8-1991

Role of glycolipids on liposome biodistribution, tumor uptake and immunotargeting

Atsuhide Mori

To the Graduate Council:

I am submitting herewith a thesis written by Atsuhide Mori entitled "Role of glycolipids on liposome biodistribution, tumor uptake and immunotargeting." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology.

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I am submitting herewith a thesis written by Atsuhide Mori entitled "ROLE OF GLYCOLIPIDS ON LIPOSOME BIODISTRIBUTION, TUMOR UPTAKE AND IMMUNOTARGETING." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Leaf Huang

Leaf Huang, Major Professor

We have read this thesis
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ROLE OF GLYCOLIPIDS ON LIPOSOME BIODISTRIBUTION, TUMOR UPTAKE AND IMMUNOTARGETING

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Atsuhide Mori

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Dedication

This thesis is dedicated to my mother,

Kyoko Mori [森　京子],

who has given me invaluable educational opportunities.

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I would like to thank my major professor, Dr. Leaf Huang, for his guidance and patience. I would also like to thank the other committee members, Dr. Stephen J. Kennel and Dr. Daniel M. Roberts, for their comments and assistance. I also wish to thank my collaborators, Dr. Aleksander L. Klibanov, Dr. Dexi Liu, Dr. Kazuo Maruyama and Dr. Vladimir P. Torchilin, for their helpful suggestions and criticism. Finally, I wish to thank Barbara McGirl and the other members of my laboratory for their technical help and suggestions. The work was supported by National Institutes of Health grants CA 24553 and AI 29893.

Abstract

Several variables important in the construction of liposomes with reduced affinity to the reticuloendothelial system (RES) and thus prolonged circulation time were examined. Studies using a series of dioleoyl N-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine (PEG-PE) of different polymer chain lengths showed that both the activity of PEG-PE in prolonging the circulation time of liposomes and the relative steric barrier activity of the amphipathic polymer, measured by a liposome agglutination assay, were directly proportional to the polymer chain length (PEG5000-PE > PEG2000-PE > PEG750-PE). These results strongly support the hypothesis that PEG-PE exerts its activity in prolonging the circulation time of liposomes by presenting a steric barrier on liposome surfaces such that interactions of liposomes with the RES were reduced. In contrast, the activity of GM_1 in prolonging the circulation time of liposomes did not correlate with its relatively weak steric barrier activity, suggesting a different mechanism for this glycolipid. Systematic size-dependent biodistribution study with GM_1-containing liposomes revealed that there was a relatively restricted size requirement for the activity of GM_1 in prolonging the circulation time of liposomes. The optimal size appeared to be 70 ~ 200 nm in average diameter; larger and smaller liposomes accumulated efficiently in the spleen and the liver, respectively. The effectiveness of the long-circulating liposomes was demonstrated in both passive and active targeting studies of liposomes to non-RES tissues. Tumor-targeting studies of liposomes, using the transplantable solid tumor (EMT6) model, showed that the long-circulating liposomes were able to accumulate more efficiently than conventional liposomes in tumor. Furthermore, target binding studies of immunoliposomes using a lung endothelial model showed that, when GM_1 or PEG2000-PE was included in the lipid composition to reduce their affinity to the RES, the resulting immunoliposomes showed more efficient target binding than the ones with the conventional lipid composition. However, PEG5000-PE caused a significantly reduced target binding of immunoliposomes presumably due to its overly strong steric barrier activity which may interfere with the antibody-antigen interaction.

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List of Abbreviations

biotin-cap-PE:	biotinamidocaproyl-phosphatidylethanolamine
chol:	cholesterol
DOPE:	dioleoyl phosphatidylethamolamine
DSPC:	distearoyl phosphatidylcholine
DTPA-SA:	diethylenetriamine pntaacetic acid distearylamide complex
GM_1:	monosialoganglioside
NGPE:	N-glutaryl phosphatidylethanolamine
PBS:	phosphate-buffered saline
PC:	egg phosphatidylcholine
PEG:	polyethyleneglycol
PEG-PE:	dioleoyl N-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine
RES:	reticuloendothelial system

CHAPTER I

Introduction

Liposomes have attracted a considerable amount of interest for potential use as a drug delivery system due to their suitable characteristics (for a recent book, see Ostro, 1987). They consist of one or more concentric phospholipid bilayers enclosing an aqueous space. They are biocompatible, biodegradable, and normally nonimmunogenic. More importantly, they are capable of loading both hydrophilic and hydrophobic drugs in the aqueous and bilayer phase, respectively. Drugs encapsulated in the liposomes are protected from enzymatic degradation and other inactivation processes.

There are basically two different modes in liposome targeting: passive and active targeting. The former takes advantage of the fact that systemically injected liposomes are rapidly and efficiently taken up by phagocytic cells of the reticuloendothelial system (RES) located mainly in the liver and the spleen (for a review, see Hwang, 1987). This natural homing activity of liposomes provides an effective targeting system for the liver and the spleen, and various studies have been carried out with this targeting mode (for a review, see Alving, 1986). However, this predominant uptake of liposomes by the RES and a resulting rapid clearance from the circulation have been a major obstacle in any attempt to deliver liposomes to cells, tissues or organs other than the RES, which is referred to as "active targeting". One of the exciting approaches in active targeting of liposomes takes advantage of a specific ligand-receptor interaction. Liposomes are covalently or noncovalently linked to a suitable ligand for the target specificity. Antibody directed liposomes, or so-called "immunoliposomes", have been extensively studied due to their high degrees of specificity and versatility (for a review, see Wright and Huang, 1989). A number of methods for covalent conjugation of antibody on the liposome surface have been developed. While the rationale of this approach has been well established in various

in vitro systems, successful *in vivo* studies of immunoliposome targeting have been carried out only recently. Several studies have shown that a considerable amount of immunoliposomes accumulate in the liver and the spleen, showing the difficulty arising from the vivid RES competition for liposome uptake (for a review, see Peeters *et al.*, 1987). If the rate of immunoliposome binding to the target is less than the rate of uptake by the RES, little if any target binding would be expected.

One of the current efforts in active targeting of liposomes is to overcome the above described kinetic barrier by manipulating the liposome characteristics such that the affinity of liposomes to the RES is reduced and the circulation time of liposomes is thus increased. In this theses, details of the construction and optimization of liposomes for prolonged circulation will be described (Chapter III). Applications of this novel type of liposomes in attempts to improve localization of liposomes in the solid tumor (Chapter IV) and in enhancing target binding of immunoliposomes (Chapter V) in a model system will then be described.

CHAPTER II

Materials and Methods

A. Materials

1. Lipids and Chemicals

Egg phosphatidylcholine (PC), distearoylphosphatidylethanolamine (DSPC), and dioleoylphosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL) and cholesterol (chol) was from Sigma (St. Louis, MO). Bovine monosialoganglioside (GM_1) was obtained from Calbiochem (San Diego, CA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was obtained from Sigma, and N-hydroxysulfosuccinimide (Sulfo-NHS) was from Pierce (Rockfold, IL). All other chemical used were obtained from Sigma. ^{111}In and ^{125}I were obtained from New England Nuclear. Biotinamidocaproyl-phosphatidylethanolamine (biotin-cap-PE) (Bayer *et al.*, 1979), diethylenetriamine pentaacetic acid distearylamide complex (DTPA-SA) (Kabalka *et al.*, 1987), N-glutaryl phosphatidylethanolamine (NGPE) (Weissig *et al.*, 1986), and dioleoyl N-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine (PEG-PE) (Klibanov *et al.*, 1990) were synthesized as previously described. Radiolabeling of DTPA-SA with ^{111}In has been described elsewhere (Klibanov *et al.*, 1991).

2. Antibody

The monoclonal antibody 34A (MoAb 34A) was obtained from Dr. Stephen J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN). The MoAb 34A was radiolabeled with ^{125}I using Iodo-Gen method (Pierce, Rockfold, IL) and purified using spin column on BioGel P-4 (Bio-Rad, Richmond, CA).

3. Tumor Cells

EMT6 cells were grown as a monolayer culture in Waymouth's medium containing 20% fetal calf serum and antibiotics (Rockwell, 1977). Cells were detached from the plate by trypsin-EDTA treatment and washed with the medium. EMT6 tumor-bearing mouse were established by injecting 1 x 10^6 tumor cells in subcutaneous space at the left-hind leg of mouse (Balb/c, 4 ~ 6 week old). Mice were tested 2 ~ 3 weeks after tumor inoculation at which the local tumor implants weighed between 0.7 and 2.0 g. EMT6 tumor was maintained by alternate passage in mice and in culture.

B. Liposome Preparation

Large unilamellar liposomes composed of phospholipid (PC, DSPC, or DOPE) and chol (2:1, mol/mol) additionally containing 6.3 mol% of either PEG-PE or GM_1 were prepared by the extrusion method. A thin film of solvent-free lipid mixture containing 1 mol% of ^{111}In-labeled DTPA-SA, as a lipid marker (Kabalka *et al.*, 1987), was hydrated with phosphate buffered saline (PBS) (pH 7.5) overnight. The liposome suspension was extruded several times through various size of Nucleopore membranes to generate liposomes with homogeneous size distribution. Liposomes containing DSPC were heated to above its phase transition temperature (~ 50 °C) during the hydration and extrusion processes. Small unilamellar liposomes (< 70 nm in average diameter) were prepared by extensive sonication using a bath sonicator (Laboratory Supplies, Hicksville, NY), followed by fractionation using chromatography on Sepharose 4B-CL column. Liposome size was determined by dynamic laser light scattering using a Coulter N4SD instrument (Hialeah, FL).

