Review

In Vivo Targeting by Liposomes

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This review deals with the current status of newly developed pendant-type PEG-immunoliposomes (Type C), carrying monoclonal antibodies or their fragments (Fab′) at the distal ends of the PEG chains. In terms of target binding of Type C, two different anatomical compartments are considered. They are mouse lung endothelium as a readily accessible site *via* the intravascular route and the implanted solid tumor as a much less accessible target site reached *via* extravasation. Distearoyl phosphatidylethanolamine derivatives of PEG with a carboxyl group (DSPE-PEG-COOH) and dipalmitoyl phosphatidylethanolamine derivatives of PEG with a maleimidyl group (DPPE-PEG-Mal) at the PEG terminus were newly synthesized. Small unilamellar liposomes (90—130 nm in diameter) were prepared from phosphatidylcholine and cholesterol (2 : 1, m/m) containing 6 mol% of DSPE-PEG-COOH or DPPE-PEG-Mal. For targeting to the vascular endothelial surface in the lung, 34A antibody, which is highly specific to mouse pulmonary endothelial cells, was conjugated to PEG-liposomes (34A-Type C). The degree of lung binding of 34A-Type C in BALB/c mouse was significantly higher than that of 34A-Type A, which is an ordinary type of immunoliposome (without PEG derivatives). For targeting to solid tumor tissue, 21B2 antibody (anti-human CEA) and its Fab′ fragment were used. The targeting ability of Fab′-Type C was examined by using CEA-positive human gastric cancer strain MKN-45 cells inoculated into BALB/c nu/nu mice. Fab′-Type C showed low RES uptake and a long circulation time, and enhanced accumulation of the liposomes in the solid tumor was seen. The small Fab′-Type C predominantly passed through the leaky tumor endothelium by passive convective transport. These studies offer important insights into the potential of Type C liposomes for target-specific drug delivery.

Key words drug delivery system; liposome; targeting

1. INTRODUCTION

It was the German bacteriologist Paul Ehrlich who, in the late nineteenth century, coined the term "magic bullet," meaning a chemical that travels through the body and selectively kills diseased cells without harming neighboring healthy ones.[1] Since then, the field of site-specific drug delivery has been continuously explored to develop formulations with a high degree of specificity. Many different approaches using various physical and biochemical principles have been proposed and examined to develop systems with a therapeutically acceptable degree of target specificity.[2—5] Among the different approaches to drug delivery, immunoliposomes using an antibody as a targeting ligand and a lipid vesicle as a carrier for both hydrophobic and hydrophilic drugs have attracted much attention. It has been demonstrated that specific delivery of drugs to the target cells is far more efficient with immunoliposomes than with liposomes lacking antibody.[6] The success of *in vitro* delivery to target cells using immunoliposomes (corresponding to Type A in Fig. 2) has prompted similar experiments *in vivo*. However, targeting of immunoliposomes *in vivo* is far more complicated. Studies *in vivo* have revealed that bound antibodies lead to enhanced uptake of the immunoliposomes by the reticuloendothelial system (RES),[7—9] and the targeting efficiency depends on the antibody density on the surface.[10] The rapid uptake of liposomes by the RES and the endothelial barriers separating blood and tissues largely prevent immunoliposomes from reaching their target cells. Thus, highly efficient targeting and a relatively low level of RES uptake of the immunoliposomes are apparently mutually exclusive. As systemic administration is the most practical route for treatment, immunoliposomes overcoming these physiological barriers are highly desirable. The development of liposomes with RES-avoidance is a necessary first step in this direction.

