophages that migrated to the lungs of these tumor mice.

With the type of liposomes (REV) employed in this study we were not able to confirm an earlier suggestion by ourselves and observations by Gregoriadis *et al.* (41) and Hwang *et al.* (42) who reported that liposomes composed of sphingomyelin as the main phospholipid constituent, were cleared more slowly than vesicles containing phosphatidylcholine instead of sphingomyelin. Intravenously injected REV with phosphatidylcholine as the main phospholipid constituent showed a dose dependence of elimination from the blood and tissue distribution, very similar to the sphingomyelin vesicles (data not shown). A different intrahepatic disposition of small and larger vesicles, as discussed above may provide an explanation for this discrepancy.

In mice intraperitoneally injected liposomes were transported from the peritoneal cavity to the blood relatively quickly. We observed earlier in rats (32) that after intraperitoneal injection uptake of liposomes in liver and spleen was considerably slower than after intravenous injection. It has been reported that the disappearance from the peritoneal cavity of intraperitoneally administered red blood cells varies between species (43). Parker *et al.* (44) demonstrated the presence of intraperitoneally injected liposomes in thoracic duct lymph in rats. These authors suggested that not the lymph flow, but possibly another process, such as transport of liposomes across the wall of lymph vessels is governing the rate of lymphatic absorption of liposomes from the peritoneal cavity. This would be in agreement with our observation in mice that 2 h after intraperitoneal injection of ^{125}I-poly (vinylpyrrolidone)-containing REV (50 μmol/kg) levels of radioactivity in peritoneal cavity and blood were reasonably similar, irrespective of the volume of the injected dose (0.1 or 1.0 ml).

Fundamental studies on the in vivo fate of liposomes are of importance

for the design of treatment schedules with encapsulated drugs and also for the interpretation of antitumor effects of encapsulated drugs. From this study it is clear that, by simply varying the dose of liposomes, the elimination from the blood and uptake by liver and spleen can be influenced significantly. The presence of tumor cells influences the elimination from the blood and tissue distribution of liposomes. One day after intraperitoneal injection of 10^6 tumor cells, the elimination from the blood and the hepatic uptake of liposomes are inhibited. This is probably due to a transient depression of reticuloendothelial activity, caused by the tumor cells as was indicated by the simultaneous inhibition of carbon clearance. Six days after injection of tumor cells the mice have recovered from the depression of reticuloendothelial activity. The elimination from the blood and the hepatic uptake of liposomes in these animals is comparable to those in control animals and carbon clearance is even increased. However, because in the six-day tumor mice liver and spleen weights are increased due to the presence of substantial amounts of tumor cells in these tissues, the accumulation of liposomes per g tissue weight is decreased. This indicates that uptake of liposomes by tumor cells *per se* does not contribute to a significant extent to total tissue accumulation. This is compatible with results described elsewhere in this thesis.

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CHAPTER IV

REVERSIBLE DEPRESSION OF THE RETICULOENDOTHELIAL SYSTEM BY LIPOSOMES

SUMMARY

The effect of various doses of different types (reverse phase evaporation vesicles and small unilamellar vesicles) of intravenously injected liposomes on reticuloendothelial activity, as measured by the blood clearance rate of intravenously injected carbon, was investigated. Also the effect of preteatment with reverse phase evaporation vesicles on the elimination from the blood and the tissue distribution of a second dose of similar vesicles was determined. For all concentrations used reverse phase evaporation vesicles caused a reduction in reticuloendothelial activity at least up to four hours after injection. Twentyfour hours after administration the rate of carbon clearance returned to the control level. On the contrary, small unilamellar vesicles did not block reticuloendothelial activity. Pretreatment with reverse phase evaporation vesicles (250 μmol/kg) caused an increased blood level and a decreased hepatic uptake of a second dose of the vesicles, injected one hour after the first dose. This seems to be due to a depression of reticuloendothelial activity and not to a depletion of opsonins. Pretreatment with small unilamellar vesicles (250 μmol/kg) had no significant influence on the tissue distribution of a second dose of vesicles. Our results clearly indicate that reverse phase evaporation vesicles cause a reversible depression of reticuloendothelial activity and this depression seems to be induced by a saturation of reticuloendothelial cells with liposomes.

INTRODUCTION

Liposomes are of interest as drug-carriers for cancer chemotherapy, since they are non-toxic under therapeutic conditions, biodegradable, can encapsulate a wide variety of pharmacological agents and because the lipid bilayer can accomodate molecules important in biological recognition processes, thus providing a possibility for targetting vesicles to specific cells (1,2). The uses of liposomes, especially as drug carriers have been recently

reviewed by Kimelberg and Mayhew (3) and Papahadjopoulos (4).

Although there is good evidence that liposomes do interact with cells of the reticuloendothelial system (RES) (5) little is known about the details of such interaction. For example liposomes are taken up by macrophages in the liver (5) but it is not known whether all vesicles that become associated with liver and spleen are taken up by reticuloendothelial cells. It is possible that both uptake by, and adsorption to, these cells and/or other cells will occur simultaneously (6).

Investigation of these interactions is of importance since clearance of liposomes by the RES may limit their usefulness in chemotherapy of tumors not located in liver and spleen, as uptake of drug-containing liposomes by the RES will result in a reduced interaction of the drug, or the drug-containing liposomes, with the target tissue and may also result in undesirable toxicity to the RES. Saturation of liposome binding-sites has been suggested as a way of reducing the blood clearance rate and altering the tissue distribution of drug-containing liposomes (7,8,9), producing higher blood levels of the encapsulated drug for a prolonged period of time.

The blood clearance rate of colloidal particles can be reduced by giving a high dose which causes a reduction in the rate of clearance through a saturation of the reticuloendothelial cells, or through a depletion of opsonins; plasma proteins which play a role in the recognition of foreign particles by reticuloendothelial cells (10). A similar concentration dependence has been reported for liposomes (7,8,11). However, it is not known whether a reduction in the rate of clearance at high liposome doses is due to a saturation of reticuloendothelial cells or to a depletion of opsonins from the plasma or possibly to a saturation of other liposome binding-sites *e.g.* vascular endothelial cells.

In this study we investigated the effect of liposomes on RES function as measured by the classical carbon clearance assay. We examined the effect of various doses of liposomes of different size at different times after injection and also measured the effect of pretreatment with liposomes on the clearance and tissue distribution of a second dose.

MATERIALS

Cholesterol was obtained from A.B. Fluka (Buch, Switzerland) and was recrystallized twice from methanol. Unipore polycarbonate filters were from Biorad. Sepharose 4B was purchased from Pharmacia. ^{14}C-labeled inulin (spec. act. 1.8 mCi/mmol; molecular weight 5000) was obtained from New England Nuclear. The tissue solubilizer NCS and the scintillation solution OCS were obtained from the Radiochemical Centre Amersham. All organic solvents used were reagent grade. 10-12 weeks old female DBA 2/CR mice were used.

METHODS

Preparation of lipids

The preparation of the phospholipids was described previously (12,13).

Preparation of Reverse Phase Evaporation Vesicles (REV)

The vesicles were prepared essentially as described by Szoka and Papahadjopoulos (15). A chloroform solution of 6 μmol of phosphatidylglycerol, 24 μmol of phosphatidylcholine and 30 μmol cholesterol was evaporated to dryness on a rotary evaporator. The dry lipid film was dissolved in 3 ml of ether. In order to remove peroxides, the ether was distilled from sodium bisulfite immediately before use. To the lipid solution was added 1 ml of phosphate-buffered saline and the mixture was sonicated for 5 min under a nitrogen atmosphere in a bath type sonicator. This way a homogeneous suspension of inverted micelles was formed. The ether was removed in a rotary evaporator and the vesicles thus obtained were extruded through a Unipore polycarbonate filter with a pore size of 0.4 μm (16). When ^{14}C-labeled inulin containing REV were prepared the aqueous phase consisted of a 5 mM ^{14}C-labeled inulin solution in phosphate buffered saline. Encapsulated inulin was separated from non-encapsulated material by gel chromatography on Sepharose 4B.

Preparation of Small Unilamellar Vesicles (SUV)

A chloroform solution of 6 μmol of phosphatidylglycerol, 24 μmol of phosphatidylcholine and 30 μmol of cholesterol was evaporated to dryness on a rotary evaporator. 1 ml of phosphate-buffered saline was added to the dry lipid film. The lipids were dispersed by mechanical agitation at 37^{o}C and

the mixture was sonicated in a bath type sonicator under N_2 at 37°C until the suspension was clear. For the tissue distribution experiments the SUV were composed of dipalmitoylphosphatidylglycerol, dipalmitoylphosphatidylcholine and cholesterol in a molar ratio 1 : 4 : 5. The lipid bilayer marker used was ^{125}I-BPE (p-hydroxybenzidine phosphatidylethanolamine). BPE was synthesized by condensation of p-hydroxybenzamidate with dipalmitoylphosphatidylethanolamine (13) and was iodinated by a modification of the method of Greenwood *et al.* (14). The molar proportion of ^{125}I-BPE in the liposomes was 0.1 percent. In stability studies it was found that this marker exchanged with plasma proteins approximately at the same rate as ^{3}H-dipalmitoylphosphatidylcholine (13).

RES function assay

RES activity was measured by determining the rate of clearance of intravenously injected carbon. Mice received 0.1 ml of India Ink 4415 (Higgins Ink Co., NJ) diluted five fold with phosphate-buffered saline to give a 1.1% suspension. One and five min after injection of carbon, groups of mice were bled via a cardiac puncture. 0.1 ml of blood was diluted with 2.9 ml of distilled water and the carbon concentration was measured by determining the absorbance value at 620 nm. The phagocytic index was calculated according to Donald and Tennent (17).

Platelet counting

0.1 ml of blood, obtained via a cardiac puncture, was added to 10 µl of a 3.2% sodium citrate solution. Blood was diluted 100 fold in Unopette vials (recorder no. 5855, Becton-Dickinson, Rutherford, NJ) containing: ammonium oxalate, phosphate buffer and thimerosal and was incubated for 10-15 min at room temperature, resulting in lysis of red blood cells. Platelets were counted in a hemacytometer.

Opsonization of liposomes

Opsonization of the liposomes was accomplished by incubation with 50% homologous mouse serum for 15 min at 37°C immediately prior to injection of the vesicles.

Tissue distribution of liposomes

Non labeled liposomes or liposomes containing ^{14}C-labeled inulin were

injected i.v. in the lateral tail vein of 10-12 week old female DBA 2/CR mice, weighing approximately 20 g and various times thereafter animals were anesthetized with chloroform, blood was obtained via a cardiac puncture and tissues were collected. For the tissue distribution of ^{14}C-labeled inulin-containing liposomes, blood and tissue samples were processed in the following way. 0.1 ml of the blood was digested at room temperature in 1.2 ml of NCS. In order to bleach the digests, they were incubated for 30 min at 50°C with 0.4 ml of a freshly prepared benzoylperoxide solution in toluene (1 g of benzoylperoxide was dissolved at 60°C in 5 ml of toluene and the solution was filtered after cooling to room remperature). Tissues were collected, rinsed in phosphate-buffered saline and blotted dry on filter paper. Weighed samples of approximately 50 mg were digested in 1 ml of NCS at 50°C. The digests were bleached by incubation with 0.3 ml of the benzoylperoxide solution for 30 min at 50°C. After cooling to room temperature the digests were mixed with 13 ml of the xylene based scintillation solution OCS and radioactivity was counted in a Packard Tri-carb scintillation counter. Counting efficiency was calculated with an external standard. For the tissue distribution of ^{125}I-labeled SUV, tissues were rinsed in phosphate-buffered saline and counted directly in a Packard γ counter.

RESULTS

Effect of liposomes on platelet numbers

In order to ensure that we were measuring the phagocytic activity of the RES we used a low dose of carbon at which phagocytosis was the major mechanism of clearance (17). Since changes in platelet numbers have been shown to influence the blood clearance of carbon we measured the effect of liposomes on the number of circulating platelets. As is shown in Table 1 platelet numbers did not change after injection of liposomes, so in our experiments blood clearance of carbon can be taken as a measure of RES activity.

Effect of liposomes on RES activity

The effects of different doses of REV and SUV on RES activity are shown in Table 2. Since RES activity varied from experiment to experiment, control animals were included in each experiment. It can be seen that REV reduced

TABLE 2

EFFECT OF REV AND SUV ON RES ACTIVITY IN MICE

Various doses of REV and SUV (50, 250 and 1250 μmol/kg) were injected i.v. in DBA2/CR mice and 1, 4, 24 or 48 h later RES activity was measured as described in the methods section. The number of animals used at each time are given in parenthesis.

Type and dose (μmol/kg) of vesicles	time after injection of vesicles (h)	optical density at 620 nm (± S.E.) 1 min	5 min	phagocytic index*
Control		0.67 ± 0.05 (4)	0.38 ± 0.04 (4)	0.062
REV 50 μmol/kg	1	0.70 ± 0.03 (5)	0.48 ± 0.01 (3)	0.041
	4	0.75 ± 0.02 (4)	0.57 ± 0.04 (5)	0.030
	24	0.76 ± 0.05 (4)	0.39 ± 0.05 (4)	0.073
Control		0.67 ± 0.03 (5)	0.49 ± 0.02 (5)	0.034
REV 250 μmol/kg	1	0.66 ± 0.08 (4)	0.70 ± 0.02 (3)	0.000
	4	0.70 ± 0.04 (3)	0.69 ± 0.05 (4)	0.002
	24	0.77 ± 0.03 (5)	0.49 ± 0.02 (4)	0.049
Control		0.70 ± 0.01 (4)	0.45 ± 0.03 (4)	0.048
REV 1250 μmol/kg	1	0.65 ± 0.09 (5)	0.59 ± 0.05 (5)	0.011
	4	0.89 ± 0.02 (4)	0.67 ± 0.02 (5)	0.031
	24	0.70 ± 0.03 (5)	0.46 ± 0.02 (5)	0.046
Control		0.77 ± 0.03 (3)	0.61 ± 0.03 (3)	0.025
SUV 50 μmol/kg	1	0.75 ± 0.03 (4)	0.50 ± 0.03 (4)	0.044
	4	0.80 ± 0.03 (4)	0.64 ± 0.07 (4)	0.024
	24	0.70 ± 0.04 (3)	0.57 ± 0.08 (3)	0.022
Control		0.70 ± 0.01 (3)	0.52 ± 0.05 (3)	0.033
SUV 250 μmol/kg	1	0.78 ± 0.03 (5)	0.55 ± 0.06 (4)	0.038
	4	N.D.**	N.D.**	
	24	0.71 ± 0.02 (4)	0.55 ± 0.03 (5)	0.028
Control		0.76 ± 0.05 (4)	0.59 ± 0.02 (4)	0.028
SUV 1250 μmol/kg	1	0.72 ± 0.04 (3)	0.51 ± 0.10 (4)	0.038
	4	0.75 ± 0.04 (4)	0.57 ± 0.05 (5)	0.030
	24	0.70 ± 0.03 (5)	0.50 ± 0.05 (4)	0.037
Control		0.74 ± 0.05 (4)	0.47 ± 0.04 (4)	0.049
REV 50 μmol/kg	48	0.80 ± 0.01 (3)	0.50 ± 0.03 (4)	0.051
250 μmol/kg	48	0.74 ± 0.02 (5)	0.43 ± 0.03 (5)	0.047
1250 μmol/kg	48	0.76 ± 0.03 (5)	0.48 ± 0.02 (4)	0.050

* phagocytic index equals $\frac{\log_{10} \text{o.d.}_{1\ \text{min}} - \log_{10} \text{o.d.}_{5\ \text{min}}}{t_5 - t_1}$

** not determined

TABLE 1

NUMBER OF CIRCULATING PLATELETS AFTER INJECTION OF LIPOSOMES

The vesicles were injected i.v., blood was obtained via a cardiac puncture and platelets were counted as described in the methods section.