C. Immunoliposome Preparation

1. Conjugation of antibody with NGPE

Solvent free NGPE was solubilized with octylglucoside in MES buffer (10 mM MES/150mM NaOH, pH 5.0) (NGPE:octylglucoside = 0.06:1, mol/mol). EDC (10 μmol in water) and Sulfo-NHS (4 μmol in water) were added to 200 nmol of the above NGPE

solubilized with octylglucoside and then incubated for 10 min at room temperature. The mixture was neutralized with 100 mM HEPES buffer (pH 7.5) and 1N NaOH to pH 7.5. Antibody with a trace amount of ^{125}I-labeled antibody were then added (antibody:NGPE = 1:14, mol/mol). The resultant mixture was incubated for 8 hr at 4 °C with gentle stirring to generate antibody conjugated to NGPE.

2. Incorporation of amphipathic antibody into liposome

Immunoliposomes composed of PC and chol (1:1, mol/mol) additionally containing 7.0 mol% of either PEG-PE or GM_1 were prepared by the detergent-dialysis method (Holmberg *et al.*, 1989). A thin film of solvent free lipid mixture containing 1 mol% of ^{111}In-labeled DTPA-SA was solubilized with octylglucoside (100 mM in PBS, pH 7.5) (lipid:octylglucoside = 1:5, mol/mol). The resultant solution was mixed vigorously with the above NGPE-conjugated antibody (lipid:antibody = 1:4, wt./wt.), and the detergent was then removed by dialysis against PBS (pH 7.5) for 12 ~ 18 hr at 4 °C. The resulting immunoliposomes were extruded several times through stacked 0.4 and 0.2 μm Nucleopore membranes to generate immunoliposomes with about 200 nm in average diameter. The extruded immunoliposomes were then chromatographed on a BioGel A1.5M column to remove the unbound antibody. The peak liposome fractions were pooled, and the amount of antibody incorporated into liposomes and the final ratio of antibody-to-lipid in the immunoliposomes were calculated from the specific radioactivities of ^{125}I for the antibody and ^{111}In for the lipid. Normally, 35 ~ 50% of the initial amount of antibody was incorporated into liposomes.

D. Liposome Agglutination Assay

Liposomes used in this study were prepared, using the extrusion method, from PC and chol (1:1, mol/mol) additionally containing 2.5 mol% of biotin-cap-PE and various amount (2, 5, 10 mol%) of either PEG-PE or GM_1. The agglutination was initiated by mixing the liposome suspension (60 μg phospholipid in 560 μl PBS, pH7.5) with 10 μg

streptavidin in a microcuvette. Increase in turbidity was monitored by optical density at 440 nm.

E. Liposome Biodistribution Study

^{111}In-labeled Liposomes were i.v. injected into either normal or EMT6 tumor-bearing mice (Balb/c, 6 ~ 8 week old) at a dose of 0.2 ~ 0.7 mg lipid in 200 μl per mouse. At various time intervals, mice were anesthetized lightly and bled by eye puncture. The mice were then sacrificed by cervical dislocation and dissected. Blood and major organs including spleen, liver, lung, heart, kidney, and tumor were collected, weighed, and counted for ^{111}In radioactivity in a gamma counter. The data were expressed as percent of total injected dose in each organ, except for tumor (expressed as percent injected dose per gram of tissue). The total radioactivity in the blood was determined by assuming that the blood volume of mouse is 7.3% of the body weight.

CHAPTER III

Liposomes with Prolonged Circulation Time

The biodistribution of liposomes *in vivo* has been exhaustively studied in the last decade (for a review, see Senior, 1987). Many studies have been focused on the mechanism underlying the efficient uptake of liposomes by the RES. It is now generally understood that the mononucleophagocytes of the RES, principally the Kupffer cells of the liver and secondarily the splenic macrophages, are responsible for the clearance of liposomes from the circulation (Segal *et al.*, 1974; Scherphof *et al.*, 1986). In addition, the parenchymal cells of the liver are also involved in liposome uptake (Gregoriadis and Ryman, 1972). Furthermore, serum factor(s) (opsonins) coating the liposome surfaces are believed to promote the specific uptake of liposomes by the liver and the spleen, although these molecules have not been identified (Moghmi and Patel, 1989). These interactions obviously depend on the physical and chemical characteristics of the liposomes. Thus, our effort has been to optimize some variables in the construction of liposomes such that the affinity of the liposomes to the RES is reduced and the circulation time is prolonged. Two such variables, lipid composition and liposome size, have been optimized in this regard.

A. Optimization of Lipid Composition

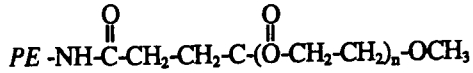
Liposomes can be prepared from a variety of lipid components. Some parameters of liposomes, such as the bilayer rigidity and surface charge, depend largely on the lipid composition and affect the stability of liposomes. The basic lipid composition of liposomes consists of a mixture of phospholipid and chol. Inclusion of chol results in an enhanced structural stability of liposomes in serum or plasma, resulting in a relatively prolonged circulation time as compared to the chol-free liposomes (Patel *et al.*, 1983). A major breakthrough, however, is the recent finding that inclusion of a glycolipid such as GM_1,

hydrogenated phosphatidylinositol, sulfatides (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen *et al.*, 1989a; Liu and Huang, 1990) and amphipathic polyethyleneglycol (PEG) (Klibanov *et al.*, 1990 & 1991; Blume and Cevc, 1990; Senior *et al.*, 1991) in the lipid composition results in a significantly prolonged circulation time of liposomes. The mechanism of action of the above amphiphiles in prolonging the circulation time of liposomes has not been fully elucidated. However, it has been postulated that an increased hydrophilicity (Senior *et al.*, 1991) and/or steric barrier on the liposome surface (Klibanov *et al.*, 1990 & 1991; Blume and Cevc, 1990) may prevent or reduce interactions of liposomes with serum constituents, thus resulting in an enhanced stability of liposomes and a reduced rate of RES interaction. To examine the importance of the steric barrier on the liposome surface, provided by an additional amphiphile, in prolonging the circulation time of liposomes, a series of PEG-PE with different polymer chain length and GM_1 (Figure 1) was used to prepare liposomes to examine the relationship between the steric barrier activity and activity in prolonging the circulation time of liposomes.

1. Steric Barrier Activities of PEG-PE and GM_1

A liposome agglutination assay was used to assess the degree of the steric barrier produced on the liposome surface by PEG-PEs and GM_1. This assay takes advantage of the fact that the agglutination of liposomes containing biotin-cap-PE, mediated by streptavidin, requires a close apposition of the neighboring liposomes (Figure 2) (Klibanov *et al.*, 1989). Thus, a decrease in liposome turbidity compared to that of control liposomes directly reflects the relative steric barrier activity on liposome surfaces. Data were expressed as the ratio of relative turbidity of the test liposomes to that of the control liposomes (composed of PC and chol) at 12 min after the incubation. At this time, the turbidity increases showed plateaus and the values were plotted as a function of the amphiphile concentration (Figure 3). With all PEG-PEs tested here, the relative turbidity decreased with increasing concentration of the polymer. Also, the longer the chain of PEG, the greater the decrease in the relative turbidity. At 10 mol%, liposome agglutination was inhibited by 14%, 55%, and 100% for PEG750-PE, PEG2000-PE, and PEG5000-

(a)



n = 15	PEG750-PE
n = 45	PEG2000-PE
n = 110	PEG5000-PE

(b)

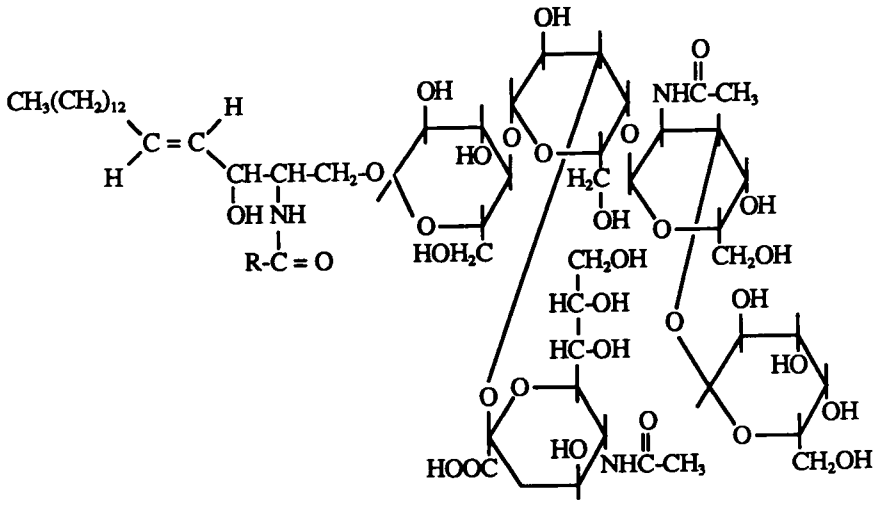


Figure 1. Structure of PEG-PE (a) and GM_1 (b)

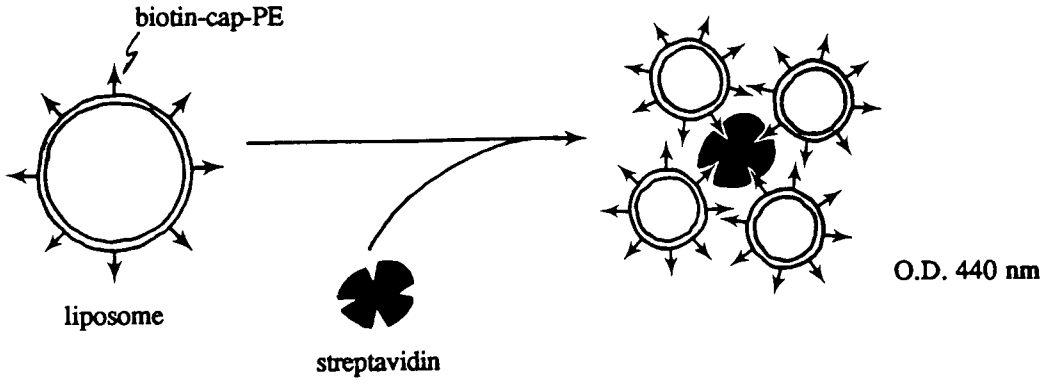


Figure 2. Schematic presentation of the streptavidin-induced agglutination of liposomes containing biotin-cap-PE.