Liposomes have been extensively tested in experimental animals as carriers for drug delivery. A major development in the last few years has been the development of liposomes with a prolonged circulation time in blood, commonly called long-circulating or sterically stabilized liposomes. Liposomes containing either monosialoganglioside G_{M1}[11] or polyethylene glycol derivatives of phosphatidylethanolamine[12—15] are not readily taken up by the macrophages in the RES, and hence remain in the circulation for a relatively long period of time. In addition to the basic components of phosphatidylcholine and cholesterol (Chol) for conventional liposomes, these long-circulating liposomes usually contain a small percentage (5—10 mol%) of amphipathic molecules containing bulky hydrophilic head groups. The molecules found to be active in prolonging liposome circulation time include amphipathic polyglycerol derivatives[16] and others.[17,18] Pharmacokinetic analysis and therapeutic studies with tumor-bearing mice revealed that these long-circulating liposomes have considerable potential as drug carriers for cancer therapy.[19—22] Elevated liposome accumulation has been found the tumor-bearing mice. Results from preclinical studies with doxorubicin encapsulated into PEG-liposomes revealed an increased therapeutic efficacy compared to the free drug or drugs encapsulated in conventional liposomes. These new formulations of long-circulating liposomes should allow the development of immunoliposomes with both long survival times in the circulation and effective target recognition *in vivo*.

Given a suitable antibody with high specificity and affinity

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for the target antigen, the critical factor is the accessibility of target cells to the immunoliposomes. Efficient target binding of the injected immunoliposomes occurs only when the target cell is in the intravascular compartment or is accessible through leaky vascular structures. Thus, in terms of targeting drug delivery by immunoliposomes, two anatomical compartments can be considered. One is a readily accessible intravascular site, such as the vascular endothelial surface, T cells, B cells or a thrombus. The other is a much less accessible extravascular site, such as a solid tumor, an infection site, or an inflammation site, where the vascular structure is leaky.

In this review, the current status of newly developed immunoliposomes, constructed by using functionalized PEG and monoclonal antibody, will be described. To characterize the key parameters for immunoliposome targeting *in vivo*, two different targeting models in mice were employed; the mouse lung targeting model as a readily accessible site and the implanted mouse tumor model as a less accessible extravascular site.

2. TARGETING TO A READILY ACCESSIBLE SITE (LUNG ENDOTHELIAL SURFACE)

A model system using immunoliposomes containing monoclonal antibodies specific for mouse pulmonary endothelium was used. The antibody 34A binds to a surface glycoprotein (gp112) which is expressed at high concentration in the mouse lung.[23] It has been demonstrated that mouse lung contains large amounts of gp112 (500 to 700 ng/mg protein), whereas other organs have very low (spleen, uterus) or undetectable (liver) levels. Immunogold electron microscopy of the lung, using 34A antibodies, shows that the gold particles are concentrated along the luminal membrane of endothelial cells.[23] There are about 3.8×10^6 antigen sites per pulmonary artery endothelial cell grown in culture.[24] The anatomy and physiology of the lung favor such a model system for a number of reasons; all the blood circulates through the lung capillaries, the flow rate of blood in the lung is relatively slow for the purpose of exchange of oxygen and carbon dioxide, and the largest capillary bed in the body is found in this organ. This convenient model has allowed us to investigate the effect on the target binding of various physical parameters of immunoliposomes, such as the antibody-to-lipid ratio, liposome size, and injection dose.

a. Characterization of *in Vivo* Immunoliposome Targeting to Pulmonary Endothelium Liposomes composed of egg PC, Chol and NGPE with a molar ratio of 5 : 5 : 1 were conjugated to the antibodies *via* NGPE.[25] The resulting 34A-immunoliposomes (corresponding to Type A in Fig. 2) were then injected into mice (Balb/c, 6—8 weeks old, male) *via* the tail vein. Table 1 shows that the 34A-immunoliposomes accumulated in the target organ, lung, up to about 32% of the injected dose. The percent dose/g of tissue weight for the lung was about 12—22 fold greater than that for the liver. Liposomes containing no antibody, or containing an irrelevant antibody, 14 (matching the subtype of 34A), did not accumulate in the lung at any time after injection. The accumulation of 34A-immunoliposomes at the lung was immunospecific because the accumulation could be completely inhibited by the preinjection of a large dose of specific antibody (Table 2). Preinjection of control antibody 14 had no effect on the immunoliposome binding. It is worth noting that when lung binding was inhibited, the uptake of 34A-immunoliposomes by liver and spleen increased significantly, suggesting that liver and spleen are still the major organs for nonspecific immunoliposome uptake.