Type of vesicle	dose (μmol/kg)	time after injection of vesicles (h)	platelets/mm^3 ($X10^{-3}$ ± S.E.)
Control			970 ± 110
SUV	1250	1	1090 ± 90
SUV	1250	4	1040 ± 40
REV	250	1	880 ± 140
REV	250	4	940 ± 70

the RES activity during at least four hours after injection, for all concentrations used. Twenty four hours after injection the rate of carbon clearance returned to the control level. However, SUV did not reduce RES activity; on the contrary there was a consistent increase in the clearance rate at one hour after injection of the vesicles.

Effect of pretreatment with non-labeled liposomes on the tissue distribution of a second dose of labeled liposomes

Table 3 shows the effect of pretreatment with a high dose of non-labeled REV on the subsequent distribution of a second, low dose of ^{14}C-labeled inulin containing REV (column A and B). The first and second doses were separated by one hour and the tissue distribution was determined one hour after injection of the labeled vesicles. It can be seen that at 1 h after injection of the labeled vesicles the blood level of labeled vesicles in pretreated animals was increased several fold and hepatic uptake was decreased two fold. Increased blood levels and decreased hepatic uptake could result not only from a reduction in clearance due to reticuloendothelial depression, but also from a dilution of the vesicles in the blood, since the first, high, dose of non-labeled vesicles was not completely cleared

TABLE 3

EFFECT OF PRETREATMENT WITH REV ON THE TISSUE DISTRIBUTION OF A SECOND DOSE OF SIMILAR VESICLES

Non-labeled REV (250 μmol/kg) were injected i.v. and 1 h later ^{14}C-labeled inulin containing vesicles (50 μmol/kg) were injected. The tissue distribution was determined 1 h after the inulin containing vesicles were administered. The data given represent mean ± S.D. of 4 mice. The results are expressed as % of dose per organ.

	A	B	C	D	E
Pretreatment dose (μmol/kg)		250			250
Test dose (μmol/kg)	50	50	250	50	50
				opsonized liposomes	
Blood	7.9 ± 2.9	40.1 ± 4.1	15.3 ± 1.0	1.3 ± 0.2	24.5 ± 10.8
Liver	52.6 ± 2.0	25.8 ± 4.8	28.2 ± 7.6	45.7 ± 5.4	30.0 ± 6.6
Spleen	8.0 ± 3.7	6.3 ± 0.4	8.9 ± 0.4	7.3 ± 2.8	11.3 ± 1.2
Lung	0.14 ± 0.05	0.39 ± 0.10	0.66 ± 0.11	0.14 ± 0.01	0.56 ± 0.17
Kidney	0.21 ± 0.04	0.89 ± 0.08	0.62 ± 0.06	0.19 ± 0.05	0.78 ± 0.06
Small intestine	0.73 ± 0.29*	0.78 ± 0.22*	1.11 ± 0.13*	0.22 ± 0.05*	0.12 ± 0.06*

* % of dose per g tissue

upon injection of the second dose of vesicles (Table 3, column C). Pretreatment with non-labeled vesicles has a qualitatively similar effect on the elimination from the blood and uptake by liver and spleen, of opsonized vesicles (Table 3, column D,E).

Pretreatment with 250 μmol/kg of non-labeled SUV had no significant influence on the distribution of a second dose of labeled SUV, as is shown in Table 4, when the doses were separated by one hour. Blood levels in the pretreated animals were slightly increased. This again is likely to be due to dilution of the vesicles since SUV are cleared very slowly (18).

TABLE 4

EFFECT OF PRETREATMENT WITH SUV ON THE TISSUE DISTRIBUTION OF A SECOND DOSE OF SIMILAR VESICLES

Non-labeled SUV (250 μmol/kg) were injected i.v. and 1 h later ^{125}I-labeled SUV (50 μmol/kg) were injected. The tissue distribution was determined 4 h after administration of the labeled vesicles. The data given represent mean ± S.D. of 3 mice. The results are expressed as % of dose per organ.

Pretreatment dose (μmol/kg)		250
Test dose (μmol/kg)	50	50
Blood	45.9 ± 5.0	61.0 ± 9.2
Liver	20.0 ± 1.1	18.4 ± 3.1
Spleen	1.7 ± 0.3	1.1 ± 0.2
Lung	1.7 ± 0.2	3.7 ± 2.7
Kidney	2.1 ± 0.2	2.2 ± 0.5
Small intestine	2.1 ± 0.3	1.7 ± 0.1

DISCUSSION

The results indicate that REV cause a reversible depression of RES activity, where as SUV do not. Large liposomes are cleared more quickly than small liposomes (18), so that the total amount of vesicle lipid taken up by reticuloendothelial cells within a few hours after injection of equal doses of REV and SUV will be significantly larger upon administration of REV. The simplest explanation of both depression of the RES activity by REV and the inability of SUV to generate such a depression, is a saturation of reticuloendothelial cells by REV due to the large amount of vesicle lipid taken up in a short period of time. The rapid recovery of phagocytic activity indicates that the saturation is transient, probably because the lipid vesicles are metabolized, as has been shown in other studies (19,20).

RES depression by REV was most effective at 250 μmol/kg but even 50 μmol/kg brought about a reduction in the clearance rate of carbon. Both 50 μmol/kg and 250 μmol/kg are doses that have been used in animal studies (18 and Mayhew *et al.* unpublished data). As previously mentioned a reduction in RES activity could be mediated by depletion of opsonins or by a saturation of reticuloendtohelial cells by the ingested particles (10). A great deal of specificity exists in the interaction of opsonins with the surfaces of foreign particles. Several authors have reported that they could not demonstrate RES depression by one colloid when a second, dissimilar colloid was used as the test material (21,22,23). This observation was attributed to the fact that the initial RES depression was caused by depletion of opsonins, specific for the colloid used for induction, but not for the test colloid. Altough we cannot completely rule out the possibility, it is unlikely that a reduction in carbon clearance after administration of REV was due to a depletion of opsonins. Firstly, since SUV of a similar lipid composition and at equal lipid doses, giving the same surface area as REV and similar surface properties, did not block carbon clearance and secondly since we demonstrated a depression of RES activity using carbon, which is a different type of test particle.

At 1250 μmol/kg REV caused less reduction in the clearance rate of carbon than at 250 μmol/kg. At present we do not have an explanation for this observation.

The dilution of labeled liposomes in the blood of pretreated mice could account to a considerable extent for the decreased association of liposomes with the liver. On the other hand, it has been demonstrated that a dose of liposomes as large as that used for the pretreatment causes a depression of RES activity, as measured by carbon clearance and that in the liver liposomes are taken up by reticuloendothelial cells (5). This would make it likely, that apart from any dilution, the decrease in liposome accumulation in the liver is at least partially caused by the preload of liposomes. Since adsorption of liposomes to cells is a well known phenomenon, it is conceivable that the residual "uptake" represents adsorption of liposomes to Kupffer or other cells. That mechanisms other than Kupffer cell phagocytosis play a role in the hepatic uptake of liposomes was also suggested by results of Souhami *et al.* (24).

The results of the tissue distribution experiments with opsonized test vesicles are consistent with the hypothesis that the reduction of RES activity is not due to a depletion of opsonins. The low recoveries in our tissue distribution experiments are probably due to leakage of inulin from the liposomes. It is possible that pretreatment of the animals with unlabeled liposomes depletes the plasma of leakage inducing factors, thus resulting in a slower clearance of inulin from the blood. However, we found a persistent difference in blood clearance between pretreated and control animals when the test vesicles were incubated with serum (opsonized) prior to injection, which would indicate that the decreased clearance is not due to a depletion of leakage inducing factors from serum. Obviously, it cannot be excluded that leakage is induced by factors other than serum proteins, *e.g.* cell surfaces (25). If this is the case, the inhibitory effect of pretreatment on liposome accumulation in the liver is underestimated, because of the higher specific activity of the vesicles in the pretreated animals.

Our results are in agreement with findings of Abra *et al.* (26) who showed that pretreatment with a high dose of multilamellar vesicles resulted in a transient decrease of blood clearance and hepatic uptake of a second low dose of similar vesicles.

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CHAPTER V

METABOLISM OF LIPOSOME-ENCAPSULATED Ara-C IN DOG AND MOUSE TISSUES

SUMMARY

The effect of liposome-encapsulation on the metabolic activation (phosphorylation) and degradation (deamination) of arabinofuranosylcytosine (Ara-C) in liver and spleen of dogs and mice was investigated. Ara-C in free or liposome-encapsulated form was administered intravenously to dogs and DBA_2/CR mice bearing leukemia L_{1210}. At various times after injection the concentration of Ara-C and Ara-C metabolites in the blood, liver and spleen was measured. It was shown that liposome-encapsulation results in an increased Ara-C/Ara-U ratio in the liver and spleen of dogs and leukemic mice and that encapsulated Ara-C generates a sustained level of arabinofuranosylcytosine triphosphate in the liver and spleen of leukemic mice. These results clearly indicate that (1) encapsulated Ara-C is protected against deamination (2) encapsulated Ara-C is slowly released from liposomes in liver and spleen and (3) that liposomes may act as a local depot for Ara-C in these tissues.

INTRODUCTION

1-β-D arabinofuranosylcytosine (Ara-C) is an S-phase specific antitumor agent with activity against some animal and human tumors (1-4). The active metabolite arabinofuranosylcytosine triphosphate (Ara-CTP) inhibits DNA replication (5-8). The efficacy of Ara-C depends on the maintenance of therapeutic levels in blood and tissues for a period long enough to inhibit the DNA synthesis of nearly all tumor cells (9). Ara-C is rapidly deaminated to the inactive metabolite 1-β-D arabinofuranosyluracil (Ara-U) by the enzyme pyrimidine nucleoside deaminase, the tissue distribution and kinetic characteristics of which vary widely among species (10-12).

Several strategies have been employed to evaluate the role of deamination in the therapeutic efficacy of Ara-C. One approach has been through the synthesis of potent inhibitors of the deaminase, such as tetrahydrouridine (13). Administration of tetrahydrouridine prior to Ara-C has been shown to

increase Ara-C levels in blood and tissues with a concomitant decrease in Ara-U levels (13-16). However, the combination of tetrahydrouridine and Ara-C did not result in an increased therapeutic index in most animal tumors tested (15). Another approach involved the use of agents which are not susceptible to deamination. An example of this is cyclocytidine (2,2'-anhydro-1-β-D arabinofuranosylcytosine) (17). Cyclocytidine can only exert its antitumor activity after hydrolysis to Ara-C (18,19). It serves as an Ara-C depot and has activity against animal and human tumors (20-23).

A more recent approach to the protection of drugs against metabolic degradation is the use of macromolecular drug carriers such as liposomes. Encapsulation in liposomes reduces metabolism and excretion of Ara-C (24,25), and it has been shown that liposome-entrapped Ara-C is more effective against L_{1210} leukemia in mice than free Ara-C (26-29). This effect is thought to be due to a slow release of Ara-C from the liposomes, resulting in a prolonged availability of the drug at therapeutically effective concentrations (7,29,30). Liposomes are taken up mainly by liver and spleen, where a substantial proportion becomes associated with cells of the reticuloendothelial system (31-33).

In this study we compared the metabolic degradation of free and liposome-encapsulated Ara-C in the liver and spleen of dogs and we measured degradation and activation of free and encapsulated Ara-C in liver and spleen of leukemic mice. The potential usefulness of liposomes as drug carriers in the treatment of tumors in liver and spleen is discussed.

MATERIALS AND METHODS

Materials

Cholesterol was obtained from A.B. Fluka (Buch, Switzerland) and was recrystallized twice from methanol. Unipore polycarbonate filters were from Biorad. [5-^{3}H] Cytosine arabinoside (spec. act. 10.2 Ci/mmol), the tissue solubilizer NCS and the scintillation solutions OCS and Liquiscint were purchased from the Radiochemical Centre Amersham. [2-^{14}C] Cytosine arabinoside (spec. act. 55 μCi/mmol) was obtained from Moravek Biochemicals (Calif). All organic solvents used were reagent grade.

Methods

Preparation of phospholipids

The preparation of phospholipids has been described previously (28).

Preparation of Reverse Phase Evaporation Vesicles (REV)

The vesicles were prepared essentially as described by Szoka and Papahadjopoulos (34). A chloroform solution of phosphatidylglycerol, phosphatidylcholine and cholesterol (molar ratio 1 : 4 : 5) was evaporated to dryness on a rotary evaporator. The dry lipid film was dissolved in diethylether to a final concentration of 20 mM. The diethylether was redistilled from sodium bisulfite immediately before use. To 3 ml of the lipid solution was added 1 ml of a 345 mM Ara-C solution in 10% phosphate buffered saline (containing labeled Ara-C). The mixture was sonicated for 5 min under a N_2 atmosphere in a bath type sonicator. In this way a homogeneous suspension of inverted micelles was formed. The ether was removed in a rotary evaporator and the vesicles thus obtained were extruded through a Unipore polycarbonate filter with a pore size of 0.4 μm (35). The vesicles were dialyzed overnight against 3 changes of phosphate buffered saline to remove non-encapsulated Ara-C.

Animals

A. Dogs. Well fed, dewormed mongrel dogs weighing approx. 20 kg were anesthetized and mechanically ventilated. A generous thoraco-abdominal incision allowed access both to the right pleural cavity and intra-abdominal vascular structures to be infused or sampled. The right internal jugular vein was exposed, a catheter inserted and the tip positioned in the right atrium. Eighteen-gauge plastic catheters were placed in the portal vein, right suprahepatic vein and femoral vein, for injection (portal vein) and sequential sampling (right atrium, suprahepatic vein and femoral vein). A total of three dogs received a 50 ml bolus of liposome-encapsulated and free Ara-C, 10 mg/kg of both. The first dog received a mixture of ^{14}C labeled free Ara-C (500 μCi) and ^{3}H labeled encapsulated Ara-C (1 mCi), the second dog received ^{3}H-labeled encapsulated Ara-C (1 mCi) and the third dog received ^{3}H-labeled free Ara-C (1 mCi). All dogs received 900 μmol of liposomal lipid. In the first dog, both the urinary bladder and gall bladder were cannulated for sampling and biopsies from the liver were taken during the

course of the experiment. Sodium heparin (1.5 mg/kg) and normal saline solution were used to prevent clotting and maintain normovolemia respectively. Blood, bile and urine samples were collected in tubes containing 1 mM tetrahydrouridine. At the conclusion of the experiment, the animals were sacrificed and biopsies were taken from the major organ sytems.