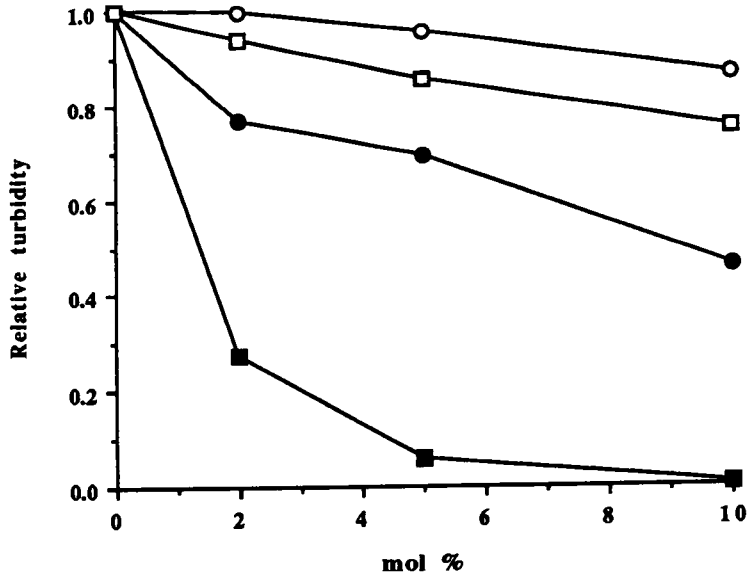


Figure 3. Effect of increasing concentrations of PEG-PE and GM_1 on streptavidin-induced agglutination of liposomes containing biotin-cap-PE. Increase of turbidity (O. D. 440 nm) was measured 12 min after incubation for liposomes composed of PC and chol (1:1, mol/mol) additionally containing PEG750-PE (○), PEG2000-PE (●), PEG5000-PE (■) or GM_1 (□). Liposome agglutination was normalized with respect to the control liposomes (PC-chol) and was plotted as a function of amphiphile concentration. Data taken from Mori *et al.* (1991).

PE containing liposomes, respectively. Thus, the steric barrier activity of PEG-PE is directly proportional to the polymer chain length: the longer the polymer chain, the stronger the steric barrier. Also shown in Figure 3 is the steric barrier activity of GM_1. The inhibition of liposome agglutination was also concentration dependent; however, GM_1 provided only a relatively weak steric barrier which was less than that of PEG2000-PE.

2. Activities of PEG-PE and GM_1 in prolonging the circulation time of liposomes

To test whether the steric barrier activity of PEG-PE and GM_1 correlates with the activity in prolonging the circulation time of liposomes and with the interaction of liposomes with the RES, the biodistribution of i.v. injected liposomes containing PEG-PE or GM_1 was examined at different times after injection. Liposomes used in this study were prepared, using the extrusion method, from PC and chol (2:1, mol/mol) additionally containing 6.3 mol% PEG-PE or GM_1. Data were expressed as percent injected dose in blood and the RES (liver and spleen) and were plotted as a function of time after injection (Figure 4). At 3 hr after injection, liposomes composed of PC and chol were almost completely cleared from the circulation and accumulated exclusively in the liver and the spleen. Inclusion of PEG-PE or GM_1 in the lipid composition resulted in a reduced clearance rate from the circulation presumably due to a decrease in the rate of RES uptake. The estimated half-life for liposome blood clearance increased from 0.5 hr to 0.7 hr, 1.7 hr, 6.2 hr, and 3.4 hr by the inclusion of PEG750-PE, PEG2000-PE, PEG5000-PE, and GM_1, respectively. Activities of the above amphiphiles in prolonging the circulation time of liposomes were concentration dependent, plateauing at 5 mol% of the total lipid (except for PEG750-PE which plateaus at 10 mol%) (data not shown). These results clearly indicate that, among the various PEG-PEs tested, the activity of PEG-PE in prolonging the circulation time of liposomes is directly proportional to the chain length of the PEG polymer which is in turn proportional to the relative steric barrier activity of the PEG-PE, as assessed by the liposome agglutination assay (Figure 3).

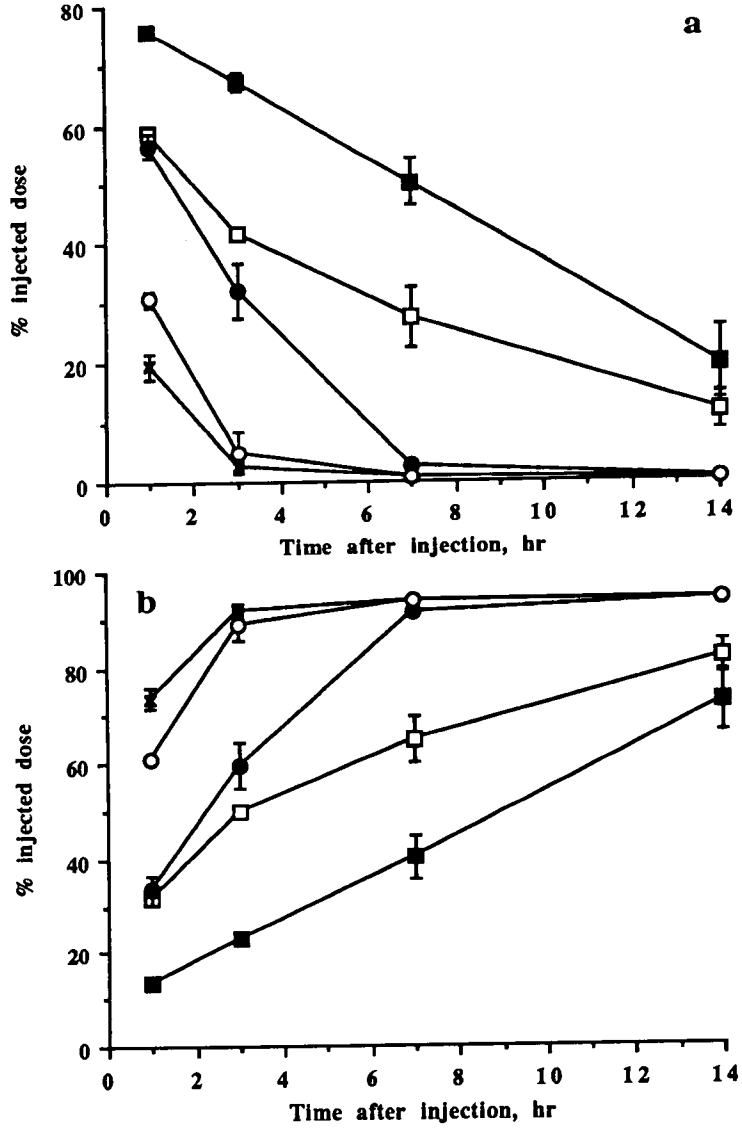


Figure 4. Effect of various PEG-PEs and GM_1 on (a) blood clearance and (b) the RES (liver and spleen) uptake of liposomes. ^{111}In-labeled liposomes were i.v. injected into mice (0.4 mg lipid per mouse), and biodistribution was examined at indicated time intervals. Bars represent S.D. (n = 3). Lipid composition and the average diameter of liposomes were: (×) PC-chol (10:5, mol/mol), 201 nm; (○) PC-chol-PEG750PE (10:5:1, mol/mol), 244 nm; (●) PC-chol-PEG2000PE (10:5:1, mol/mol), 189 nm; (■) PC-chol-PEG5000PE (10:5:1, mol/mol), 194 nm; (□) PC-chol-GM_1 (10:5:1, mol/mol), 193 nm. Data taken from Mori *et al.* (1991).

3. Mechanism of action of PEG-PE and GM_1 in prolonging the circulation time of liposomes

PEG is a linear polymer which exhibits considerable conformational flexibility and hence provides a reduced steric barrier activity when compared to a rigid polymer. However, it has been reported that the PEG polymers on liposome surfaces appeared to be in a partially extended conformation, as studied by X-ray analysis (Needham and McIntosh, 1991), suggesting that the steric barrier activity is a function of the polymer chain length. The above results of the liposome agglutination assay and the biodistribution study of liposomes containing various PEG-PEs clearly indicated that both the activity of PEG-PE in prolonging the circulation time of liposomes and its steric barrier activity are proportional to the chain length of amphipathic polymer. Thus, the mechanism by which PEG-PE reduces the RES uptake of liposomes can be attributed at least partially to the steric barrier presented by these polymers on the liposome surface. Other study using radiolabeled streptavidin showed that the inhibitory effect of PEG5000-PE on the binding of streptavidin to liposomes containing biotin-cap-PE is also concentration dependent (Klibanov *et al.*, 1991). However, no significant inhibitory effect was observed at low concentration of PEG-PE, indicating that a protein molecule, i.e. streptavidin, is able to penetrate a relatively weak steric barrier. These results suggests two independent modes of action of PEG-PE. Firstly, the presence of PEG may increase the hydrophilicity of the liposome surface. Secondly, the steric barrier of PEG may prevent opsonins from direct interaction with liposomes. Maximal activity of PEG-PE in prolonging the circulation time of liposomes can be obtained when sufficient concentration and polymer chain length are used so that interaction of liposomes with the RES is minimized.