Table 1. Biodistribution of Immunoliposomes and Liposomes in Major Organs of Mice

Organ	Percent injected dose		
	34A-LP	14-LP	BLP
Lung	32.7±2.3	0.5±0.1	0.6±0.1
Blood	4.7±0.2	5.4±0.6	11.7±2.1
Liver	57.8±1.5	52.1±0.5	46.9±5.6
Kidney	2.0±0.1	3.0±0.1	2.6±0.7
Spleen	6.4±1.5	2.3±0.1	4.2±0.1
Heart	0.9±0.0	1.0±0.2	0.3±0.1

Liposomes (egg PC : Chol : NGPE=5 : 5 : 1 molar ratio) containing trace amounts of ^{125}I-BHPE were prepared by the reverse-phase evaporation method followed by extrusion through a 0.4 μm Nuclepore membrane. Immunoliposomes were then prepared by conjugating antibodies to NGPE and the unconjugated antibodies were removed by gel filtration. The average size of liposomes determined by a sub-micron particle size analyzer (Coulter N4SD, Hialeah, FL) was about 250 nm in diameter. Immunoliposomes containing specific antibody against gp112 (34A-LP) (200 μg of lipid, 19 μg of protein), containing nonspecific antibody (14-LP) (200 μg of lipid, 19 μg of protein) or bare liposomes (BLP) (200 μg of lipid) were injected intravenously. Biodistribution was measured at 15 min post injection and is presented as percent injected dose per organ. Data represent the mean±S.D. of 3 mice.

Table 2. Effect of Preinjection of Ascites Fluid on Biodistribution of 34A-Immunoliposomes

Organ	Percent injected dose		
	PBS	34A-LP	14-LP
Lung	22.3±1.7	0.3±0.0	21.5±1.8
Blood	1.7±0.3	2.4±0.2	1.9±0.1
Liver	51.3±0.9	65.7±2.2	49.8±2.8
Kidney	1.0±0.2	0.7±0.1	1.2±0.1
Spleen	5.0±1.1	8.0±1.1	3.7±0.5

Immunoliposomes containing 34A (250 nm in diameter, 200 μg of lipid, 14 μg of protein) were injected into the mice 1 h after the injection (i.p.) of ascites fluid containing *ca.* 500 μg of the indicated monoclonal antibody. Mice were then sacrificed 15 min post-injection (i.v.) of liposomes.

Among several parameters examined, the most important one seems to be the antibody-to-lipid ratio of the immunoliposomes. As shown in Table 3, a series of immunoliposomes with 34A was prepared with various initial antibody-to-lipid ratios. It is clear that there was a direct relationship between the antibody density of the immunoliposomes and the extent of their binding to the lung target. Approximately 60% of the injected dose was found in the lung within 15 min after injection when the immunoliposomes contained an average of 935 antibody molecules per liposome. Uptake by the liver and spleen followed an opposite trend from that of the lung, *i.e.* the accumulation of liposomes in the liver and spleen decreased as the antibody-to-lipid ratio increased, suggesting that uptake of immunoliposomes by the liver and spleen might limit the binding of immunoliposomes to the lung target. Thus, antibody density is an important factor for target binding in the blood. Furthermore, this animal model system revealed important differences between immunoliposome binding *in vivo* and *in vitro*. *In vitro* binding, under the static non-flow condition, is characterized by a diffusion-limited

Table 3. Effect of Antibody-to-Lipid Ratio of Immunoliposomes on Biodistribution

Characteristic	BLP	34A-LP			
Init. Ab: lipid (w/w)	—	1:50	1:10	1:5	1:1
Conjugation (%)	—	60	53	57	48
Size (nm)	250	224	236	234	247
# Ab molec/liposome	0	24	101	219	935
Organ		Percent injected dose			
Lung	0.4±0.0	3.0±0.3	20.0±1.2	35.3±2.4	59.8±0.4
Blood	10.9±0.7	7.9±0.8	4.8±0.2	2.9±0.0	2.1±0.1
Liver	49.7±1.6	55.3±1.7	41.7±1.8	34.2±2.5	12.1±0.3
Kidney	1.2±0.0	1.2±0.1	1.0±0.1	1.1±0.0	1.3±0.1
Spleen	4.3±0.6	3.7±1.0	3.3±0.2	1.8±0.3	0.6±0.1