B. Mice. Female DBA_2/CR mice (10-12 weeks old) were inoculated intravenously with 10^6 L_{1210} cells. Tissue distribution and metabolism studies of free and liposome-encapsulated Ara-C were performed three days after inoculation, when there was a leukemic infiltration in liver and spleen (9). The mice received 70 mg/kg of free or encapsulated ^3H-labeled Ara-C (66 μCi ^3H Ara-C and 5 μmol of liposomal lipid per mouse) and were sacrificed at 30 min or 3 h after injection. Blood was obtained via a cardiac puncture under light chloroform anesthesia. Tissues were collected and rapidly frozen in a mixture of methanol and dry ice.

Blood clearance and tissue distribution

Blood samples (0.1 ml) were dissolved in 1.2 ml NCS and weighed samples (10-50 mg) of various tissues were dissolved in 1 ml NCS. The solutions were bleached with 20% benzoyl peroxide in toluene (0.3 ml or 0.4 ml was added to tissue or blood samples respectively, followed by a 30 min incubation at 50°C).

Ara-C metabolism

A. Analysis of blood samples. Blood samples were centrifuged at 500 x g for 3 min and 0.5 ml plasma was extracted with 0.3 ml 6% perchloric acid and centrifuged at 1000 x g for 5 min. The supernatant was neutralized with 2 M KOH to pH 7.0. Aliquots of the acid soluble fractions were analyzed by high pressure liquid chromatography (HPLC). Ara-C plus phosphorylated derivatives were separated from Ara-U on a μ Bondapak C18 reverse phase column, 30 cm x 3.9 mm (inside diameter), 10 μm particle size (Waters Associates, Milford, MA). The column was eluted with 2.5 mM potassium phosphate buffer pH 7.0, containing 0.75% acetonitril. The retention times for Ara-C plus phosphorylated derivatives and Ara-U were 7.9 and 12.1 min respectively. The effluents were collected directly into counting vials.

B. Analysis of tissues samples. Frozen samples of liver and spleen were

homogenized with 1 ml of 12% perchloric acid per gram of tissue and centrifuged at 25000 x g (at 4°C) for 10 min. The acid soluble fraction was neutralized with 4 M KOH to pH 7.0. Separation of Ara-U from Ara-C plus phosphorylated derivatives was as described above. In the mouse experiments the intracellular nucleotide pools of Ara-C were separated using an HPLC method previously described (36). A Dupont model 830 equiped with an ABX anion exchange column (1 m x 2.1 mm, inside diameter) and a 254 and 280 nm detector was utilized for the separation and quantitation of Ara-CTP pools. The column was eluted with a phosphate buffer gradient (2.5 mM KH_2PO_4, pH 3.0 to 0.5 M KH_2PO_4, pH 4.5) at a rate of 0.3%/min and at a constant pressure of 600 psi, yielding a flow rate of 0.5 ml/min. Under these conditions the retention times of Ara-CMP, Ara-CDP and Ara-CTP were 4.1, 15.3 and 24.8 min respectively. The effluents were collected directly into counting vials. Radioactivity was measured in a Packard Tricarb Model 330 liquid scintillation spectrometer. The levels of Ara-C, Ara-U, Ara-C nucleotides and Ara-CTP in the tissues were based on the molar ratio between the metabolites as determined by HPLC and on radioactivity per gram of tissue.

RESULTS

Blood clearance of free and liposome-encapsulated Ara-C in dogs following intravenous injection

Figure 1 shows the clearance of radioactivity (Ara-C and metabolites) from the suprahepatic vein, after injection of free and liposome-encapsulated Ara-C into the portal vein. The corresponding levels of Ara-C plus phosphorylated derivatives and Ara-U are shown in Figure 2. The clearance of radioactivity from right atrium and femoral vein (not shown) followed essentially the same pattern. It can be seen that liposome-encapsulated Ara-C disappeared from the blood more slowly than free Ara-C. The concentration of Ara-U was low at all times for both free and encapsulated Ara-C and did not change greatly during the first hour after injection.

Tissue distribution of radioactivity following injection of free and liposome-encapsulated Ara-C in dogs

The time course of the uptake of radioactivity (Ara-C plus metabolites)

Fig. 1. (Upper panel). Blood clearance of free and liposome-encapsulated Ara-C in dogs. Blood levels of radioactivity (Ara-C and metabolites) were measured at various times after injection. The dogs received a mixture of 10 mg/kg free Ara-C and 10 mg/kg encapsulated Ara-C. The first dog (A) received a mixture of ^{14}C-labeled free Ara-C (500 μCi) and ^{3}H-labeled encapsulated Ara-C (1 mCi). The second and third dog (B) received ^{3}H-labeled encapsulated Ara-C (1 mCi) and ^{3}H-labeled free Ara-C (1 mCi) respectively. All dogs received 900 μmol of liposomal lipid. ●——●, encapsualted Ara-C; X——X free Ara-C.

Fig. 2. (Lower panel). Blood levels of Ara-C plus phosphorylated derivatives and Ara-U were measured at various times after injection of free and liposome-encapsulated Ara-C. For administered doses see legend to Fig. 1. Ara-C plus phosphorylated derivatives and Ara-U were extracted and quantitated as described in the methods section. ●——●, encapsulated Ara-C; X——X, free Ara-C; ●- -●, Ara-U from encapsulated Ara-C; X- - X, Ara-U from free Ara-C.

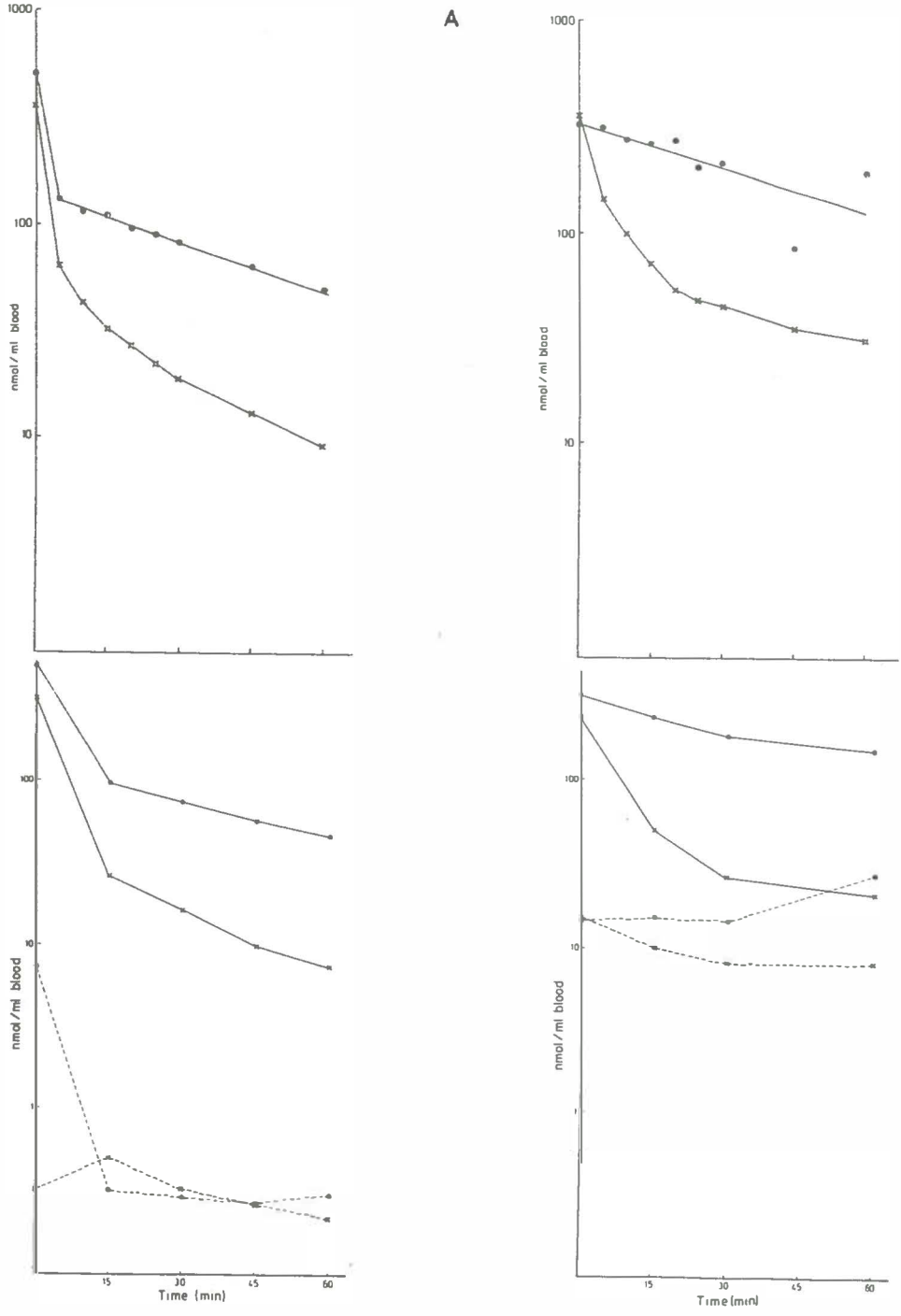
A
B
1000
100
10
1
nmol / ml blood
15
30
45
60
Time (min)

TABLE 1

TIME COURSE OF THE UPTAKE OF RADIOACTIVITY IN THE LIVER AFTER I.V. INJECTION OF FREE AND LIPOSOME-ENCAPSULATED Ara-C IN DOGS

The dog received a mixture of free ^{14}C-labeled Ara-C (10 mg/kg; 500 μCi) and ^{3}H labeled encapsulated Ara-C (10 mg/kg; 1 mCi; 900 μmol of liposomal lipid). At various times after injection liver biopsies were taken and analyzed for radioactivity (Ara-C and metabolites) as described in the methods section.

Tissue	Time after injection	Encapsulated Ara-C* (nmol/g) Dog I	Free Ara-C* (nmol/g) Dog I
Liver	15 min	244.5	159.3
	30 min	312.2	74.8
	1 h	249.9	40.1
	2 h	174.0	42.2
	3 h	170.8	24.0
	4 h	165.0	23.6

* The total of Ara-C and Ara-C metabolites

in the liver is shown in Table 1. The liposome-encapsulated Ara-C reached a maximum of 312.2 nmol per g at 30 min after injection and fell rapidly to 174 nmol per g at 2 h. After that, the decrease in radioactivity was very slow, about 4-5 nmol per gram per hour. Following injection of free Ara-C radioactivity was taken up by the liver quickly, the maximum level was reached before the first biopsy was taken and after that disappeared rapidly. The tissue distribution of radioactivity at 1 and/or 4 h after injection is shown in Table 2. The uptake of encapsulated Ara-C is highest in the liver (165.0 and 197.3 nmol per g) and spleen (546.0 and 719.9 nmol per g). After injection of free Ara-C the highest level is found in the kidneys (190.5 nmol per g).

Deamination of free and liposome-encapsulated Ara-C in the liver and spleen of dogs

The time course of the levels of Ara-C plus phosphorylated derivatives and Ara-U in the liver of the first dog is shown in Figure 3. After

TABLE 2

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER I.V. INJECTION OF FREE AND LIPOSOME-ENCAPSULATED Ara-C IN DOGS

The dogs received a mixture of free and liposome-encapsulated Ara-C; 10 mg/kg of both. The first dog (I) received free ^{14}C labeled Ara-C (500 μCi) and ^{3}H labeled encapsulated Ara-C (1 mCi), the second dog (II) received ^{3}H labeled encapsulated Ara-C (1 mCi) and the third dog (III) received ^{3}H-labeled free Ara-C (1 mCi). All dogs received 900 mol of liposomal lipid. The tissues were analyzed for radioactivity (Ara-C and metabolites) as described in the methods section. The numbers in parentheses refer to the dogs.

	Enc. Ara-C* (nmol/g)	Free Ara-C* (nmol/g)	
Time after injection	4 h	1 h	4 h
Liver	165.0 (I) 197.3 (II)	40.1 (I) 56.0 (III)	23.6 (I)
Spleen	546.0 (I) 719.9 (II)	35.8 (III)	1.8 (I)
Lung	14.0 (I)		21.1 (I)
Small Intestine	14.0 (I)		26.1 (I)
Kidney	71.6 (I)	190.5 (III)	
Pancreas	25.1 (I)	50.8 (III)	
Heart	26.5 (I)	46.5 (III)	
Muscle	17.2 (I)	67.7 (III)	

* The total of Ara-C and Ara-C metabolites

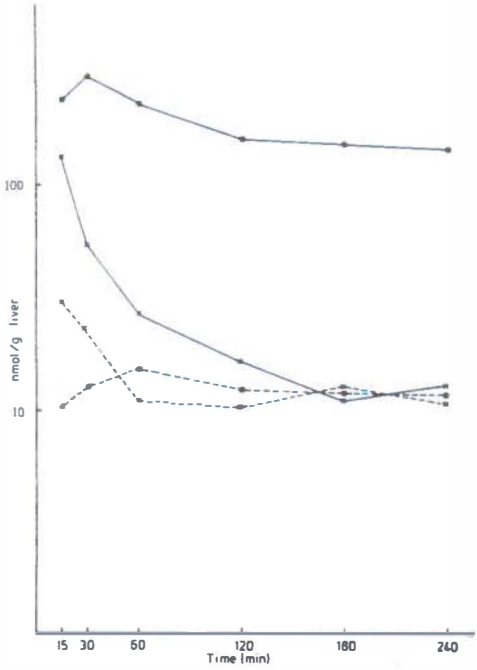

Fig. 3. Time course of Ara-C plus phosphorylated derivatives and Ara-U concentrations in the liver after injection of free and encapsulated Ara-C. Samples of the liver were taken at various times after injection of free and encapsulated Ara-C. For the administered dose see legend to Fig. 1. Ara-C plus phosphorylated derivatives and Ara-U were extracted and quantitated as described in the methods section. ●——● encapsulated Ara-C, X——X free Ara-C, ●---● Ara U from encapsulated Ara-C, X---X Ara-U from free Ara-C.

injection of the encapsulated Ara-C the highest level of Ara-C plus phosphorylated derivatives was reached at 30 min and the highest Ara-U level at 60 min. Following administration of the free Ara-C both Ara-C plus phosphorylated derivatives and Ara-U reached a maximum concentration before the first biopsy was taken. The fraction of radioactivity associated with Ara-U was higher after injection of the free Ara-C, and when encapsulated Ara-C was administered the production of Ara-U in the liver was delayed. Table 3 summarizes the levels of Ara-C plus phosphorylated derivatives and Ara-U in the liver and spleen of the dogs at 1 and/or 4 h after injection. It can be seen that the fraction of radioactivity associated with Ara-U is lower for

TABLE 3

LEVELS OF Ara-C PLUS PHOSPHORYLATED DERIVATIVES AND Ara-U IN LIVER AND SPLEEN OF DOGS AFTER I.V. INJECTION OF FREE AND LIPOSOME-ENCAPSULATED Ara-C

The dogs received a mixture of free and liposome-encapsulated Ara-C; 10 mg/kg of both. The first dog received ^{14}C-labeled free Ara-C (500 μCi) and ^{3}H-labeled encapsulated Ara-C (1 mCi). The second and third dog received ^{3}H-labeled encapsulated Ara-C (1 mCi) and ^{3}H-labeled free Ara-C (1 mCi), respectively. All dogs received 900 μmol of liposomal lipid. Tissue samples were extracted and Ara-C plus phosphorylated derivatives and Ara-U were separated and quantitated as described in the methods section.