In view of the steric barrier hypothesis, the relationship between the steric barrier activity of GM_1 and its activity in prolonging the circulation time of liposomes is ambiguous. Needham and McIntosh (1991) have estimated, using X-ray analysis, the steric barrier of GM_1 to be 2.5 nm thick, compared to 6 nm for amphipathic PEG1900. Thus, GM_1 provides only a weak steric barrier compared with PEG1900 in agreement with the data from the liposome agglutination assay (Figure 3). However, the activity of GM_1 in prolonging the circulation time of liposomes is greater than that of PEG2000-PE

(Figure 4). In other words, the activity of GM_1 in prolonging the circulation time of liposomes does not correlate with its steric barrier activity. Allen *et al.* (1985) have shown that incorporation of gangliosides in liposomes results in their enhanced stability in the presence of serum, as shown by the reduced leakage of aqueous markers. The stabilizing effect of gangliosides depends on the number of sialic acid residues; the higher order the ganglioside, the greater the activity in enhancing the stability of liposomes. However, among various gangliosides, GM_1 is unique in terms of having a strong activity in prolonging the circulation time of liposomes. Other gangliosides such as GD_{1a} and GT_{1b} exhibit only little or no activity (Allen *et al.*, 1989b), although these gangliosides contain larger oligosaccharide headgroups than GM_1 and would be expected to be better steric barriers for liposomes. This and our results from the liposome agglutination assay indicate that the stabilizing effect of GM_1 via the steric barriers provided by the oligosaccharide residues is not likely a major mechanism in prolonging the circulation time of liposomes.

B. Optimization of Liposome Size

In addition to the lipid composition, liposome size is another variable in the construction of liposomes. To examine the role of liposome size in determining the biodistribution of liposomes, GM_1-containing and GM_1-free liposomes were prepared, from PC and chol (2:1, mol/mol), with different average diameters. Liposomes were i.v. injected into mice, and the biodistribution was examined 4 hr after injection. Figure 5 shows the effect of liposome size on the biodistribution of GM_1-containing liposomes. Clearly, the activity of GM_1 in prolonging circulation time is restricted to a relatively narrow size range. Optimal liposome sizes for prolonged circulation range from 70 to 200 nm in average diameter. The larger liposomes have an increased tendency to accumulate in the spleen, whereas the smaller liposomes accumulate in the liver very efficiently. In contrast, the size-dependent accumulation in the spleen and the liver was less profound for the liposomes containing no GM_1, although a relatively reduced accumulation of GM_1-free liposomes in the liver with a concomitant increase in the circulation level were observed in liposomes with average diameter of 135 nm (Figure 6). The preferential accumulation of GM_1-containing large and small liposomes in the spleen and the liver, respectively, can be

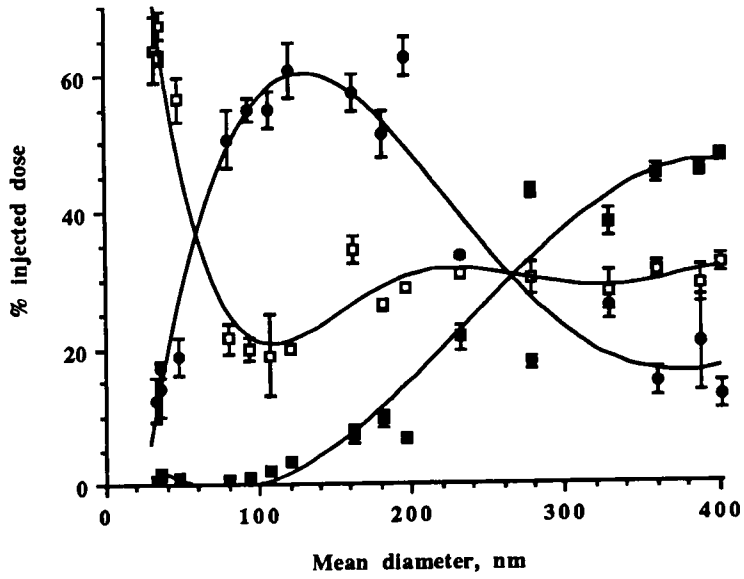


Figure 5. Effect of liposome size on biodistribution of GM_1-containing liposomes. ^{111}In-labeled liposomes composed of PC, chol and GM_1 (10:5:1, mol/mol) with defined diameter were i.v. injected into mice (0.7 mg lipid per mouse). Biodistribution was examined 4 hr after injection. Data are expressed as percent injected dose of liposomes in (●) blood, (■) spleen, and (□) liver. Bars represent S. D. (n = 3). Data taken from Liu *et al.* (1991b) with modification.

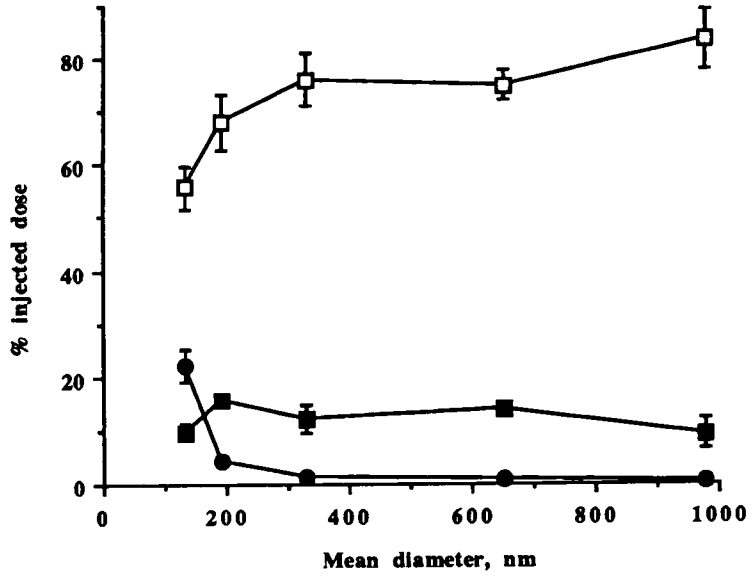


Figure 6. Effect of liposome size on biodistribution of GM_1-free liposomes. ^{111}In-labeled liposomes composed of PC, chol (10:5, mol/mol) with defined diameter were i.v. injected into mice (0.7 mg lipid per mouse). Biodistribution was examined 4 hr after injection. Data are expressed as percent injected dose of liposomes in (●) blood, (■) spleen, and (□) liver. Bars represent S. D. (n = 3). Data taken from Maruyama *et al.* (1991b).

attributed to the anatomical structures of these organs in addition to their lower affinity to the RES, in particular the Kupffer cells of the liver. Since GM_1-containing liposomes show a reduced affinity to the liver Kupffer cells, they have an increased chance of staying in the circulation. When liposomes are large enough (> 300 nm in diameter), they are retained by the splenic sinusoidal filter, thus resulting in enhanced accumulation in the spleen. On the other hand, sufficiently small liposomes (< 70 nm in diameter) can extravasate to the liver parenchyma through the holes (approximately 100 nm in average diameter) located in the fenestrae of the liver sinus (Wisse, 1970), thus resulting in enhanced accumulation in the liver. Thus, the mechanisms responsible for the biodistribution of small and large liposomes are basically different. The highest circulation level of GM_1-containing liposomes can be obtained using liposomes of appropriate size, i.e. between 70 and 200 nm in diameter; the total uptake of these liposomes by the spleen and the liver is minimized. On the other hand, since GM_1-free liposomes may have a high affinity to the liver Kupffer cells, they rapidly accumulate in the liver and are cleared from the circulation irrespective of liposome size. Thus, only a small fraction of liposomes is available for the accumulation in the liver parenchyma and the spleen, and thus size-dependent biodistribution of GM_1-free liposomes is less profound. Although we specifically discussed the importance of liposome size using GM_1-containing liposomes, it is likely that the activities of other amphiphiles such as the amphipathic PEG in prolonging the circulation time of liposomes are also liposome size-dependent.

CHAPTER IV

Tumor Targeting with Long-Circulating Liposomes

In the previous chapter, the construction and optimization of liposomes for prolonged circulation were described. An immediate application using this novel type of liposome is to deliver liposomal drugs to the cells, tissues or organs other than the RES. Especially, a successful application of liposomes in cancer therapy requires an efficient targeting of liposomal drugs to the malignant areas, i.e., tumor cells. Various attempts have been made to improve accumulation of liposomes in tumors. Proffitt *et al.* (1983) have reported that, by a preinjection with unlabeled "empty" liposomes to block the saturable RES uptake of liposomes, an enhanced gamma-imaging of solid tumor was resulted from an increased accumulation of subsequently injected ^{111}In-labeled liposomes in the tumor. A hypothesis, derived from these tumor-targeting studies of liposomes, is that if the liposomes can stay in the circulation for a prolonged period of time, they have an improved chance to interact with tumor cells, and thus an enhanced accumulation of liposomes in the tumor would be expected. In this chapter, the potential of long-circulating liposomes for a drug delivery to solid tumor will be described.

A. EMT6 Tumor System

Mouse mammary adenocarcinoma, EMT6, was used as a model tumor system (Figure 7). The growth rate of the tumor cells in mice varied widely. Two weeks after inoculation, the weight of tumor ranged from 0.5 to 2.0 g. In larger tumors, exceeding 2.0 g, an extensive necrotic area was observed. In this study, the mice with tumor weighing between 0.8 and 1.5 g were used for biodustribution studies of liposomes.