Immunoliposomes with different antibody-to-lipid ratio were prepared by employing different input ratios of antibody to lipids. The number of antibody molecules per liposome was calculated from the known conjugation efficiency for each preparation of immunoliposomes and the average diameter of the liposomes. The number of lipid molecules per liposome at a given diameter (*ca.* 250 nm) was estimated according to H. G. Enoch and P. Strittmatter (*Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194—4198 (1978)). Data represent the biodistribution of liposomes in the mice 15 min after liposome administration (i.v.). The percent of injected dose value for each ratio of antibody to lipid is the average±S.D. of three mice.

on-rate and a slow off-rate which is sensitive to the valency of the multivalent binder. *In vivo* binding, on the other hand, is likely to be strongly influenced by the blood flow rate and the rate of liposome passage through the target site. The uptake by liver and spleen is high for any immunoliposomes which have not bound to the lung. Immunoliposomes which are not bound to the lung during the first few passages through the lung capillaries are quickly taken up by the liver and spleen and no longer have a chance to bind to the target site. Any means to reduce the affinity of the liposomes to the liver and spleen should allow a longer circulation time for the unbound immunoliposomes and thus enhance the chance of target binding at later passages.

b. The Influence of Prolonged Circulation Time of Liposomes on the Efficiency of Immunoliposome Targeting Conventional liposomes are avidly taken up by the phagocytic cells in the RES, mainly represented by the liver's Kupffer cells and splenic macrophages.[26] Coating liposomes with intact immunoglobulin molecules often leads to enhanced uptake of the immunoliposomes by the RES.[7,8] Highly efficient target binding and relatively low levels of RES uptake of the immunoliposomes seem inconsistent with this finding. Nevertheless, long-circulating liposomes have been prepared by coating the liposome surface with amphipathic molecules containing bulky hydrophilic head groups, such as G_{M1} or PE-PEG; this coating allows the liposomes to evade RES uptake and remain in the systemic circulation for a long period of time.[11—15] We have examined the role of these molecules in 34A-immunoliposome binding to the lung target in the above animal model system.

(1) Ganglioside G_{M1}: With the work of Allen and Chonn,[11] and Gabizon and Papahadjopoulos,[20] it became clear that the rate of liposome uptake by the RES can be reduced by including G_{M1} in the liposome membrane, resulting in a prolonged circulation time. The efficiency of binding to the lung of 34A-immunoliposomes containing G_{M1} was compared with that of 34A-immunoliposomes containing the same amount of PS, which is known to allow elevated uptake of liposomes by the RES.[27,28] The time-dependent accumulation of liposomes with various amounts of the antibody on the surface is shown in Fig. 1. It is clear that liposomes without the antibody did not accumulate in the lung at all. The

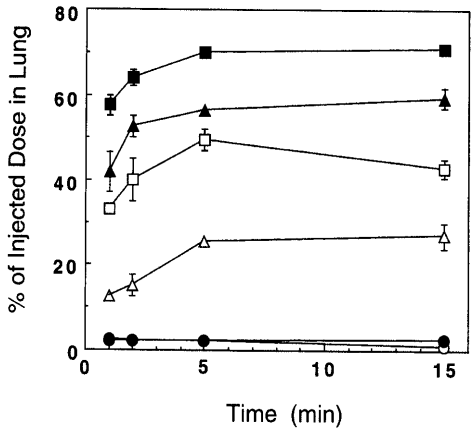

Fig. 1. Effect of Liposome Composition on the Accumulation of Liposomes in the Lung

34A-immunoliposomes composed of PC/Chol and either G_{M1} or PS with a molar ratio of 10:5:1 were prepared by a detergent dialysis method (E. Holmberg, K. Maruyama *et al.* (*Biochem. Biophys. Res. Commun.*, **165**, 1272—1278 (1989)). ■, 34A immunoliposomes containing G_{M1} with an antibody-to-lipid ratio of 1:11. ▲, 34A immunoliposomes containing G_{M1} with an antibody-to-lipid ratio of 1:37. ●, G_{M1} containing liposomes without 34A. □, 34A immunoliposomes containing PS with an antibody-to-lipid ratio of 1:8. △, 34A immunoliposomes containing PS with an antibody-to-lipid ratio of 1:31. ○, PS-containing liposomes without 34A.