		Dog I Encapsulated nmol/g		Dog I Free nmol/g		Dog II Encapsulated nmol/g		Dog III Free nmol/g	
		Ara-C and phosphorylated derivatives	Ara-U	Ara-C and phosphorylated derivatives	Ara-U	Ara-C and phosphorylated derivatives	Ara-U	Ara-C and phosphorylated derivatives	Ara-U
Liver:	1 h	234.9	15.0	26.1	14.0	N.D.*	N.D.	26.1	29.9
	4 h	153.6	11.5	12.5	11.1	167.5	29.7	N.D.	N.D.
Spleen:	1 h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	30.1	5.7
	4 h	534.9	11.1	1.4	0.4	690.9	28.6	N.D.	N.D.

* N.D.: Not Determinated.

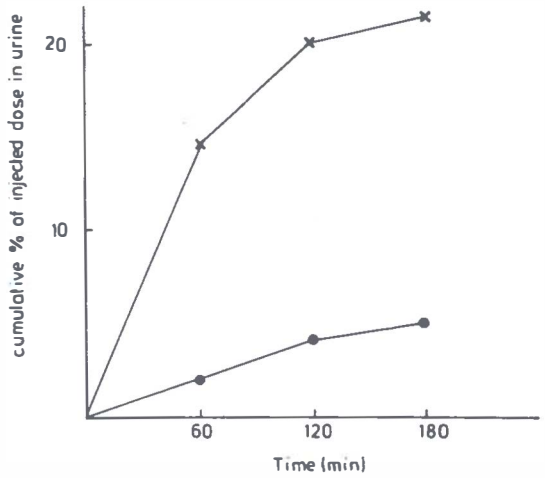

Fig. 4. Radioactivity excreted in Urine after injection of free and liposome encapsulated Ara-C. Urine was collected at various times after injection of free and encapsulated Ara-C and was analysed for radioactivity. For the administered dose see legend to Fig. 1. ●——● encapsulated Ara-C, X——X free Ara-C. The results are expressed as cumulative % of the injected dose.

the encapsulated Ara-C than for the free Ara-C. At 4 h after injection about 10% of the radioactivity of the encapsulated Ara-C in the liver is associated with Ara-U, while for the free Ara-C 50% is associated with Ara-U. Due to the excessive uptake of encapsulated Ara-C however, the absolute levels of Ara-U in the liver are reasonably similar.

Excretion of radioactivity in urine and bile

The excretion of radioactivity in the urine is shown in Figure 4. Three hours after injection of free Ara-C a total of 23% of the radioactivity was found in the urine, as compared to only 5% for the encapsulated Ara-C. For both free and encapsulated Ara-C small amounts of radioactivity were recovered from the bile (Fig. 5). It must be noted that excretion of radio-

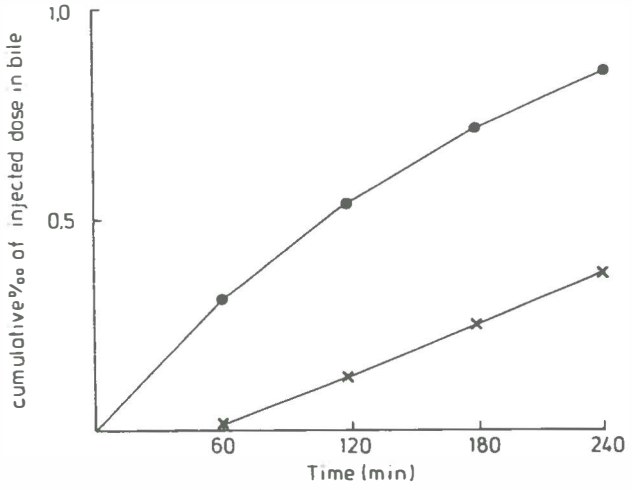

Fig. 5. Radioactivity excreted in bile after injection of free and liposome-encapsulated Ara-C. Bile was collected at various times after injection of free and encapsulated Ara-C and was analysed for radioactivity. For the administered dose see legend to Fig. 1. ●——● encapsulated Ara-C, X——X free Ara-C. The results are expressed as cumulative ‰ of injected dose.

activity in the bile, following administration of the encapsulated Ara-C continued during the period when the decline in the level of radioactivity in the liver was very slow (2-4 hrs after injection). More than 70% of the radioactivity in the bile was associated with Ara-C plus phosphorylated derivatives, as determined by HPLC, the rest represented Ara-U.

Tissue distribution and metabolism of free and liposome-encapsulated Ara-C in L_{1210} *bearing mice*

In order to examine whether liposome-encapsulated Ara-C in liver and spleen becomes available for phosphorylation to Ara-CTP, we measured Ara-C, Ara-U and Ara-CTP levels in mouse liver and spleen, infiltrated with Ara-C sensitive leukemic cells. Table 4 shows the tissue distribution of radio-

TABLE 4

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER I.V. INJECTION OF FREE AND LIPOSOME-ENCAPSULATED Ara-C IN LEUKEMIC MICE

The mice received an i.v. injection of 70 mg/kg free or liposome-encapsulated Ara-C (66 μCi ^{3}H-Ara-C and 5 μmol of liposomal lipid per mouse) and were sacrificed at 30 min or 3 h after injection. The tissues were analyzed for radioactivity (Ara-C and metabolites) as described in the methods section. The numbers are mean values ± S.D. of 3 mice

		REV Ara-C* (nmol/g)	FREE Ara-C* (nmol/g)
Blood	30 min	530.1 ± 23.4	65.2 ± 9.4
	3 h	155.8 ± 89.0	4.5 ± 0.8
Liver	30 min	1496.0 ± 90.0	351.0 ± 72.6
	3 h	975.5 ± 259.1	57.4 ± 17.2
Spleen	30 min	4002.9 ± 1920.6	417.7 ± 212.0
	3 h	2478.0 ± 826.7	39.7 ± 5.5
Lung	30 min	1407.1 ± 180.0	763.8 ± 230.8
	3 h	366.5 ± 125.5	40.6 ± 6.6
Kidney	30 min	1219.3 ± 17.2	829.0 ± 254.6
	3 h	342.8 232.1	38.1 ± 5.7
Small intestine	30 min	493.6 ± 27.5	499.8 ± 105.0
	3 h	203.4 ± 77.9	81.6 ± 40.2

* The total of Ara-C and Ara-C metabolites.

activity (Ara-C plus metabolites) at 30 min and 3 h after injection of free or liposome-encapsulated Ara-C. It can be seen that the uptake of Ara-C and metabolites by liver and spleen is several times higher after injection of encapsulated Ara-C. The Ara-C, Ara-U, and Ara-CTP levels in liver and spleen are shown in Table 5. After injection of encapsulated Ara-C, the fraction of

TABLE 5

Ara-C, Ara-U, Ara-C NUCLEOTIDES AND Ara-CTP LEVELS IN LIVER AND SPLEEN OF LEUKEMIC MICE AFTER INJECTION OF FREE OR LIPOSOME-ENCAPSULATED Ara-C

The mice received an i.v. injection of 70 mg/kg free or liposome-encapsulated ^{3}H-labeled Ara-C (66 μCi, ^{3}H-Ara-C, 5 μmol of liposomal lipid per mouse) and were sacrificed at 30 min or 3 h after injection. Tissues were extracted as described in the methods section. The numbers are mean values ± S.D. of 3 mice.

		Ara-C nmol/g	Ara-U nmol/g	Nucleotides nmol/g	Ara-CTP nmol/g
REV Ara-C	30 min	1261.7 ± 76.8	171.1 ± 13.1	66.2 ± 4.5	35.4 ± 6.3
Liver	3 h	849.3 ± 209.2	65.5 ± 43.4	61.1 ± 18.3	22.5 ± 6.7
FREE Ara-C	30 min	124.3 ± 27.7	110.6 ± 44.3	75.6 ± 15.4	16.1 ± 3.8
Liver	3 h	5.9 ± 1.2	12.8 ± 3.5	38.8 ± 13.0	3.6 ± 0.7
REV Ara-C	30 min	3592.3 ± 1232.4	262.8 ± 87.5	147.5 ± 62.0	52.1 ± 6.4
Spleen	3 h	2220.7 ± 722.4	139.2 ± 60.9	117.2 ± 44.4	42.9 ± 21.8
FREE Ara-C	30 min	169.3 ± 82.1	154.3 ± 72.9	94.0 ± 53.1	44.1 ± 22.3
Spleen	3 h	10.7 ± 7.0	9.7 ± 2.0	19.4 ± 3.8	3.2 ± 0.4

radioactivity in liver and spleen, associated with Ara-U and Ara-CTP, is lower but the Ara-CTP concentrations are higher than after injection of free Ara-C. Moreover the encapsulated Ara-C generates a sustained level of Ara-CTP in the liver and spleen.

DISCUSSION

Ara-C in dogs is cleared by the kidneys and deaminated in the liver, although deamination is not as fast as in monkey or human liver (11,13,14,36). Our data clearly indicate that liposome-encapsulated Ara-C in dogs is to a considerable degree protected against deamination in the liver and from excretion in the urine. As has been shown previously in mice (24,37), liposome-encapsulated Ara-C is cleared from the blood more slowly than free Ara-C and the uptake of encapsulated Ara-C is highest in liver and spleen.

There was a considerable difference between the dogs, in the disappearance of liposomes from the blood. It is known that large liposomes are cleared more quickly than small liposomes (38). However, it is not very likely that this would explain the difference between the dogs in our experiment, since the liposomes we used were passed through a Unipore polycarbonate filter (0.4 μm) and, therefore, were of a relatively uniform size (35). The dogs weighed 17 kg and 20.4 kg with liver weights of 550 g and 300 g, respectively. Biozzi *et al.* (39) reported on the positive correlation between liver and spleen size and the rate of clearance of colloidal particles from the blood. Thus, the differences in liver size between the dogs could account for the observed difference in clearance rate of liposome-encapsulated Ara-C. This implies that if liposomes are to be used clinically the effect of the relative liver and spleen size on the clearance rate of colloidal particles has to be taken into account.

The relative Ara-U production in the liver was delayed after injection of liposome-encapsulated Ara-C as compared to free Ara-C. There was a good agreement between the dogs as far as protection against deamination was concerned. In all probability the rate at which Ara-C is released from the vesicles is the slow step regulating the *in vivo* metabolism and pharmacokinetics of encapsulated Ara-C. The delayed production of Ara-U, the continuous appearance of radioactivity in the bile and the existence of a steady state Ara-U level

in the liver all suggest that Ara-C is gradually released from the vesicles. It is only after Ara-C is released that it becomes available for deamination and/or would be able to be taken up and phosphorylated to the active metabolite Ara-CTP if sensitive tumor cells were present.

The existence of two phases in the disappearance of encapsulated Ara-C plus phosphorylated derivatives from the liver, a rapid fall in concentration between 30 min and 2 h and a very slow decrease after 2 h may indicate that liposomes are bound by the liver at two different locations and as a consequence release their Ara-C at different rates. Although it is clear that liposomes are taken up by Kupffer cells, (31,32,33) there is evidence that Kupffer cell phagocytosis is not the only mechanism that is responsible for the retention of liposomes by the liver (40). Data not shown here indicate that part of the liposomes might be adsorbed to cell surfaces, without actually being phagocytosed. If indeed liposomes at different locations release their Ara-C at different rates, then the balance between uptake of vesicles by Kupffer cells and adsorption to Kupffer or other cells will determine the overall rate at which the encapsulated drug is released from the vesicles. Uptake of liposomes by Kupffer cells provides an extra barrier between the cytotoxic agent and the tumor cells and also may result in toxicity to the macrophages, although this would not be so for Ara-C, since Ara-C is toxic only to cells synthesizing DNA and Kupffer cells do not have a very active DNA synthesis (41). The balance between uptake and adsorption of liposomes in the liver can presumably be influenced by the dose of liposomes or by saturating the macrophages with empty liposomes before injection of the drug containing liposomes (40,42). The rate at which Ara-C or any other drug encapsulated in liposomes becomes bioavailable, will also depend on the type and lipid composition of the vesicles (43).

Hunt *et al.* (37) observed a rapid excretion of Ara-C (or Ara-C metabolites) in the urine during the first hour after injection of liposome-encapsulated Ara-C in mice. After that the excretion rate declined. As a possible explanation for this phenomenon the authors mention the interaction of liposomes with proteins, resulting in a rapid leakage of Ara-C from the outer few aqueous layers of multilamellar vesicles, where the trapped volume per layer is largest, followed by a slow equilibration of inside and leaky

outside layers. Since we used reverse phase evaporation vesicles which are largely uni- or oligolamellar, and contain most of their trapped volume in the inner compartment and as a consequence probably release their entrapped Ara-C all at once our observations on Ara-C levels in the liver cannot be explained by a similar reasoning.

In order to inhibit 90% of the DNA synthesis of L_{1210} cells growing in the liver of mice, the tissue level of Ara-CTP has to be 1 nmol/g (5). In our experiments the rate of decrease in the level of Ara-C (or phosphorylated derivatives) in the dog liver, after 2 h is approximately 4-5 nmol/g per h. If we assume that (1) 4-5 nmol/g per h becomes available for deamination and interaction with tumor cells, (2) the data for L_{1210} tumor cells hold for other Ara-C sensitive tumor cells as well and (3) most of the Ara-C taken up by Ara-C sensitive tumor cells is phosphorylated to the active metabolite Ara-CTP, then a release of 4-5 nmol/g per h would result in a significant inhibition of DNA synthesis of tumor cells in the liver, over a period as long as this release would continue, even if part of the Ara-C was deaminated. The aim would be to deliver a substantial cytotoxic level of Ara-C in the liver over a period long enough so that all cells in the susceptible tumor cell population would be inhibited as they underwent DNA synthesis. It should be noted that the dose used in our experiments with dogs, 10 mg of encapsulated Ara-C/kg bodyweight, is a dose succesfully used in the treatment of L_{1210} bearing mice (27-29).

The sustained levels of liposomal Ara-C, Ara-U, Ara-C nucleotides and Ara-CTP in leukemic mouse liver and spleen and the small fraction of liposomal Ara-C that becomes phosphorylated (compared to free Ara-C) concomitant with a marked protection against deamination all indicate that Ara-C is gradually released from the liposomes and subsequently becomes available for deamination and phosphorylation. Rustum *et al.* (44) recently reported that it was not possible to overcome Ara-C resistance in L_{1210} cells with encapsulated Ara-CTP, indicating that the cells do not internalize liposomes. In all probability the Ara-C in leukemic mouse liver and spleen is released from the liposomes either extracellularly (*i.e.* adsorbed to tumor cells or other cells) or inside cells other than tumor cells (*i.e.* Kupffer cells in the liver, macrophages in the spleen).