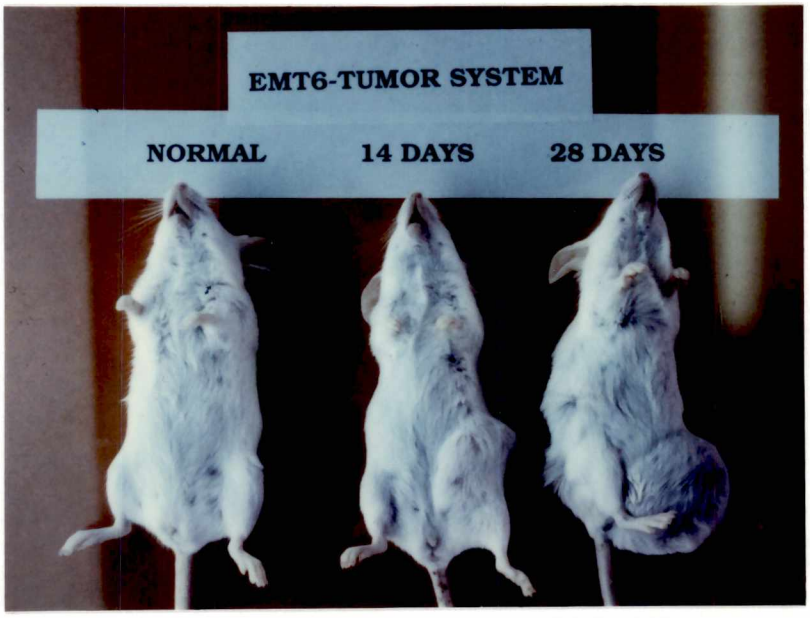


Figure 7. EMT6 tumor system

B. Effect of Prolonged Circulation on Liposome Accumulation in Tumor

1. Biodistribution of GM_1-containing liposomes in tumor-bearing mice

The first step of this study was to examine the biodistribution of GM_1-containing and GM_1-free liposomes in EMT6 tumor-bearing mice. Three different liposome formulations, differing in a phospholipid component, were used in this study. Liposomes were prepared from phospholipid (PC, DSPC, or DOPE) and chol (2:1, mol/mol) with or without 6.3 mol% GM_1 and having average diameter ranging from 92 to 130 nm. Biodistribution of liposomes in tumor-bearing mice was examined 24 hr after injection. Data were expressed as percent injected dose in tumor (per gram), blood and the RES (liver and spleen) (Table 1). As observed in the normal mouse experiment, a majority of liposomes injected into tumor-bearing mice accumulated in the RES. However, a significant increase in tumor uptake was observed in each GM_1-containing PC-, DSPC-, and DOPE-based liposomes, as compared to the respective GM_1-free liposomes. Accumulation of GM_1-containing DSPC-based liposomes in tumor reached 9.2% of total injected dose per gram of tumor, followed by 7.9% for GM_1-containing PC-based liposomes. Accumulation of GM_1-containing DOPE-based liposomes appeared to be relatively low (2.6%), which was nevertheless greater than that of the GM_1-free liposomes (1.4%). A relatively high accumulation of liposomes in tumor generally correlated with a relatively high amout of liposomes in circulation. Also shown in Table 1 is data on the RES uptake of liposomes. The RES uptake of liposomes was considerably reduced when GM_1 was included in the lipid composition irrespective of the phospholipid component. Preliminary studies showed that incorporation of PEG-PE, another amphiphile having an activity in prolonging the circulation time of liposomes, also resulted in an enhanced accumulation of PC-based liposomes in tumor (data not shown). These results clearly indicate that the lipid composition exhibiting a reduced affinity to the RES and thus prolonged circulation is an important requirement for an enhanced accumulation of liposomes in tumor.

Figure 8 shows the time-dependent biodistribution data for GM_1-containing PC-based liposomes (125 nm in average diameter) in tumor-bearing mice. Within the initial 4 hr after injection, a rapid clearance of liposomes from the circulation was observed,

Table 1. Biodistribution of GM1-containing and GM1-free liposomes in tumor bearing mice [a]

Lipid Composition	Mean diameter (nm)	% injected dose [b]			
		Tumor [c,d]	Blood	RES	Total
PC-chol (10:5)	130	2.9 (0.3)[e]	0.3 (0.1)	71.6 (2.5)	79.3 (2.7)
PC-chol-GM1 (10:5:1)	92	7.9 (2.5)[f]	3.1 (1.0)	51.0 (2.2)	68.2 (1.6)
DSPC-chol (10:5)	130	5.4 (1.3)[g]	0.4 (0.1)	41.2 (7.9)	52.2 (7.5)
DSPC-chol-GM1 (10:5:1)	118	9.3 (1.0)[h]	4.1 (1.1)	34.1 (4.4)	54.4 (2.2)
DOPE-OA (10:5)	113	1.4 (0.5)[i]	0.3 (0.1)	73.7 (6.7)	78.3 (4.6)
DOPE-OA-GM1 (10:5:1)	110	2.6 (0.4)[j]	0.6 (0.0)	66.6 (2.8)	75.1 (2.3)

[a] 111-In-labeled liposomes with the indicated lipid composition and size were i.v. injected into EMT6-tumor bearing mice. Percent injected dose of liposomes in tumor, blood, and the RES was examined 24 hr after injection.

[b] Data were expressed as mean (S.D.), n = 3.

[c] Data were expressed as percent injected dose per gram of tumor

[d] Statistical analysis (Student t-test): e vs. f, $p < 0.01$; g vs. h, $p < 0.01$; i vs. j, $p < 0.01$.

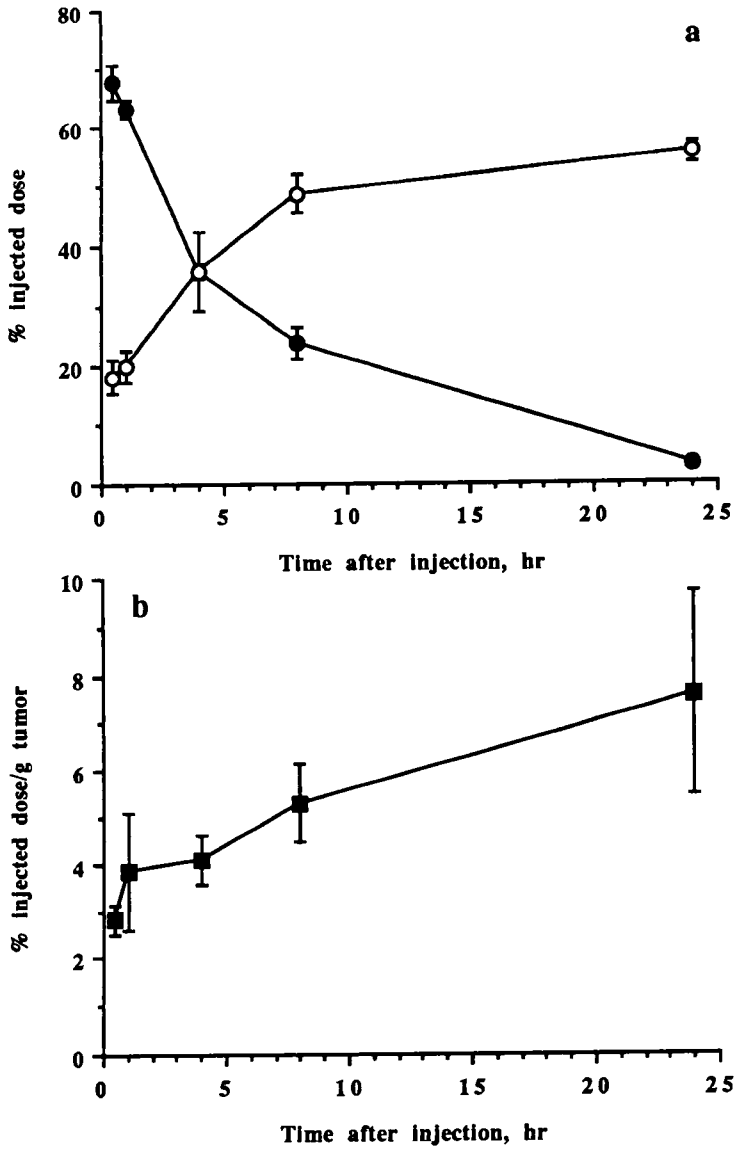


Figure 8. Time-dependent biodistribution of GM_1-containing liposomes in tumor-bearing mice. ^{111}In-labeled liposomes composed of PC, chol and GM_1 (10:5:1, mol/mol) with average diameter of 125 nm were i.v. injected into mice bearing EMT6 tumor (0.7 mg lipid per mouse). Biodistribution was examined at indicated time intervals. Data were expressed as perent injected dose of liposomes in (■) tumor, (●) blood, (O) RES (liver and spleen). Bars represent S. D. (n = 3).

followed by a relatively gradual clearance up to 24 hr after injection (Figure 8a). The data also indicate that liposomes cleared from the circulation accumulated mainly in the RES. Accumulation of liposomes in tumor was also time dependent, reaching about 8% of total injected dose per gram of tumor at 24 hr after injection (Figure 8b). The enhanced accumulation of GM_1-containing liposomes in tumor was not due to blood contamination of tumor, since only a small fraction (3%) of liposomes remained in the circulation at this time. On the contrary, the liposome level in lung, heart, and kidney rapidly decreased in accordance with the rapid clearance of liposomes from the circulation (data not shown).

The effect of injected lipid dose on the biodistribution of GM_1-containing liposomes in tumor was then studied. In this study, an increasing amount, ranging from 0.5 to 5.0 mg lipid, of liposomes composed of PC, chol and GM_1 (10:5:1, mol/mol) was injected into tumor-bearing mice, and biodistribution was examined 24 hr after injection. Data were expressed as the amount of lipid per gram of tumor, blood, liver and spleen. It is clear from the data in Figure 9a that the accumulation of liposomes in tumor was not a saturable process. As the injected dose of liposomes increased, the amount of lipid accumulating in tumor also increased. The amount of lipid in the circulation increased with increase in the amount of liposomes injected (Figure 9a). Thus, the amount of lipid accumulating in tumor correlated well with that in the circulation. Similarly, no saturable uptake of liposomes by the RES (liver and spleen) was observed in this range of lipid dose (Figure 9b). These results indicate that accumulation of liposomes in tumor is through a nonspecific interaction with the tumor mass, which is driven by the concentration of the liposome in circulation.