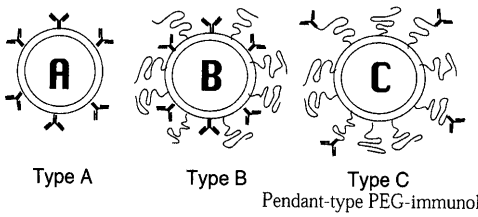

Fig. 2. Schematic Illustration of Immobilization of Antibody on Liposomes

Type A: PEG-free immunoliposomes with antibody covalently linked to the short anchor NGPE; Type B: PEG-immunoliposomes with antibody covalently linked to NGPE; Type C: new type of PEG-immunoliposomes with antibody attached to the distal terminal of DSPE-PEG-COOH, so-called pendant-type PEG-immunoliposomes.

binding of immunoliposomes to the lung is relatively rapid; it reached the steady state within 5 min after injection. Lung accumulation of immunoliposomes was dependent on the number of antibody molecules per liposome. For 34A-G_{M1}-immunoliposomes (corresponding to Type B in Fig. 2), with an antibody-to-lipid ratio of 1:11 (w/w), lung accumulation

was about 70% of the injected dose, whereas those with a ratio of 1 : 37 (w/w) only accumulated to the extent of 60% of the injected dose. For immunoliposomes containing PS with an antibody-to-lipid ratio of 1 : 8, lung accumulation was about 50% and for those with a ratio of 1 : 31, it was only about 25% of the injected dose. More importantly, 34A-G_{M1}-immunoliposomes allowed a significantly higher level of binding to the lung target than 34A-immunoliposomes containing PS. Thus, although the antibody density on the liposomes is important for target binding (Table 3), the inclusion of G_{M1} elevated the blood concentration and enhanced the target binding of immunoliposomes. G_{M1} acts a weak steric barrier, as was clearly indicated by the liposome agglutination assay,[29,30] although G_{M1} coexists with the targeting molecules, 34A antibodies, on the liposome surface. However, since it is difficult to obtain large quantities of G_{M1} either by extraction from natural sources or by synthesis, G_{M1}-liposomes are impractical for therapeutic applications.

(2) Amphipathic PEG (PE-PEG): Amphipathic PEG, such as PEG conjugated to PE, can also significantly prolong the circulation time of liposomes in blood if the conjugate is incorporated into the liposome membrane.[12—15] PEG is very useful because of its ease of preparation, relatively low cost, controllability of molecular weight and linkability to lipids or protein (including antibody) by a variety of methods, as compared with G_{M1} molecules. Immunoliposomes containing amphipathic PEG were designed as shown in Fig. 2.[31] Type A is PEG-free immunoliposomes with the antibody covalently linked to a short anchor. Type B is PEG-immunoliposomes with the antibody covalently linked to the short anchor. Antibody molecules and PEG molecules coexist on the liposome surface. Type C is a new type of PEG-immunoliposomes with the antibody or fragments attached to the PEG terminus, so-called pendant-type PEG-immunoliposomes. A scanning electron micrograph of Type C is shown Fig. 3.[32] The location of antibodies on the surface of liposome can be clearly identified in the backscatter electron image. For the preparation of Type C, several functionalized PE-PEG derivatives were newly synthesized as shown in Fig. 4. Ideally, the coupling method should be both simple and rapid, producing a stable, non-toxic bond. A wide range of antibody densities should be achievable at the liposome surface in order to optimize immunoliposome binding to the target cells. During conjugation, the antibody should retain antigen recognition and the liposomes should not lose their structural integrity.