Our results show that liposomes protect Ara-C from deamination and might act as a local depot for Ara-C in both liver and spleen. However, their usefulness in the treatment of Ara-C sensitive tumors in liver and spleen, remains to be established. Although we have in this report only investigated Ara-C, in principle similar local depot effects could be expected for the treatment of tumors in liver and spleen, sensitive to different drugs.

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CHAPTER VI

EFFECT OF LIPOSOME-ENCAPSULATION ON ANTITUMOR ACTIVITY OF ADRIAMYCIN AND ARABINOFURANOSYLCYTOSINE IN THE TREATMENT OF LYMPHOSARCOMA BEARING MICE

SUMMARY

The antitumor activities of free and liposome-encapsulated adriamycin (ADM) and arabinofuranosylcysotine (Ara C) were examined in lymphosarcoma-bearing mice. The antitumor activity of free and entrapped ADM was measured as the effect on the weights of tumorous liver and spleen seven days after inoculation of 10^6 tumor cells and on the survival time of the tumor mice. Liposome-encapsulation resulted in a *decrease* of the antitumor activity of ADM measured as the effect on weights of tumorous liver and spleen and on the survival time. Free nor encapsulated Ara C had any effect on the survival of lymphosarcoma-bearing mice, while *in vitro* uptake of the drug in the lymphosarcoma cells was very low. This is taken to indicate that the resistance to this drug is caused by an insufficiency in Ara C uptake, which could not be overcome by liposome-encapsulation. *In vivo*, evidence was obtained that lymphosarcoma cells do not take up liposomes: after intravenous injection liposome-entrapped peroxidase activity could never be observed in tumor cells. It is concluded therefore that antitumor effects of encapsulated drugs on this tumor must be attributed to drug being released from the liposomes and subsequently taken up by tumor cells rather than to internalization of liposomes by tumor cells *per se*.

INTRODUCTION

Liposomes have attracted a great deal of attention as a carrier system for antineoplastic agents over the past decade (1-3). Only in a few instances, however, systemic application of liposome-encapsulated drugs resulted in an enhanced therapeutic index compared to a similar dose of free drug, *e.g.* the treatment of L_{1210} leukemia in mice with arabinofuranosylcytosine

(Ara C) and adriamycin (ADM) (4-9).

Adriamycin, an anthracycline antibiotic, exhibits antitumor activity against a wide variety of animal and human tumors. Its usefulness, however, is limited by its potentially fatal toxicity. The drug has two kinds of toxic effects: an acute high dose effect involving myelosuppression (10) and a chronic low dose effect which is cumulative and results in irreversible cardiomyopathy (11).

Coupling of ADM to a macromolecular carrier such as DNA (12,13) or encapsulation of ADM in liposomes (9,14) has been shown to alter its distribution and to result in a decreased accumulation in cardiac tissue (9,15). This is likely to be the reason why encapsulation in liposomes results in a protection against both acute and chronic toxicity of ADM. In mice the LD_{50} was increased two-fold for both kinds of toxicity (8), while the antitumor activity against L_{1210} leukemia was not altered (8,14), thus resulting in an increased therapeutic index. In another study the activity of encapsulated adriamycin against L_{1210} leukemia was even shown to be increased (9).

Ara C is a cell cycle specific (S phase) antitumor drug with activity against animal and human tumors. The drug is converted intracellularly to the active metabolite arabinofuranosylcytosine triphosphate (Ara CTP) which inhibits DNA replication (15). The efficacy depends on the maintenance of sufficiently high levels of Ara CTP in the target cells for a period sufficiently long to inhibit DNA synthesis of (nearly) all tumor cells.

In this chapter preliminary experiments are presented on the antitumor activity of free and encapsulated ADM and Ara C against a murine lymphosarcoma. Earlier, Konings and coworkers (16) showed that in vivo treatment with free ADM or Ara C causes a substantial inhibition of the incorporation of ^{3}H-thymidine into the DNA of these tumor cells in the spleen. To gain more insight into the mechanism of antitumor activity of drug-containing liposomes, we investigated the capacity of lymphosarcoma cells to take up liposomes, *in vitro* as well as *in vivo*.

MATERIALS AND METHODS

Cholesterol was obtained from A.B. Fluka (Buch, Switzerland) and was recrystallized twice from methanol. Dipalmitoylphosphatidylcholine and dipal-

mitoylphosphatidylglycerol were from Avanti. Egg phosphatidylcholine and egg phosphatidylglycerol were a gift from Dr. Mayhew and were prepared as described in ref. 6. α-Tocopherol was purchased from Supelco (Bellefante, Pa.). Bovine brain sphingomyelin was from Sigma. Unipore polycarbonate filters were from Biorad. Ficoll and Sepharose 2B-Cl were from Pharmacia. Arabinofuranosylcytosine was obtained from Sigma and adriamycin from Farmitalia (Milan, Italy). 5-^{3}H arabinofuranosylcytosine (spec. act. 10.2 Ci/mmol) and 5-^{3}H tymidine (spec. act. 17.7 μCi/mmol) were from the Radiochemical Centre Amersham. RPMI 1640 medium was from Flow Laboratories. Horse-radish peroxidase grade II and 3,3'-diaminobenzidine tetrahydrochloride (DAB) grade II were from Sigma. The diammonium salt from 2,2'-azino-di-(3-ethylbenzthiazolin sulphonic acid) (ABTS) was from Boehringer.

Preparation of liposomes

Adriamycin-containing multilamellar liposomes

The vesicles were prepared essentially as described by Olson *et al.* (8). ADM was dissolved in methanol and was mixed with egg phosphatidylglycerol, egg phosphatidylcholine, cholesterol and α-tocopherol dissolved in a 1:4:4:0.09 molar ratio in chloroform. α-Tocopherol was added to protect the lipid against oxidation (18). Per 60 μmol of total lipid 1.5 mg of adriamycin was added. The lipid mixture with ADM was dried on a rotary evaporator. To the dry lipid film 1 ml of phosphate buffered saline (PBS, 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) was added per 60 μmol total lipid and the mixture was shaken overnight in a 37^{o}C waterbath. The liposomes thus formed were extruded through polycarbonate membranes with 0.4 μm pores (18). The liposomes were dialyzed overnight at 37^{o}C against three changes of PBS to remove non-encapsulated ADM. The amount of encapsulated ADM was determined by fluorescence (19).

Ara-C containing reverse phase evaporation vesicles

The vesicles were prepared essentially as described by Szoka and Papahadjopoulos (20). A chloroform solution of dipalmitoylphosphatidylglycerol, dipalmitoylphosphatidylcholine and cholesterol (molar ratio 1:4:5) was evaporated to dryness on a rotary evaporator. The dry lipid film was dissolved to a final concentration of 10 mM in a 1:1 mixture of di-isopropylether and

chloroform. The di-isopropylether was redistilled in the presence of sodium-bisulfite immediately before use. Per 6 ml of the organic phase 1 ml of a 345 mM Ara C solution in 10% PBS was added, (containing a trace amount of ^{3}H-labeled Ara C). The mixture was sonicated for 5 min under a N_2 atmosphere in a bath type sonicator (at 0^oC). Thus a homogeneous suspension of inverted micelles was formed. The organic phase was removed in a rotary evaporator and the vesicles obtained were extruded through polycarbonate membranes with 0.4 μm pores (21). The vesicles were dialyzed overnight at 4^oC against three changes of PBS to remove non-encapsulated Ara C. The amount of encapsulated Ara C was calculated from liposome-associated radioactivity.

Horse-radish peroxidase containing reverse phase evaporation vesicles

A chloroform solution of sphingomyelin/cholesterol/phosphatidylserine (4:5:1) was evaporated to dryness. The dry lipid was dissolved to a final concentration of 20 mM in diethylether. The diethylether was redistilled in the presence of sodiumbisulfite immediately before use. Per 3 ml ether 1 ml of Tris-buffered saline (0.15 M NaCl, 5 mM Tris, pH 7.4) was added, containing 30 mg horse-radish peroxidase per ml. The mixture was sonicated under N_2 in a bath type sonicator, at room temperature, until an apparently stable suspension was formed. The organic phase was removed on a rotary evaporator and the vesicles obtained were extruded through polycarbonate membranes with 0.4 μm pores. Peroxidase containing liposomes were separated from non-entrapped enzyme by gel chromatography on Sepharose 2B-Cl. Peroxidase activity associated with the liposomes was measured by a modification of the method of Steinman and Cohn (22) as described by Roerdink *et al.* (23). ABTS was used as a chromogen. Latency of the entrapped enzyme is defined as:

$$\frac{\text{free enzyme activity (measured in the absence of Triton X-100)}}{\text{total enzyme activity (measured in the presence of 1\% Triton X-100)}} \times 100\%$$

The latency found for the vesicles used was 94%.

In vitro studies

L_{1210} cells, maintained by intraperitoneal passage in DBA_2/CR mice, were used on day six after injection of 10^6 cells. Lymphosarcoma cells were used on day

seven after injection of 10^6 cells (see below). Tumor cells were incubated at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2% HEPES (pH 7.4). Ara C uptake and phosphorylation in L_{1210} cells and in lymphosarcoma cells were measured as follows. Tumor cells (10^7/ml) were incubated in medium (5 ml) containing 5 µg/ml Ara C and 4 µCi/ml ^{3}H-Ara C. After 30 min the cells were pelleted and washed twice by centrifugation with ice-cold PBS. The cell pellet was extracted with 100 µl 6% perchloric acid. The acid-soluble fraction was assayed for radioactivity (total uptake of Ara C including metabolites). The extract was then neutralized with 2 M KOH and was analyzed by HPLC for Ara CTP as described in chapter V.

The effect of free and encapsulated Ara C on the ^{3}H-thymidine incorporation into DNA was measured as follows (24). Lymphosarcoma cells ($5x10^6$/ml) were incubated in medium (1 ml) with or without various concentrations of free Ara C or Ara C encapsulated in REV (composed of bovine brain phosphatidylserine/egg phosphatidylcholine/cholesterol 1:4:5). The final liposomal lipid concentration in all incubations was 0.2 µmol total lipid/ml. In control incubations and in incubations with free Ara C "empty" liposomes were added not containing any Ara C. At 30 or 60 min 0.5 µCi ^{3}H-thymidine was added (30 min was found to be a sufficiently long period for Ara C to penetrate into these cells (16)). The ^{3}H-thymidine incorporation was stopped 15 min later by addition of 1 ml ice-cold Tris-buffered saline, containing 2 mM unlabeled thymidine (Sigma) and followed by immediate centrifugation at 4°C. The pellet was resuspended in 6% perchloric acid and was washed three times with 6% perchloric acid. The precipitate was dissolved in Lumasolve (Baker Chemicals, Holland) and assayed for radioactivity with Lipoluma (Baker Chemicals) as a scintillation fluid.

Leakage of Ara C from the liposomes during the incubation was measured as follows. ^{3}H-Ara C containing liposomes (0.2 µmol total lipid/ml) were incubated at 37°C for 30 min or 60 min in medium with $5x10^6$ lymphosarcoma cells/ml. The cell suspension was then layered on a 20% Ficoll solution in Tris-buffered saline and centrifuged for 10 min at 1400xg. The supernatant with the liposomes and the free Ara C was chromatographed on Sepharose 2B-Cl.

Tumor model

A lymphosarcoma which originated spontaneously in a C57Bl mouse (25) was transplanted weekly. The lymphosarcomatous spleen was excised and squeezed through nylon gauze into ten parts of ice-cold PBS. The single-cell suspension thus obtained was used for tumor transplantation. Mice received 10^6 cells intraperitoneally. For a detailed description of this tumor model the reader is referred to chapter I.

Therapeutic effect of free and encapsulated adriamycin and arabinofuranosylcytosine.

Groups of C57Bl/6J mice were inoculated intraperitoneally with 10^6 tumor cells and were treated 48 h later with either PBS, various doses of free or encapsulated ADM (female mice) or various doses of free or encapsulated Ara C (male mice). Half the number of the mice from the groups treated with ADM were sacrificed seven days after inoculation and liver and spleen were excised and weighed. For trivial reasons, in the experiments with Ara C male mice were used, while the ADM experiments were done with female mice. Mice were observed for 75 days and the day of death was recorded.

Microscopic studies

Horse-radish peroxidase containing REV were injected intravenously into 3 months old (C57Bl x Gr)F_1 mice. The mice received 2.8 μmol of liposomal lipid, containing 0.13 mg peroxidase. Two types of controls were included: (1) mice receiving neither liposomes nor peroxidase and (2) mice receiving liposomes mixed with free peroxidase. After 2 h the mice were anesthetized with ether and the liver was perfused *in situ* (flow rate 2 ml/min), at room temperature, via the portal vein, first with cacodylate buffer (0.1 M with 2% polyvinylpyrrolidone and 0.4% $NaNO_2$, pH 7.4) for 1 min to remove blood, then with 2% glutaraldehyde in 0.1 M cacodylate buffer for 7-8 min to fix the tissue and finally again with cacodylate buffer to remove the fixative. From the liver 30-40 μm vibratome sections (Oxford vibratome (R) sectioning system) were cut. The sections were thoroughly washed with PBS and incubated for peroxidase reaction with 0.075% DAB and 0.001% H_2O_2 in PBS, for 30 min in the dark at 37°C. The sections were washed in PBS and cacodylate buffer (0.1 M with 6.8% sucrose, pH 7.4) and post-fixed with 1% O_sO_4 in cacodylate

buffer, dehydrated in an ethanol series and embedded in Epon 812. Semithin (1 μm) and ultrathin sections were cut (LKB ultratome). Semithin sections were stained with toluidine blue. From these sections areas were selected for ultrathin sectioning. Ultrathin sections were double-stained with uranyl acetate and lead citrate and examined in a Philips EM 300 microscope.

RESULTS

Antitumor activity of free and encapsulated adriamycin

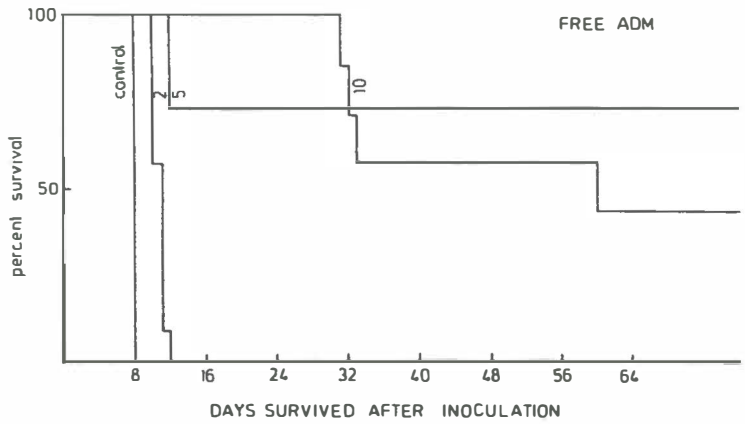
Lymphosarcoma bearing mice were divided into seven groups of 14 animals and injected intraperitoneally with free or liposome-encapsulated adriamycin. The control mice received the same volume of PBS. Half the number of mice of each group were sacrificed seven days after tumor inoculation and liver and spleen were excised and weighed. Table 1 shows that the lymphosarcoma cells were very sensitive to the free drug. A dose as low as 2 mg/kg already showed an effect on tumor growth in liver and spleen. The weights of the organs in the treated mice were clearly below those in the untreated controls. In the groups treated with 5 and 10 mg/kg the effect was even more

TABLE 1

EFFECT OF FREE AND ENTRAPPED ADRIAMYCIN ON THE GROWTH OF LYMPHOSARCOMA CELLS IN LIVER AND SPLEEN.