2. Effect of Liposome Size on Accumulation in Tumor

The above results clearly indicate that the inclusion of GM_1 in the lipid composition results in an enhanced accumulation of liposomes in tumor. The enhanced accumulation of GM_1-containing liposomes in tumor can be attributed to their reduced affinity to the RES and thus prolonged circulation. As discussed in chapter 3, there is a relatively restricted size range for the prolonged circulation of GM_1-containing liposomes. We have thus examined the effect of liposome size on the accumulation of liposomes in tumor. In this

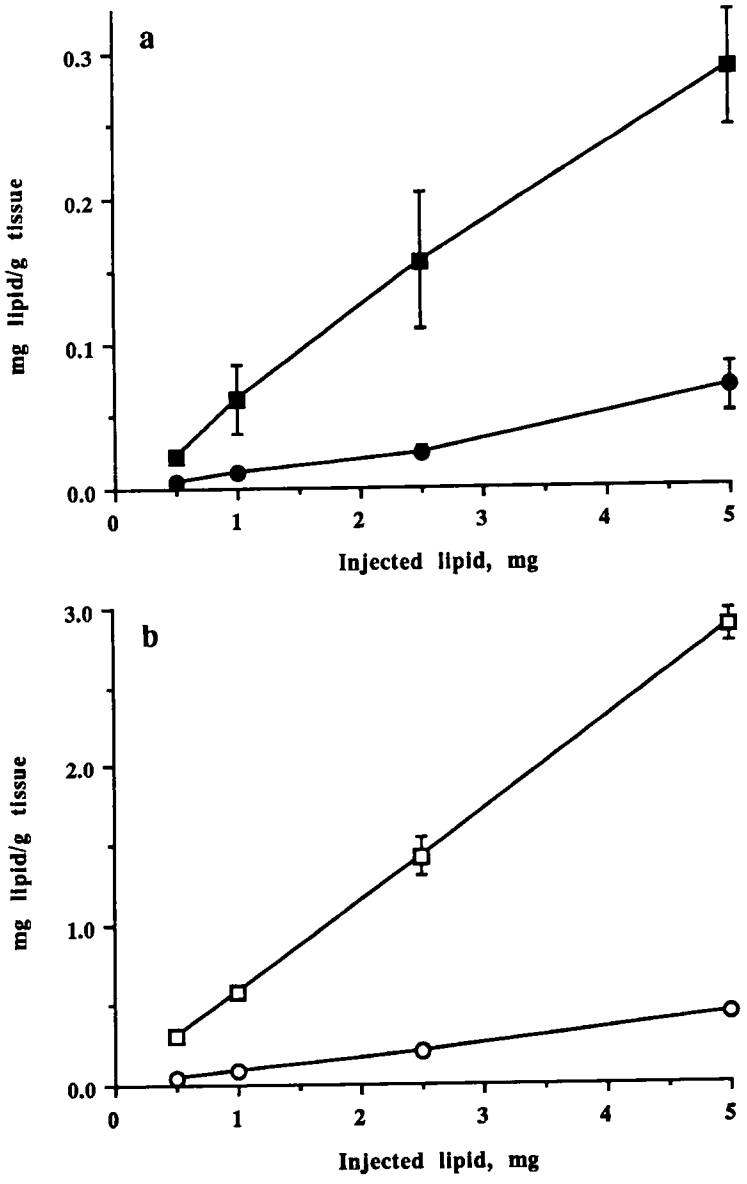


Figure 9. Effect of injected lipid dose on biodistribution of GM_1-containing liposomes in tumor-bearing mice. ^{111}In-labeled liposomes composed of PC, chol and GM_1 (10:5:1, mol/mol) with average diameter of 125 nm were i.v. injected into mice bearing EMT6 tumor at indicated lipid doses. Biodistribution was examined 24 hr after injection. Data were expressed as the amount of lipid per gram of (■) tumor, (●) blood, (□) liver and (○) spleen. Bars represent S.D. (n = 3).

study, liposomes composed of PC, chol and GM_1 (10:5:1, mol/mol) with average diameter ranging from 40 to 600 nm were used. Figure 10 shows the size-dependent accumulation of GM_1-containing liposomes in tumor 24 hr after i.v. injection, expressed as the percent injected dose per gram of tumor. Optimal accumulation (~ 8% injected dose per gram of tumor) was obtained with liposomes with average diameter of 90 and 200 nm. Liposomes with average diameter of 40 nm or larger than 200 nm did not show any significant accumulation in tumor (2 ~ 3%). This size-dependent accumulation of liposomes in tumor appeared in turn to correlate directly with the size-dependent activity of GM_1 in prolonging the circulation time of liposomes (Figure 5). These results clearly demonstrate the hypothesis that a relatively high accumulation of GM_1-containing liposomes in tumor is resulted from a relatively high level of liposomes in the circulation.

3. Tumor Imaging Study

To visualize an enhanced accumulation of GM_1-containing liposomes in tumor, a gamma imaging study of EMT6 tumor-bearing mice was performed by taking advantage of ^{111}In-label, a gamma-emitter. Liposomes used in this study were GM_1-containing and GM_1-free liposomes and have average diameter of 140 and 156 nm, respectively. Images were obtained 24 hr after injection at which the maximal accumulation of liposomes and an almost complete clearance of liposomes from the circulation was expected (Figure 8). Figure 11 shows the representative whole body images of the EMT6 tumor-bearing mice injected with GM_1-containing (a) and GM_1-free (b) liposomes. With both liposome formulations, an intense image in the upper abdomen corresponding to the liver and the spleen was obtained. However, an image in the tumor area was clearly enhanced in mice injected with GM_1-containing liposomes, as compared with mice injected with GM_1-free liposomes, in agreement with their biodistribution data.

4. Mechanism of liposome accumulation in tumor

A precise mechanism of the liposome accumulation in tumor is not known. However, a nonspecific interaction of liposomes with the tumor mass seems to be a major

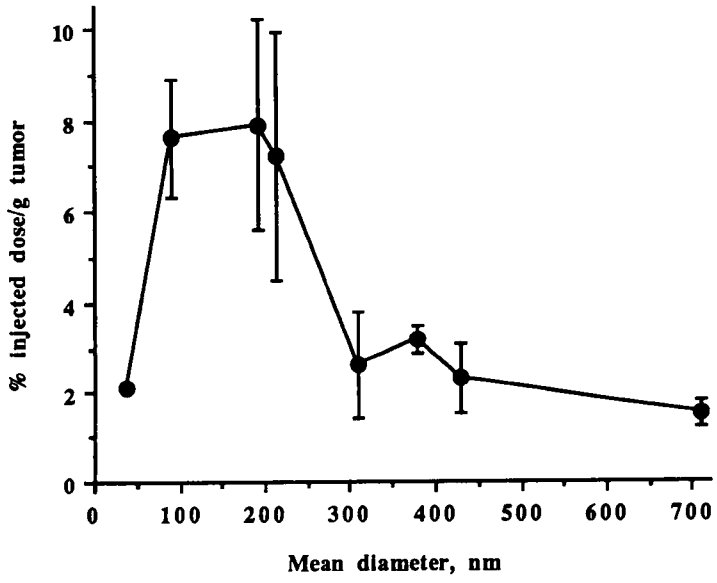


Figure 10. Size-dependent accumulation of GM_1-containing liposomes in tumor. ^{111}In-labeled liposomes composed of PC, chol and GM_1 (10:5:1, mol/mol) with difined diameters were i.v. injected into mice bearing EMT6 tumor (0.7 mg lipid per mouse). Accumulation of liposomes in tumor was examined 24 hr after inection. Data were expressed as percent injected dose of liposomes per gram of tumor. Bars repreent S.D. (n = 3).

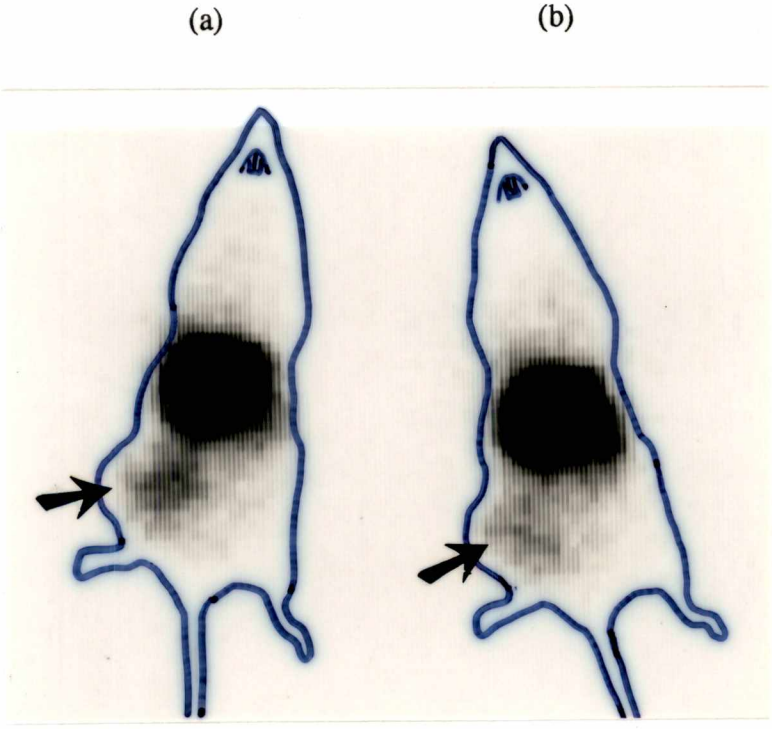


Figure 11. Gamma camera images of tumor-bearing mice injected with GM_1-containing (a) and GM_1-free (b) liposomes. ^{111}In-labeled liposomes composed of PC, chol and GM_1 (10:5:1, mol/mol, 140 nm in average diameter) or PC and chol (10:5, mol/mol, 156 nm in average diameter) were i.v. injected into mice bearing EMT6 tumor on right hind legs (5 mg lipid (2×10^7 cpm) per mouse). Images were taken 24 hr after injection. Arrows indicate the site of tumor.

mode of interaction because of the following facts: 1) There are no known tumor-specific ligands on liposome surface; 2) The liposome accumulation in tumor is a non-saturable process. The results of this study indicate that GM_1-containing liposomes accumulate more efficiently than GM_1-free liposomes. In addition, inclusion of PEG5000-PE in the lipid composition also resulted in an enhanced accumulation of liposomes in tumor (data not shown). Thus, a prolonged circulation of liposomes seems to be a general requirement for an efficient accumulation of liposomes in tumor. This hypothesis was further supported by the finding that the optimal size range of liposomes for tumor accumulation was the same as that for prolonged circulation. Several studies have shown that capillary permeability of newly formed tumor vessels is significantly greater than that of the other normal organs (Underwood and Carr, 1972; Peterson, 1979; Jain and Gerlowski, 1986). Presumably, long-circulating liposomes may have an enhanced probability of reaching these leaky vessels of tumor. It is therefore likely that this mode of liposome targeting to tumor depends largely on the degree of tumor vascularization and its permeability.