Tumor cells were inoculated intraperitoneally in female C57Bl/6J mice and the animals were injected intraperitoneally with the drugs 48 h later. The mice were sacrificed at day 7, liver and spleen were excised and weighed. The numbers are means ± S.D. of seven mice.

Group	Treatment	Liver weight (mg)	Spleen weight (mg)
1	0.2 ml PBS	2081 ± 274	706 ± 85
2	2 mg/kg free ADM	1362 ± 302	341 ± 147
3	5 mg/kg free ADM	1020 ± 138	92 ± 16
4	10 mg/kg free ADM	1007 ± 124	58 ± 8
5	5 mg/kg ADM-MLV	1361 ± 192	378 ± 50
6	10 mg/kg ADM-MLV	1108 ± 131	88 ± 24
7	20 mg/kg ADM-MLV	1007 ± 102	62 ± 6



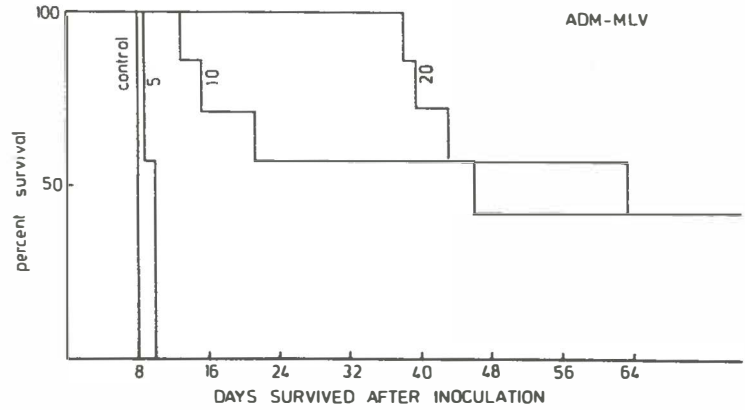


Fig. 1. Antitumor activity of free and encapsulated adriamycin. Tumor cells were inoculated intraperitoneally in female C57Bl/6J mice. The mice were treated intraperitoneally 48 h later.

pronounced. Adriamycin in multilamellar vesicles had a similar effect. However, the dose required to bring about the same effect as free adriamycin was doubled. In Fig. 1 the survival of the other seven mice in each group is shown. The control mice died eight days after tumor transplantation. The average survival times of mice treated with 2 mg/kg free adriamycin and 5 mg/kg encapsulated adriamycin were 10.7 and 9.6 days respectively. In the groups treated with the higher doses there were long-term survivors (more than 75 days).

TABLE 2

ANTITUMOR EFFECT OF FREE AND ENCAPSULATED ARABINOFURANOSYLCYTOSINE

Tumor cells (10^6) were inoculated intraperitoneally in male C57Bl/6J mice. The mice were injected intraperitoneally with the drugs 48 h later.

Group	Treatment	Average survival time (days)
1	0.2 ml PBS	9.2 ± 0.5
2	100 mg/kg free Ara C	9.4 ± 0.6
3	500 mg/kg free Ara C	10.2 ± 0.5
4	10 mg/kg REV Ara C	9.2 ± 0.5
5	20 mg/kg REV Ara C	8.8 ± 0.5
6	40 mg/kg REV Ara C	10.4 ± 1.5

Antitumor activity of free and encapsulated arabinofuranosylcytosine

Treatment with a single i.p. dose of free or encapsulated Ara C had no effect on the survival time of lymphosarcoma bearing mice (Table 2). It should be noted that the same dose of encapsulated Ara C was very effective against Ara C-sensitive L_{1210} leukemia (6). In comparison to the L_{1210} cells, the *in vitro* Ara C uptake in the lymphosarcoma cells was very low and also the Ara CTP level was almost negligible (Table 3) indicating that insufficiency in uptake is a likely cause of the observed resistance of the lymphosarcoma cells to this drug. Differences in sensitivity of various tumor cell types to Ara C have been related to differences in intracellular Ara CTP pools (24). Drug resistance due to uptake deficiency could conceivably be overcome by encapsulation of the drug in liposomes, provided that

TABLE 3

ARA C UPTAKE AND PHOSPHORYLATION IN LYMPHOSARCOMA CELLS AND L_{1210} CELLS

Tumor cells ($5x10^7$) were incubated at 37°C in 5 ml RPMI 1640, containing 10% heat inactivated fetal calf serum, 2% HEPES, 5 µg/ml Ara C and 4 µCi/ml ^3H Ara C. At 30 min the cells were washed and extracted as described in the methods section. The acid-soluble fraction was assayed for radioactivity and analyzed by HPLC for Ara CTP.

	Ara C, including metabolites (pmol/10^7 cells)	Ara CTP (pmol/10^7 cells)
lymphosarcoma cells	7.2	3.1
L_{1210} cells	208	178

the liposomes as such are taken up by the cells (26). However, the results presented in this chapter show that, at least in our tumor model, this is not the case (Table 2). The *in vivo* observations summarized in this table are consistent with results on the *in vitro* inhibition of thymidine incorporation by free and encapsulated Ara C, which demonstrated that encapsulated Ara C caused less inhibition than free Ara C (Table 4). In cells incu-

TABLE 4

INHIBITION OF ^3H-THYMIDINE INCORPORATION INTO DNA OF LYMPHOSARCOMA CELLS BY FREE AND ENCAPSULATED ARA C

Lymphosarcoma cells ($5x10^6$/ml) were incubated at 37°C in RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2% HEPES and various concentrations of free or encapsulated Ara C. At 30 min or 60 min ^3H-thymidine incorporation was measured as described in materials and methods.

		% inhibition	
	drug concentration (mM)	30 min	60 min
control	-	0	0
free Ara C	10^{-5}	91.3	91.8
	10^{-6}	49.7	57.1
	10^{-7}	8.6	26.9
REV Ara C	10^{-5}	62.6	69.0
	10^{-6}	31.3	40.3
	10^{-7}	11.7	17.2

bated with encapsulated Ara C, the inhibition of thymidine incorporation into DNA could even be partly (or completely) accounted for by Ara C released from the vesicles into the medium during the incubation. After 30 and 60 min of incubation the leakage from the liposomes was 30.3% and 33.6% respectively. Consequently, in the incubations with 10^{-5}, 10^{-6} and 10^{-7} M encapsulated Ara C, the concentration of free Ara averaged approximately 3x 10^{-6}, $3x10^{-7}$ and $3x10^{-8}$ M, respectively.

Light and electron microscopic localization of liposome-entrapped horseradish peroxidase activity

Peroxidase-containing REV were injected in normal mice and in mice one or six days after intraperitoneal injection of 10^6 lymphosarcoma cells. Fig. 2 shows the light microscopical distribution of the horse-radish peroxidase reaction product. 2 h after injection of the liposomes in a normal mouse. The reaction product was concentrated in the Kupffer cells, but could also be detected in endothelial cells and hepatocytes. When free peroxidase mixed

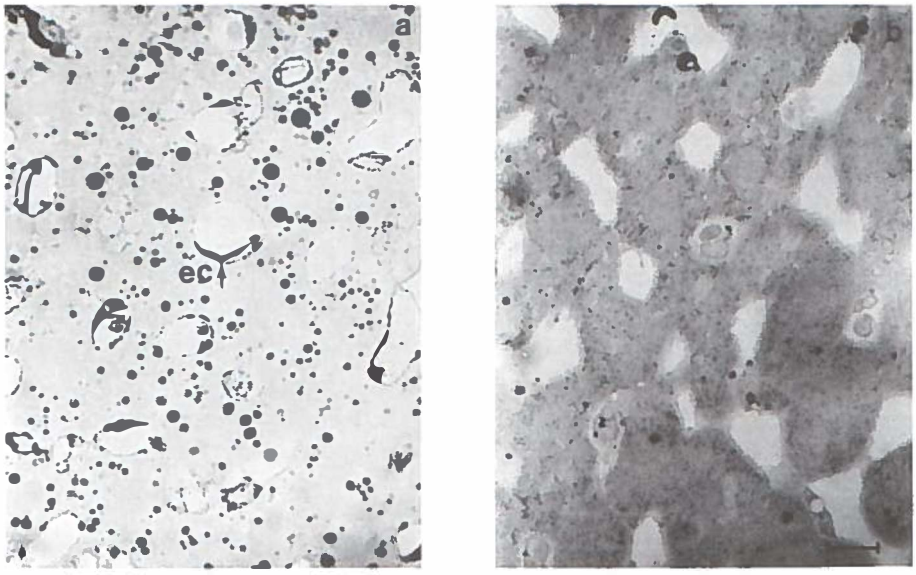


Fig. 3. Livers of normal mice 2 h after intravenous injection with "empty" liposomes mixed with free peroxidase (2.8 μmol of liposomal lipid and 0.13 mg peroxidase)(a). Peroxidase reaction product is present exclusively in endothelial cells (ec). Peroxidase reaction product is negligible in the liver of a mouse that did not receive liposomes or peroxidase (b). Bar is 10 μm (Courtesy of Dr. C.E. Hulstaert).

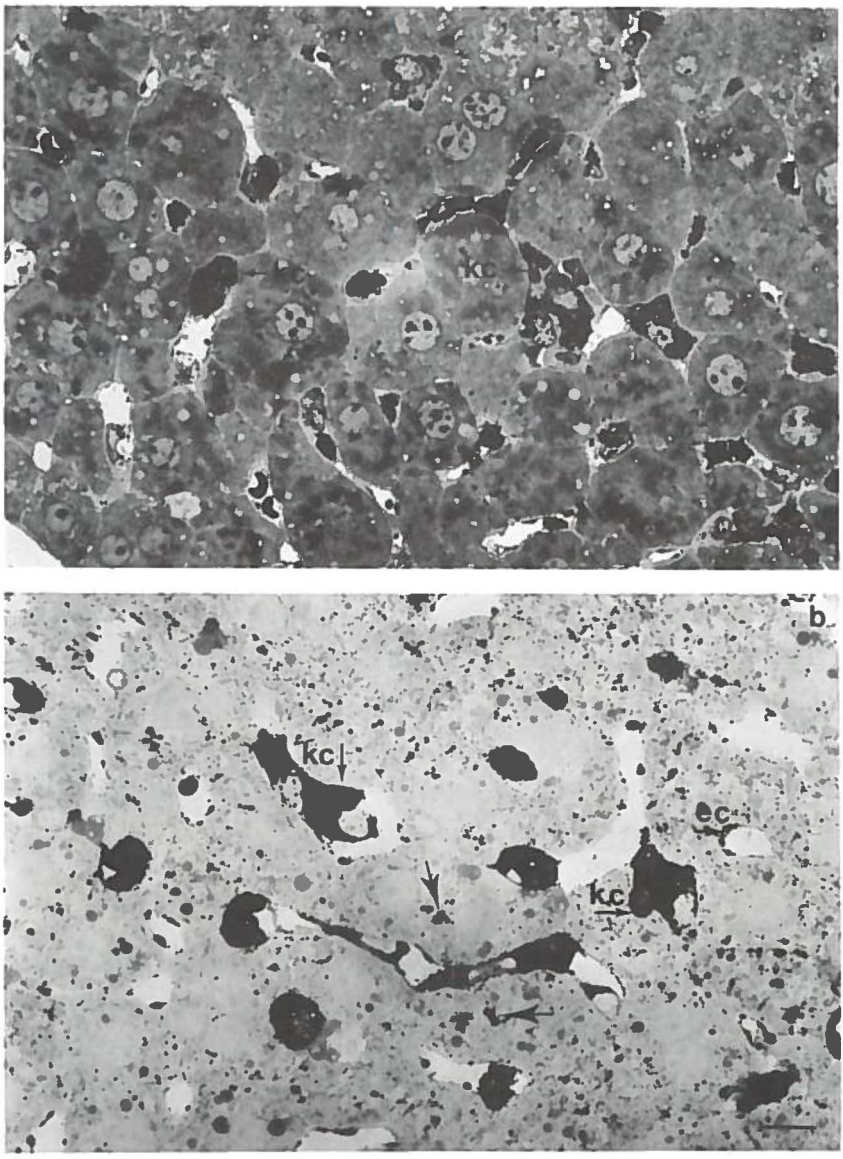


Fig. 2. Liver of a normal mouse 2 h after intravenous injection of liposomes with entrapped peroxidase (2.8 μmol of liposomal lipid containing 0.13 mg peroxidase). Peroxidase reaction product, in 1 μm sections counter-stained with toluidine blue (a), or without counter-staining (b), is conspicuously present in Kupffer cells (kc) but can also be detected in endothelial cells (ec) and in hepatocytes (arrows). Bar is 10 μm (Courtesy of Dr. C.E. Hulstaert).

with empty liposomes was injected, peroxidase reaction product was detected in endothelial cells, but not in Kupffer cells or hepatocytes (Fig. 3). The endogenous peroxidase activity of Kupffer cells can easily be distinguished from the exogenous enzyme on the basis of a negligible low staining intensity and entirely different localization (nuclear envelope, endoplasmic reticulum) of the endogenous enzyme.

Fig. 4. shows the light microscopical distribution of peroxidase reaction product in the liver of a mouse six days after injection of the tumor cells. The distribution of the reaction product is essentially similar to that observed in normal mice. Tumor cells are clearly visible in the sinusoids, but contain no peroxidase reaction product. Apparently, the tumor cells do not take up liposome-encapsulated or free peroxidase, while in Kupffer cells, located in close proximity to the tumor cells, peroxidase reaction product is abundantly present. In Fig. 5 electronmicrographs are shown of the livers of a normal and a six-day tumor mouse that received peroxidase-containing liposomes. Peroxidase activity is located in membrane-bound vacuoles in the cytoplasm of Kupffer cells. In agreement with our light microscopical observations no peroxidase activity was detected in the tumor cells. Light and electronmicroscopic observations on the location of the peroxidase reaction product in the liver of one day tumor mice (results not shown) were similar to those made on normal and six-day tumor mice.