The observation that liposome level in tumor was greater than that in circulation at 24 hr after injection suggests an existence of an active transport mechanism of liposomes into tumor from circulation. One potential mechanism may involve endocytic uptake of liposomes by tumor cells and/or binding of liposomes by capillary endothelial cells. However, these possibilities seems to be unlikely, since it has been reported that i.v. injected long-circulating liposomes localize at the interstitial space within the tumor (Papahadjopoulos, 1990). Thus, extravasation of liposomes seems to be a major mechanism of accumulation in tumors. Liposomes could extravasate either by diffusion or by convection (for a review, see Jain, 1989). Presumably, once liposomes extravasate through leaky vessels, they are retained in the tumor interstitial space due to a yet unknown mechanism(s).

CHAPTER V

Highly Targetable Immunoliposomes

Immunoliposomes have been designed to accumulate in their target site(s) through an immunospecific interaction between an antibody molecule on the liposome surface and an antigen molecule on the surface of the target cell membrane (for a review, see Wright and Huang, 1989). However, several studies of immunoliposomes have shown the difficulty of *in vivo* immunoliposome targeting due to the high affinity of liposomes to the RES (for a review, see Peeters *et al.*, 1987). Moreover, RES uptake of liposomes is often facilitated via Fc receptor mediated endocytosis due to the presence of the antibody molecules on the liposomes (Aragnol and Leserman, 1986; Derksen *et al.*, 1988; Peeters *et al.*, 1987). Thus, it is difficult to achieve "absolute targeting" of immunoliposomes *in vivo*. In this chapter, in an attempt to overcome this problem, the construction and optimization of immunoliposomes, based on the development of long-circulating liposomes, will be described.

A. A Pulmonary Endothelial Model For Liposome Targeting

One of the important factors for successful targeting of immunoliposomes *in vivo* is the choice of antibody-antigen system. Several variables including antibody specificity and affinity, antigen concentration on the target site(s), and accessibility of the antigen to the antibody are factors of the individual system to be used which directly affect the feasibility of liposome targeting. Moreover, in view of the fact that liposomes are too large to penetrate most types of endothelial cell linings of vascular walls, i.v. injected immunoliposomes would be expected to interact efficiently with the target site only within the intravascular compartment. Considering these factors, the monoclonal antibody 34A (MoAb 34A) system is a nearly ideal model for the study of *in vivo* immunoliposome

targeting. MoAb 34A is a rat IgG_{2a} which binds specifically with a glycoprotein antigen, gp112, expressed in high concentrations on the lumenal surface of the capillary endothelial cells of the mouse lung (Rorvik *et al.*, 1988; Kennel *et al.*, 1988). For preparation of immunoliposomes, the detergent-dialysis method has been developed (Holmberg *et al.*, 1989). Antibody is first conjugated to NGPE (Weissig *et al.*, 1986) in the presence of detergent. The resulting amphipathic antibody is then mixed with a lipid-detergent mixture, and the incorporation of antibody is achieved upon the removal of the detergent by dialysis. Figure 12 shows the representative biodistribution data of MoAb 34A bearing liposomes (34A-liposomes). The i.v. injected 34A-liposomes gain direct access to the lung target and accumulate primarily in the lung and secondarily in the liver, while liposomes without antibody molecules accumulate exclusively in the liver and the spleen and not in the lung (Hughes *et al.*, 1989; Maruyama *et al.*, 1990a; Holmberg *et al.*, 1990). Lung binding of 34A-liposomes was completely blocked by a preinjection of free MoAb 34A, indicating that the binding of 34A-liposomes with the lung is via immunospecific interactions. We have thus used the MoAb 34A system to study various factors affecting *in vivo* targeting of immunoliposomes. A previous study showed that the efficiency of target binding on 34A-liposomes strongly depends on the antibody density on liposomes. The higher the antibody density, the higher the rate and extent of target binding of immunoliposomes.

B. Effect of Prolonged Circulation on Target Binding of Immunoliposomes

Although 34A-liposomes bind significantly to the lung target, substantial accumulation of immunoliposomes in the liver is also observed (Figure 12). The rationale of our strategy is based on the development of liposomes with reduced affinity to the RES. By including a specific amphiphile such as GM_1 and PEG-PE in the lipid composition, immunoliposomes circulate for a prolonged period of time so that they have an increased chance to interact with the target site. This strategy has been demonstrated successfully by Maruyama *et al.* (1990b), showing that, when GM_1 is included in the lipid composition, the resulting 34A-liposomes showed much more efficient target binding than the ones containing no GM_1. Klibanov *et al.* (1991) have further shown that, although the activity

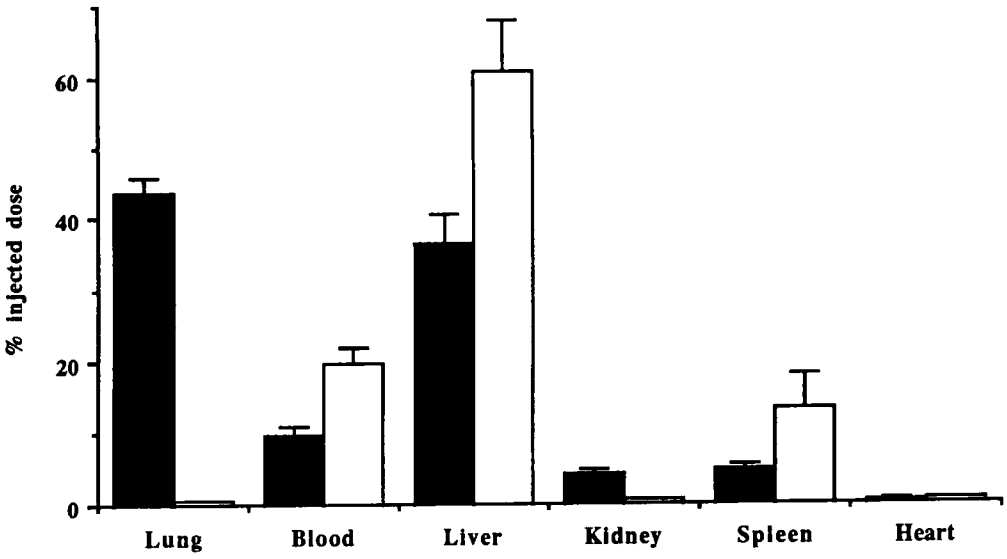


Figure 12. Biodistribution of 34A-liposomes and bare liposomes in mice. The MoAb 34A-bearing liposomes or liposomes without antibody were prepared from PC, chol and GM_1 (10:5:1, mol/mol) and were i.v. injected into mice (0.2 mg lipid per mouse). Biodistribution was examined 1 hr after injection. Bars represent S. D. (n = 3). (■) 34A-liposomes with an antibody-to lipid ratio of 1:18 (wt./wt.) and 194 nm in average diameter. (□) bare liposomes with 201 nm in average diameter.

of PEG5000-PE in prolonging the circulation time of liposomes is greater than that of GM_1, inclusion of PEG5000-PE in the lipid composition resulted in a lower level of target binding. This observation has been attributed to the overly strong steric barrier activity of PEG5000-PE, which may interfere with the antibody-antigen interaction. The results of the liposome agglutination assay indicated that PEG750-PE and PEG2000-PE provide weaker steric barriers than PEG5000-PE (Figure 3), although their activities in prolonging the circulation time of liposomes are also lower than that of PEG5000-PE (Figure 4). We have thus examined the effects of these PEG-PEs with shorter polymer chain on target binding of immunoliposomes using the MoAb 34A system.

Effect of PEG-PE on target binding of immunoliposomes

The 34A-liposomes used in this study were prepared from PC and chol (1:1, mol/mol) additionally containing 7.0 mol% of PEG-PE. The lung binding of 34A-liposomes was examined 1 hr after injection and was expressed as percent injected dose per organ (Table 2). The antibody-to-lipid ratio of 34A-liposomes used in this study ranged from 1:13.9 to 1:18.1 (wt./wt.). This range did not cause a significant change in lung binding as shown in the control 34A-liposomes with the 34A-to-lipid ratios of 1:13.9 and 1:18.1 (43% and 44%, respectively). Inclusion of PEG2000-PE in the liposomes resulted in an enhanced lung binding (53%) which was the same level as found in GM_1-containing 34A-liposomes (53%), while inclusion of PEG750-PE resulted in only a slightly enhanced binding (47%). On the contrary, PEG5000-PE caused a significantly reduced lung binding (22%). Thus, among the PEG-PEs tested, PEG2000-PE seems to be optimal for target binding of immunoliposomes. These observations can be understood on the basis of the relative steric barrier activity of each PEG-PE species. As shown in Figure 3, PEG5000-PE provides the highest steric barrier on the liposome surface which presumably prevent the antibody-antigen interaction, thus resulting in a decreased target binding of immunoliposomes. In contrast, the steric barrier activities of PEG750-PE and PEG2000-PE are not strong enough to interfere with the antibody-antigen interaction, resulting in an enhanced target binding of immunoliposomes according to their activities in prolonging the circulation time of liposomes. It should be noted that the above data were

Table 2. Effects of various PEG-PEs and GM1 on target binding of immunoliposomes[a]

Lipid composition (molar ratio)	34A:lipid (w/w)	Average diameter (nm)	% injected dose[b]		
			Lung[c]	RES	Blood
PC-chol (1:1)	1:18.1	194	43.8 (2.2)[d]	41.3 (3.5)	9.5 (1.3)
	1:13.9	194	42.9 (3.2)[d]	41.9 (2.7)	12.5 (0.2)
PC-chol-GM1 (1:1:0.15)	1:15.0	188	52.5 (2.9)[e]	35.9 (5.1)	6.3 (0.8)
PC-chol-PEG750PE (1:1:0.15)	1:16.9	192	47.1 (1.8)[f]	40.6 (4.6)	7.6 (0.3)
PC-chol-PEG2000PE (1:1:0.15)	1:17.7	176	52.6 (2.5)[g]	34.6 (1.5)	11.4 (1.1)
PC-chol-PEG5000PE (1:1:0.15)	1:15.0	163	21.5 (1.2)	21.1 (2.6)	55.0 (2.7)

[a] 111-In-labeled 34A-liposomes with the indicated lipid composition, antibody-to-lipid weight ratio, and average diameter were prepared by the detergent-dialysis method. Liposomes (0.4 mg lipid) were i.v. injected, and % injected dose in lung, RES (liver and spleen), and blood was measured 1 hour after injection.

[b] Data are expressed as mean (S.D.), n = 3.

[c] Statistical analysis (Student's t-test): d vs. e, $p < 0.01$; d vs. f, $p < 0.05$; d vs. g, $p < 0.01$; f vs. g, $p < 0.02$.

obtained using 34A-liposome with a relatively high antibody density. For 34A-liposomes with much lower antibody density, it is likely that enhanced target binding of liposomes containing GM_1 or PEG2000-PE, compared to that of the control 34A-liposomes, would be more profound.

Another *in vivo* study using liposomes bearing the rabbit anti-myosin antibody showed that inclusion of PEG5000-PE at a lower concentration (4 mol%) in the lipid composition results in a several-fold increase in the accumulation of immunoliposomes in the region of experimental myocardium infarction as compared to those containing no PEG5000-PE (Torchilin *et al.*, 1991). This is presumably due to the lower concentration of PEG5000-PE in the lipid composition which provides a weaker steric barrier and thus does not interfere with the target binding. These results suggest a rational strategy in using amphipathic PEG in the immunoliposome targeting. As can be seen in Figure 3, the degree of the steric barrier activity of amphipathic PEG is a function of both polymer chain length and concentration in the lipid composition. Thus, one can optimize target binding of immunoliposomes by altering these parameters such that the activity in prolonging the circulation time of liposomes is maximized, yet the antibody-antigen interaction is not compromised.

C. Optimization of *In Vivo* Immunoliposome Targeting

Previous studies of *in vivo* immunoliposome targeting using the MoAb 34A system indicate that accumulation of immunoliposomes at the target site is determined as a result of two kinetically competing processes: binding to the target site and uptake by the RES (Maruyama *et al.*, 1990b). The rate of target binding of immunoliposomes is directly proportional to the antibody density of liposomes, while uptake by the RES depends strongly on the lipid composition and, presumably, also the liposome size to be used. Consequently, general requirements for an efficient target binding of immunoliposomes are a sufficiently high antibody density and a reduced affinity to the RES. Maximum targeting of immunoliposomes to the target site can be achieved when these two variables are optimized.

CHAPTER VI

Conclusion and Perspective

A number of *in vitro* studies have demonstrated that liposomal drugs are much superior to the free drugs in terms of efficacy. Thus, it has been expected that liposomes may provide an excellent *in vivo* delivery system for various bioactive agents and improve their therapeutic indices. However, *in vivo* studies have indicated that an applicability of liposomes in drug delivery is severely restricted for the cells in the RES due to a predominant localization of injected liposomes in the RES and thus a rapid clearance of liposomes from the circulation. Described in Chapter III is the development of liposomes with a reduced affinity to the RES and hence with a prolonged circulation time. Two variables, lipid composition and liposome size, have been revealed to be important for the construction of such long-circulating liposomes. General requirements are the inclusion of a specific amphiphile such as GM_1 and PEG-PE in the lipid composition and the choice of appropriate liposome size, i.e., 70 ~ 200 nm in average diameter. While a steric barrier effect seems to be responsible for the activity of PEG-PE in prolonging the circulation time of liposomes, the mechanism of GM_1 in prolonging the circulation time of liposomes must involve other factors. Such information is obviously important in the optimal construction of liposomes for prolonged circulation.

The developement of long-circulating liposomes has opened new approaches using liposomes for a drug delivery. In particular, the long-circulating liposomes would be expected to provide an efficient delivery system for various bioactive agents to cells, tissues or organs other than the RES. This was demonstrated in Chapter IV, showing that long-circulating liposomes are able to accumulate much more efficiently in a transplantable tumor than are conventional liposomes. Consequently, the long-circulating liposomes would be expected to deliver bioactive agents more efficently and to reduce side effects arising from the predominant accumulation of liposomal drugs in the RES (Gabizon *et al.*,

1989). Indeed, several preliminary studies have shown the improved therapeutic indices of cytotoxic drugs using these liposomes as compared to conventional liposomes (Gabizon, 1990; Martin *et al.*, 1990; Papahadjopoulos, 1990).

Perhaps, much efforts will be concentrated in drug targeting with immunoliposome system due to its specificity and versatility. Described in Chapter V is the construction of immunoliposomes, based on the development of long-circulating liposomes, for an efficient target binding. When immunoliposomes are designed to exhibit reduced affinity to the RES by including a specific amphiphile such as GM_1 and PEG-PE in the lipid composition, they perform more efficiently than the ones with the conventional lipid composition. These studies also revealed that a co-existing molecule on the liposome surface often affects the antibody-antigen interaction through its steric barrier effect, as observed in the immunoliposomes containing PEG5000-PE. Thus, a prolonged circulation of immunoliposomes does not necessarily lead to their enhanced target binding. A basic prerequisite in the construction of immunoliposomes, derived from these observations, is that a modification of the lipid composition for prolonged circulation must be made such that the antibody-antigen interaction is not compromised. We have studied the therapeutic potential of 34A-liposomes using an experimental mouse pulmonary metastasis model (EMT6 tumor cells injected i.v.). In the concept of this organ-specific immunoliposomes, the drugs are expected to be released from the bound immunoliposomes and diffuse a short distance to reach the tumor cells in the same organ. Preliminary results showed that mean survival time of mice treated with 34A-liposomes containing lipophilic prodrugs of deoxyfluorouridine was significantly prolonged (33 days), compared to mice treated with PBS (20 days, $p < 0.01$) or free drug (15 days, $p < 0.01$) (Maruyama *et al.*, 1991a).

Although we have marginally demonstrated the effectiveness of prolonged circulation in immunoliposome targeting, it is likely that this strategy is also effective in other types of ligand-directed liposomes, such as those bearing sugars, lectins, peptide hormones, glycoproteins and glycolipids. An enhancing effect of prolonged circulation on the target binding of liposomes may, however, depend on the system to be used. It should be noted that the target antigen for the system used in this study is located at a readily accessible site, i.e., the vascular endothelial cell surface of the lung. For a much less

accessible target site, the described immunoliposome formulations may not necessarily exhibit the same high degree of target binding. For instance, the amount of blood circulating through a solid tumor is only a small fraction of the total. Furthermore, the morphology of tumor vasculature differs highly depending on the type, age, growth rate, and location of the tumor (for a review, see Jain, 1988). Thus, targeting of liposomes to tumor cell surface antigen in a solid tumor may require much stronger activity of liposomes to stay in the circulation.

The above described strategy for efficient target binding of immunoliposomes seems, however, to be effective only when the target cell is in the intravascular compartment or can be accessible through the leaky vasculature. Targeting of immunoliposomes to sites localized outside the continuous vascular compartment, such as the neurons in the brain, is still limited due to the inability of immunoliposomes to cross the continuous endothelial cell lining of the vascular wall. Although no practical strategy to overcome this anatomical barrier has been proposed, one potential approach might be to take advantage of the transcytosis function of endothelial cells. In transcytosis, certain ligand-receptor complexes are, upon endocytosis, transported across the endothelial cell barrier (Mostov and Simister, 1985; Casanova *et al.*, 1990). When such a ligand is incorporated into a liposome membrane, the liposome might be transcytosed and reach the target cells localized on the other side of endothelium.

In order for drugs carried by immunoliposomes to be effective at the target cell, several events must take place following to the binding of immunoliposomes to the target cell. These include the internalization of liposomes by the target cell and release of the drug to the cytoplasmic compartment of the target cell to exert its functional activity. Although there seems to be several mechanisms responsible for liposome internalization, evidence suggests that the receptor-mediated endocytosis is the major pathway, which in turn is a function of the target cell membrane. Thus, binding of immunoliposomes to the target cells does not necessarily result in their rapid internalization. Moreover, the destination of the internalized immunoliposomes via endocytosis is usually the lysosomes where the entrapped drugs as well as the antibody molecules and the lipids are degraded and/or inactivated. To overcome these problems, various types of liposome with a triggered

release mechanism have been developed. The pH-sensitive immunoliposomes are designed to destabilize at mildly acidic pH as found in the endosomes (Connor and Huang, 1986; Collins and Huang, 1987; Wang and Huang, 1987). The target-sensitive immunoliposomes, on the other hand, are designed to release their contents upon binding to the target cell via bilayer destabilization (Ho *et al.*, 1986). Finally, temperature-sensitive immunoliposomes release their contents at the cell surface upon heating to their characteristic transition temperature (Sullivan and Huang, 1986). The effectiveness of these novel types of immunoliposomes for cytoplasmic delivery has been well proven in various *in vitro* systems. However, the result of *in vivo* studies have indicated their rapid clearance from the circulation (Connor *et al.*, 1986). Although attempts have been made to improve the circulation time of these liposomes by inclusion of a specific amphiphile such as GM_1 and PEG-PE in the lipid composition, these modifications often resulted in a reduced sensitivity to the appropriate environmental change (Liu and Huang, 1990). Thus, the development of immunoliposomes having both reduced affinity to the RES and a target triggered release mechanism would be one of our future goals.

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