DISCUSSION

Light and electronmicroscopic observation of the livers of mice that received liposomes with entrapped peroxidase revealed that the peroxidase reaction product was concentrated in the Kupffer cells but could also be detected in endothelial cells and hepatocytes. Comparison with the adequate controls (Fig. 3) revealed that the presence of peroxidase in the Kupffer cells and presumably also in the hepatocytes could be attributed to uptake of liposome-entrapped enzyme since free peroxidase at this dose is seen only in endothelial cells. The liposomes were prepared as described by Szoka *et al.* (20,21) and according to these authors a fraction of the liposomes will have a diameter smaller than 100 nm. They could therefore, in theory, be able to pass the fenestrations in the endothelial sieve plates and subse-

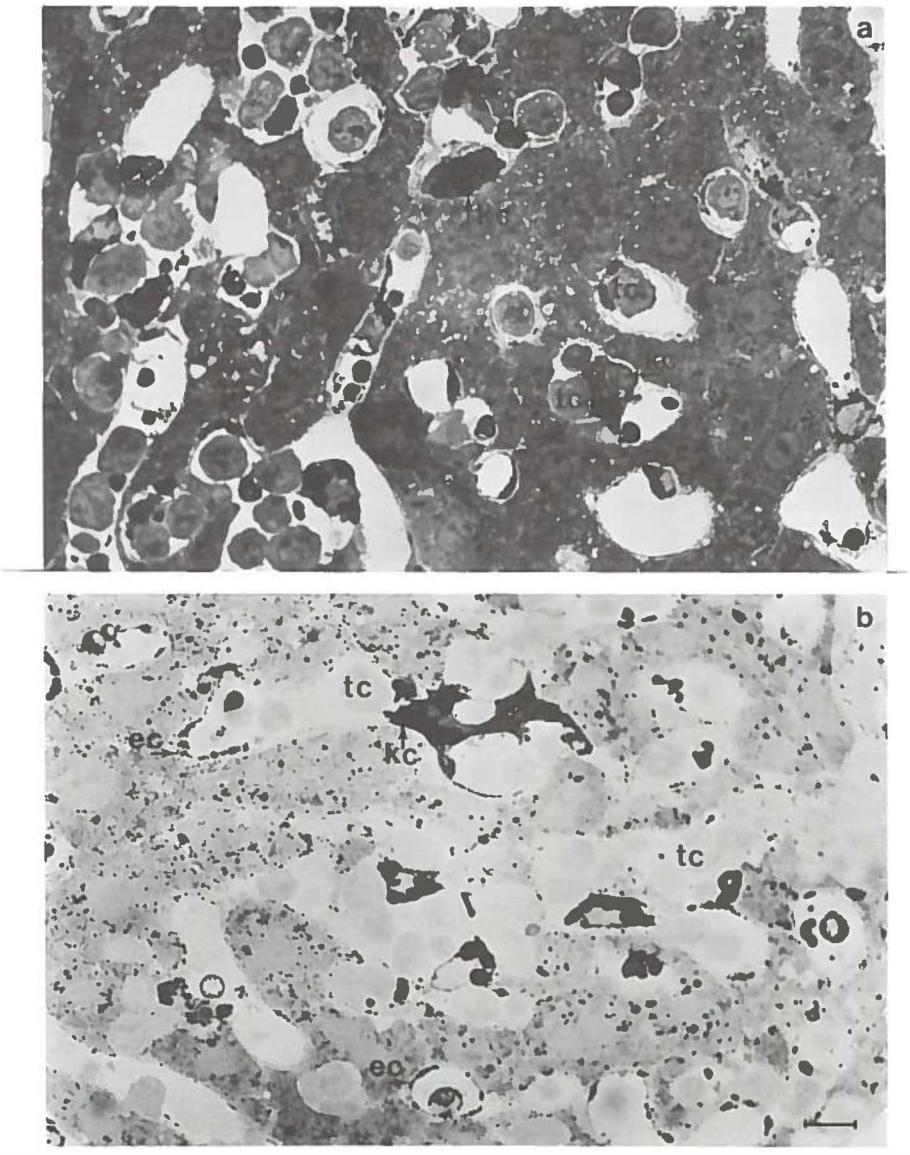
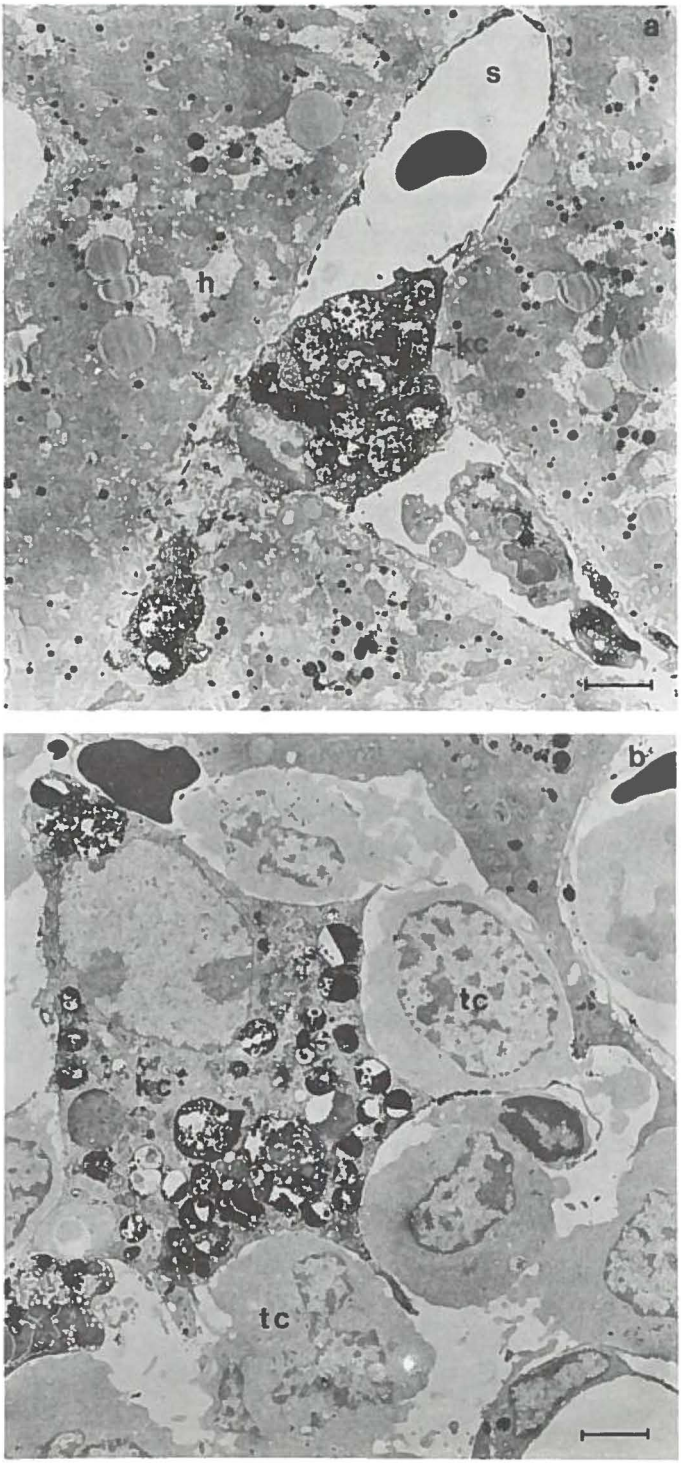


Fig. 4. Liver of a six-day tumor mouse 2 h after intravenous injection of liposomes with entrapped peroxidase (2.8 μmol of liposomal lipid containing 0.13 mg peroxidase). Peroxidase reaction product in 1 μm sections counter-stained with toluidine blue (a),or without counter-staining (b), is present in Kupffer cells (kc), but can also be detected in endothelial cells (ec) and in hepatocytes (arrows). Tumor cells (tc) are devoid of peroxidase activity. Bar is 10 μm (Courtesy of Dr. C.E. Hulstaert).

Fig. 5. Electron micrographs of the livers of mice injected with liposome-encapsulated peroxidase. (a) Normal mouse. Details as in Fig. 2. Peroxidase reaction product is present in vacuoles in the cytoplasm of the Kupffer cells (kc). h = hepatocyte, s = sinusoid. (b) Six-day tumor mouse. Details as in Fig. 4. Peroxidase reaction product is present in vacuoles in the cytoplasm of the Kupffer cell (kc). Tumor cells (tc) are devoid of peroxidase activity. Bar is 2.5 μm (Courtesey of Dr. C.E. Hulstaert).


a
s
h
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quently be taken up by hepatocytes. The observations described here are different in some respects from observations on the location of liposomes in *rat* liver reported by Roerdink *et al.* (23,27). After intravenous injection of peroxidase-containing MLV (80-150 nm), these authors did not detect significant peroxidase activity associated with hepatocytes on light and electronmicroscopical examination of the liver (23). Using a different experimental approach (isolation and separation of the main cell types of the liver in a hepatocyte fraction, a Kupffer cell fraction and an endothelial cell fraction) they found a very low association of intravenously injected liposomes (in part of the same type and size as the vesicles we used) with hepatocytes (27). The discrepancy between their results and ours might be due to differences in animal species. Macrophage activities are known to be different in rat and mouse and there may also be differences in the size of the endothelial fenestrations. In addition, the lipid dose per kg body weight used by Roerdink *et al.* was a factor ten lower than the dose per kg body weight used in our experiments. Saturation of Kupffer cells (in our experiments) and a subsequent overflow of the smaller vesicles to the hepatocytes may also contribute to the partially parenchymal localization of peroxidase in our experiments. Reaction product in the endothelial cells could be due to the uptake of free peroxidase that had leaked from the liposomes, as was also suggested by Roerdink *et al.* (23). No doubt remains as to the absolute lack of uptake of liposomes by the tumor cells.

The inhibitory activity of ADM on the growth of lymphosarcoma cells appeared to be decreased about two-fold by encapsulation. As lymphosarcoma cells fail to take up liposome-encapsulated horse-radish peroxidase *in vivo*, the effect of encapsulated ADM on tumor growth is most likely to result from drug being (slowly) released from the liposomes and subsequently taken up by the tumor cells. Since the tumor cells initially mainly proliferate in liver and spleen, tissues which also happen to have a high affinity for injected liposomes, encapsulation of ADM is likely to result in a locally high drug concentration, even if initially, the drug is mainly encapsulated. Subsequent release of the drug from liposomes deposited in these tissues could also, conceivably, result in prolonged elevated tissue levels of this

poorly water-soluble drug in liver and spleen. For Ara C we demonstrated that liposomes could, indeed, act as an effective local depot in liver and spleen (chapter V). However, ADM, in encapsulated form, is substantially less effective in this tumor model. It must be concluded that for this drug the establishment of an elevated tissue level in liver and spleen by means of liposome-encapsulation is counter-productive. The liposomes, possibly by their hydrophobic nature, apparently add to the retention of this lipophilic drug in cells/tissues other than the target tumor cells.

In general, the usefulness of liposome-encapsulation will depend on the relative effects of liposome-encapsulation on toxicity and antitumor activity. Since liposomes are mainly taken up by macrophages, it is conceivable that toxicity to the host defense system increases upon administration of encapsulated cytotoxic agents, especially in the case of ADM, since ADM is known to be toxic towards murine macrophages (28,29). This could result in a decreased therapeutic index and may contribute to the decreased effectiveness of encapsulated ADM in our tumor model. The toxicity of encapsulated ADM was reported to be decreased two-fold in normal healthy mice. However, increased toxicity of the encapsulated drug towards the host defense system may remain undetected when toxicity data are obtained in normal healthy mice as was the case in refs. 8 and 9.

Resistance to Ara C has been reported for a number of tumors (30-32) and may be due to insufficient cellular uptake (31). The inability of liposomes to overcome resistance as observed in this study would then be in agreement with our observation that the lymphosarcoma cells do not take up liposomes. These observations would also be compatible with the observation that entrapped Ara C produces a smaller degree of inhibition of thymidine incorporation than the same amount of free Ara C. It must be noted that the degree of inhibition of thymidine incorporation provides a measure of the amount of Ara C, that is intracellularly available for inhibition of DNA synthesis. It does not include any liposome-entrapped Ara C which is sequestered and/or inactivated in lysosomes. Since lymphosarcoma cells do not appear to take up any liposomes, liposome-encapsulated Ara CTP cannot be expected to overcome the type of resistance that is due to reduced levels of cytidine kinase (33) the enzyme that phosphorylates Ara C to the active metabolite

Ara CTP.

In summary, it was shown that encapsulated ADM increased the survival time of lymphosarcoma bearing mice and was effective against the growth of lymphosarcoma cells in liver and spleen, but less so than free ADM. Free nor encapsulated Ara C had any effect on the survival time of the mice. The tumor cells were shown not to take up liposomes and therefore antitumor effects of encapsulated drugs on this tumor must be attributed to drug being released from the liposomes. In view of the massive uptake of liposomes by macrophages it is to be expected that the toxicity of encapsulated drugs towards these cells will be increased.

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CHAPTER VII

CONCLUDING REMARKS

The non-selectivity of cytotoxic drugs in their action on normal and tumor cells and the development of resistance during treatment are two important factors responsible for the failure of cancer chemotherapy in many instances. When liposomes were first introduced as potential carriers for cytotoxic drugs it was hoped that liposome-encapsulation would result in a selective drug delivery to tumors and that their use could overcome resistance due to limited cellular uptake of the drug. Indeed in 1976, Poste and Papahadjopoulos (1) showed that liposome-encapsulated actinomycin-D could overcome cellular resistance against the drug in Chinese Hamster cells *in vitro*. The authors attributed this effect to the direct introduction of the encapsulated drug into the cytoplasm of the cells. Kosloski *et al.*(2), in 1978, were able to demonstrate an enhanced effect of encapsulated methotrexate in the treatment of methotrexate resistant P1798 mouse lymphosarcoma. Contrary to these promising reports evidence has been accumulating more recently that tumor cells do not usually internalize liposome-encapsulated drugs. Allen *et al.* (3) produced evidence which argues against uptake of drug-containing liposomes by various tumor cells *in vitro*. Kaye *et al.* (4) reported the failure of actinomycin-D encapsulated in liposomes to overcome drug resistance in Ridgeway osteosarcoma. Similarly Kedar *et al.* (5) reported that actinomycin-D failed to prolong the survival of mice bearing renal cell adenocarcinoma. Rustum *et al.* reported the inability of encapsulated arabinofuranosyl nucleotides to overcome drug resistance in L_{1210} leukemia cells *in vivo* (6). In this thesis indirect (chapter III) and direct (chapter VI) evidence is presented that mouse lymphosarcoma cells do not internalize liposomes.

Nonetheless an enhanced therapeutic index of liposome-encapsulated drugs has been demonstrated in several studies *in vivo* (7-9). The presently available evidence suggests that in these studies it is not a selective delivery of encapsulated drugs into tumor cells, but rather the occurrence

of changes in pharmacokinetics and tissue distribution that are responsible for the increased antitumor effects of liposome-encapsulated drugs (4,6). This will have important consequences for the applications of liposomes as drug carriers in cancer chemotherapy. The type of drug to be encapsulated and the type and dose of liposomes to be employed have to be related to the tumor to be treated.

It is conceivable that rapidly excreted and/or inactivated drugs can be protected against excretion and degradation by encapsulation in liposomes as was demonstrated for arabinofuranosylcytosine (Ara C) (chapter V). Slow release of such a drug may then add to its potential usefulness in cancer treatment. Phase-specific drugs are toxic to cells only during a specific phase of the cell cycle and the effect of treatment will, amongst other factors, depend on the time during which cytotoxic levels of the drug are present in the tumor cells. Non-phase specific drugs are equally toxic to cells in different phases of the cell cycle. For that reason liposome-encapsulation of non-phase-specific drugs could even be counter-productive since peak concentrations of the bioavailable drug in the blood will be permanently reduced when the drug is administered in entrapped form. Also, in treatment with drugs that are slowly eliminated from the body, such as adriamycin (ADM), liposome-encapsulation cannot be expected to result in such drastic improvements in therapeutic effectiveness as were found with encapsulation of Ara C in the treatment of L_{1210} leukemia. Only of course if encapsulation would result in locally high drug concentrations at the site of the tumor cells, could drug encapsulation in liposomes be beneficial in treatment with slowly excreted and/or non-phase-specific drugs.

The type and dose of liposomes that would be most suitable for the treatment of a particular tumor will depend, amongst other factors, on the location of the tumor cells. When these cells are separated from the circulation by an endothelial barrier as is the case in most tumors, an encapsulated cytotoxic drug can exert its effect only after its release from the liposomes into the blood. In such a case it may be advantageous to use small liposomes which have a relatively long circulation time, or to increase the circulation time by using a high liposome dose (chapter III). This also applies when the target tumor cells do not have an organ specific localization but

are remaining in the blood stream. Depression of the reticuloendothelial system (RES) with, for instance, $La(NO_3)_3$ (chapter III) or dextran-sulphate (10) will result in favorably increased circulation times of large liposomes. However, these agents are toxic in their effects on Kupffer cells while, in mice, recovery of hepatic phagocytosis from such treatments takes four days to be completed (11). The notion that Kupffer cells and other macrophages may play an important role in host defense mechanisms against neoplasia leaves RES depression an unfavorable appraoch to achieve longer circulation times. It would rather seem preferable to increase the liposome dose so as to saturate the RES activity. Such saturation by liposomes was shown to be readily reversible (chapter IV).

Liposomes that are taken up by liver and spleen may act as a depot for the local sustained release of encapsulated drugs (chapter V). If tumor cells are located in the liver (a target organ for metastasising tumor cells of several types) or spleen, liposomes could be useful in creating locally high and sustained levels of the drug in close proximity to the tumor cells. If liposomes are to be used for such local sustained release one would preferably not aim for a long circulation time, but rather for an extensive local accumulation of liposomes. However, the usefulness of such an approach remains to be established. The mouse lymphosarcoma used in chapters III and VI may provide an interesting model for testing liposomes in this respect since this tumor initially mainly proliferates in liver and spleen, where liposomes preferentially accumulate as well.

For ADM which is less active against the lymphosarcoma when the drug is encapsulated, the mechanism of local sustained release in liver and spleen apparently is of little value (chapter VI). Whether or not the pharmacological activity of a drug as a result of its sustained release from an encapsulated form will increase, depends on the rate of drug release into the local compartment and on the rate of redistribution of the free drug in the systemic circulation. The rate of drug release from liposomes is dependent on the type of drug (12) and the type of liposome (unilamellar versus multilamellar). Furthermore, the release may take place from liposomes in the extracellular space or, after uptake by for example a macrophage, from within such a cell. The rate of release and redistribution will ultimately

determine the concentration of bioavailable drug in the local compartment.

Toxicity of the encapsulated drug will also determine the overall effect of encapsulation on the therapeutic index. Regarding the uptake of liposomes by macrophages it is to be expected that the toxicity of encapsulated drugs towards these cells will be increased. This may be particularly disadvantageous in treatment with non-phase-specific drugs. In treatment with phase-specific drugs tissue macrophages may not suffer from the toxic effects since cells with a low DNA synthesis activity (13) will presumably not be affected.

In this connection it should be noted that it has been demonstrated repeatedly that ADM possesses a higher antitumor activity than the structurally closely related analog daunomycin (DNM) (14,15). ADM was shown to be less toxic towards murine macrophages than DNM, *in vitro* as well as *in vivo* (16,17) and several authors have put forward the hypothesis that interference with host defense mechanisms may be an important determinant of the superiority in antitumor activity of ADM over DNM (18,19).

The usefulness of liposome-encapsulation of a drug will depend on the relative extents to which overall toxicity and specific antitumor activity are influenced. Often an increased antitumor effect will be accompanied by an increased toxicity and *vice versa*. At present too little is known about the multiple factors that affect the ultimate cytocidal effect of free versus encapsulated drugs to draw generally valid conclusions. Probably only in selected cases will liposome-encapsulation result in an increased therapeutic index.

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SAMENVATTING

Het slagen of falen van een chemotherapeutische behandeling is nauw verbonden met de farmakologische eigenschappen van het gebruikte geneesmiddel. Het farmakologisch gedrag van een geneesmiddel kan worden beïnvloed door het geneesmiddel te verpakken in een drager ("carrier"). In dat geval zullen het de eigenschappen van de drager zijn die in belangrijke mate bepalend zijn voor het lot van het ingesloten geneesmiddel. Vanaf het begin van de jaren zeventig worden *liposomen* getest op hun toepasbaarheid als drager voor geneesmiddelen. Liposomen zijn mikroskopisch kleine, bolvormige partikels (met een diameter variërend van 25 nm tot enkele microns), opgebouwd uit een of meerdere, concentrisch gerangschikte, twee-molekulen dikke fosfolipide-lagen ("bilagen"), die evenzovele waterige compartimenten omsluiten. In deze compartimenten kunnen tijdens de vorming van de liposomen wateroplosbare molekulen worden ingesloten, terwijl verbindingen met een meer hydrofoob karakter in de fosfolipide-bilaag kunnen worden ingebouwd.

Indien liposomen intraveneus worden toegediend als potentiële "drug-carrier", raken ze voor een belangrijk deel geassocieerd met de lever en de milt. Hiervoor zijn naar alle waarschijnlijkheid vooral de tot het reticuloendotheliale systeem (RES) behorende makrofagen verantwoordelijk. Deze nemen de liposomen en de daarin ingesloten verbindingen op via endocytose. Dit maakt liposomen weliswaar bij uitstek geschikt als carrier voor stoffen die bedoeld zijn om in te grijpen in het metabolisme van deze makrofagen, maar vormt tevens een beperking voor de potentiële toepasbaarheid van liposomen in o.a. de chemotherapie van kanker.

Chemotherapie van kanker impliceert het handhaven van een evenwicht tussen therapeutische en toxische effekten van de gebruikte cytostatika en het is denkbaar dat relatief kleine veranderingen in de verdwijningssnelheid uit het bloed en in de weefselverdeling van een cytostatikum kunnen leiden tot een verhoogde therapeutische index. Insluiting van een cytostatikum in liposomen zal in het algemeen de klaring ervan uit het bloed vertragen en bovendien een bescherming vormen tegen een voortijdige inaktivering en zal tevens aanleiding geven tot accumulatie van het cytostatikum in de lever en de milt.

Het in dit proefschrift beschreven onderzoek handelt over verschillende aspekten van het gebruik van liposomen als "carriers" voor cytostatika. Daar de meeste dierlijke en menselijke tumor-cel typen niet van nature fagocyteren en dientengevolge waarschijnlijk geen, of slechts in geringe mate, liposomen op zullen nemen, is veel aandacht besteed aan het mogelijke funktioneren van liposomen als depot waaruit het cytostatikum langzaam vrij komt. Een dergelijke depot-werking zou vooral van nut kunnen zijn bij het gebruik van zogenaamde fase-specifieke cytostatika. Deze cytostatika oefenen alleen tijdens een speciale, kortdurende fase in de cel-cyclus een cytostatische werking uit. Een behandeling zal alleen dan effektief kunnen zijn, wanneer het cytostatikum in voldoende hoge concentraties in de tumorcellen aanwezig is en voor een periode die zo lang is dat de meeste tumorcellen de gevoelige fase kunnen bereiken.

Na een algemene inleiding over het gebruik van liposomen in medisch georiënteerd onderzoek, en een beschrijving van de karakteristieken van het in delen van dit onderzoek gebruikte tumormodel, een lymfosarcoom (hoofdstuk I), wordt in de daaropvolgende hoofdstukken II en III het lot van intraveneus en intraperitoneaal toegediende liposomen beschreven. Intraperitoneaal toegediende liposomen blijken grotendeels intakt, en niet geassocieerd met cellen, vanuit de peritoneale holte naar het bloed vervoerd te worden, waarschijnlijk met de lymfestroom. Evenals intraveneus toegediende liposomen raken intraperitoneaal toegediende liposomen dan ook voornamelijk geassocieerd met de lever en de milt, waar ze intracellulair opgenomen en ook gemetaboliseerd kunnen worden. Aangetoond wordt dat in muizen de snelheid van verdwijning van intraveneus geïnjiceerde liposomen uit het bloed dosisafhankelijk is. Hoge doses liposomen veroorzaken klaarblijkelijk een verzadiging van de fagocytotische capaciteit van de reticulo-endotheliale cellen. De aktiviteit van het RES bleek beïnvloed te kunnen worden door de aanwezigheid van tumorcellen. Eén dag na intraveneuze toediening van lymfosarcoma cellen aan muizen was de RES aktiviteit aanzienlijk geremd en dientengevolge was de verdwijning van liposomen uit het bloed vertraagd ten opzichte van die in normale, gezonde muizen. Hoewel de lymfosarcoma cellen zelf geen liposomen blijken op te nemen kan hun aanwezigheid in het proefdier kennelijk wel van invloed zijn op de klaring van liposomen uit het bloed.

In hoofdstuk IV wordt nader ingegaan op het effekt van liposomen op de RES aktiviteit. De in dit hoofdstuk beschreven resultaten duiden erop dat de door liposomen veroorzaakte remming van de RES aktiviteit reversibel is, afhankelijk is van de diameter van de liposomen en niet kan worden toegeschreven aan een depletie van fagocytose-bevorderende plasma-faktoren (opsonines).

In hoofdstuk V wordt het effekt van insluiting in liposomen op de metabole aktivering (fosforylering) en afbraak (deaminering) van het fasespecifieke cytostatikum arabinofuranosylcytosine (Ara C) beschreven. In liposomen ingesloten Ara C is beschermd tegen enzymatische deaminering en komt langzaam vrij uit o.a. met lever en milt geassocieerde liposomen. Bovendien geeft in liposomen ingesloten Ara C aanleiding tot een langdurig verhoogd arabinofuranosylcytosine trifosfaat (Ara CTP, de cytostatisch aktieve metaboliet) gehalte in de lever en milt van leukemische muizen. Liposomen kunnen klaarblijkelijk fungeren als lokaal depot van Ara C in de lever en milt en kunnen er toe bijdragen dat de concentratie van het intracellulair gevormde aktieve derivaat gedurende langere tijd verhoogd blijft.

In hoofdstuk VI wordt aangetoond dat de lymfosarcoomcellen *in vivo* geen liposomen opnemen. Antitumor effekten van in liposomen ingesloten cytostatika op deze tumor moeten dus ook op grond van deze waarneming worden toegeschreven aan vrijgekomen en vervolgens door de tumorcellen opgenomen cytostatikum en niet aan een direkte opname van het in liposomen ingesloten cytostatikum door de tumorcellen. Verder worden inleidende experimenten beschreven waarin de therapeutische aktiviteit van vrij en in liposomen ingesloten adriamycine (ADM) en Ara C in lymfosarcoom-dragende muizen wordt gemeten aan de hand van de overlevingstijd van de muizen. In liposomen ingesloten ADM veroorzaakt een verlenging van de overlevingstijd van de muizen maar is minder effektief in dit opzicht dan vrij ADM, terwijl vrij noch in liposomen ingesloten Ara C enig therapeutisch effekt heeft, waarschijnlijk tengevolge van een beperkte Ara C opname-capaciteit van de tumorcellen. Deze vorm van resistentie zal in de lymfosarcoomcellen uiteraard niet kunnen worden opgeheven door insluiting van Ara C in liposomen.

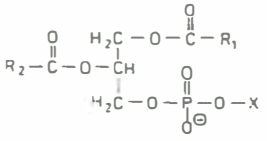
In het laatste hoofdstuk (VII) worden een aantal aspekten besproken die van belang kunnen zijn bij het gebruik van liposomen als depot van cytosta-

tika en die deels in de verschillende experimentele hoofdstukken naar voren zijn gekomen. De algemene conclusie is dat de toepassingsmogelijkheden van liposomen als carriersysteem voor cytostatika bij chemotherapie van tumoren aanzienlijk minder ruim zijn dan wel werd en wordt beweerd, maar dat er anderzijds toch in bepaalde, zorgvuldig geselecteerde gevallen een verhoging van de therapeutische index van een cytostatikum, als gevolg van insluiting in liposomen, haalbaar geacht moet worden.

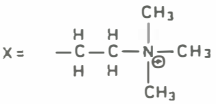
LIST OF ABBREVIATIONS

ABTS	2,2'-azino-di-(3 ethylbenzthiazolin sulphonic acid)
ADM	adriamycin
Ara-C	1-β-D-arabinofuranosylcytosine
Ara-CMP	1-β-D-arabinofuranosylcytosine monophosphate
Ara-CDP	1-β-D-arabinofuranosylcytosine diphosphate
Ara-CTP	1-β-D-arabinofuranosylcytosine triphosphate
Ara-U	1-β-D-arabinofuranosyluracil
DAB	3,3'-diaminobenzidine tetrahydrochloride
HDL	high density lipoprotein
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid
HPLC	high pressure liquid chromatography
HRPO	horse-radish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
MLV	multilamellar vesicle
PBS	phosphate buffered saline
RES	reticuloendothelial system
REV	reverse phase evaporation vesicle
RPMI	Roswell Park Memorial Institute
S.D.	standard deviation
SRBC	sheep red blood cells
SUV	small unilamellar vesicle
TLC	thin layer chromatography
Tris	tris(hydroxymethyl) aminomethane

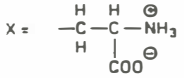
STRUCTURAL FORMULAS



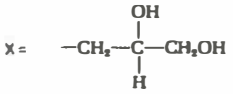
Basic structure of 3-sn-phosphoglycerides. R_1 and R_2 represent the hydrocarbon moieties of the fatty acyl chains



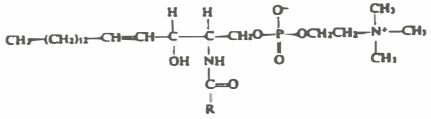
phosphatidylcholine



phosphatidylserine



phosphatidylglycerol



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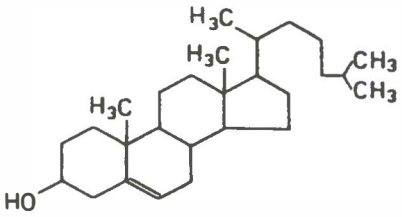

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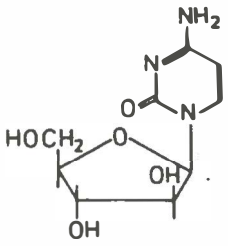
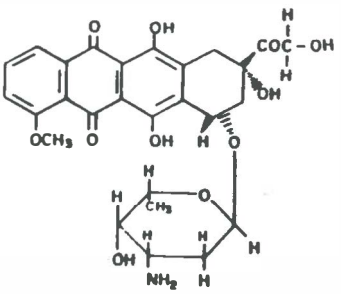


1-β-D-arabinofuranosylcytosine



adriamycin