The targetability and biodistribution of the pendant-type PEG-immunoliposomes in terms of targeting to an accessible site (the vascular endothelial surface in the lung) were studied in the lung targeting model in mice using the 34A monoclonal antibody.[31] The plain liposomes for preparing Type A or Type C immunoliposomes were composed of egg PC and Chol (2 : 1, m/m) with 6 mol% of NGPE or DSPE-PEG-COOH with an average molecular weight of 3000, respectively. The plain liposomes for Type B were composed of egg PC and Chol (2 : 1, m/m) with 6 mol% of NGPE and 6 mol% of DSPE-PEG with an average molecular weight of 3000. SUVs (90—130 nm in diameter) were prepared by the REV method followed by extrusion through Nuclepore filters (0.1 μm). The carboxyl residues in the plain liposomes were activated and coupled with 34A antibodies.

To test the effect of both the position of the antibody and

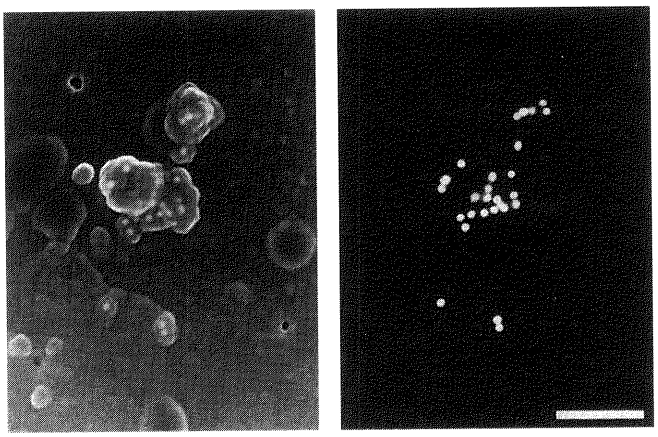

Fig. 3. Scanning Electron Image (Left) and Backscatter Electron Image (Right) of 34A-PEG-COOH Liposomes Treated with Gold Particle-Conjugated Secondary Antibody

Bar = 200 nm.

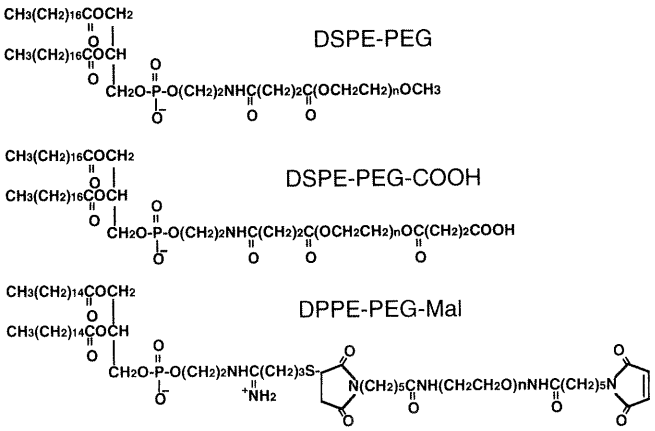

Fig. 4. Structure of Amphipathic PEG Derivatives for Coupling Antibody to the Terminus of PEG

The PE derivatives of PEG with a terminal carboxyl group (DSPE-PEG-COOH) or with a terminal maleimidyl group (DPPE-PEG-Mal) were synthesized according to K. Maruyama *et al.* (*Biochim. Biophys. Acta*, **1234**, 74—80 (1995)) and European patent publication No. 607978, respectively. The average molecular weight of PEG was 3000.

the steric hindrance of PEG chains on the behavior of immunoliposomes *in vivo*, three different types of 34A-immunoliposomes with similar numbers of antibody molecules per liposome were prepared and their targetability and biodistribution were evaluated in mice. As summarized in Table 4, Type A, B and C 34A-immunoliposomes containing approximately 35, 30 and 30 antibody molecules per liposome, respectively, were prepared and injected into mice *via* the tail vein (Fig. 5). 34A-Type A with an average of 35 antibody molecules per liposome accumulated 42.5% of the injected dose in the lung. 34A-Type B showed a lower level of target binding and a significantly higher blood level than those of Type A. In the case of 34A-Type C with 30 antibody molecules per vesicle, the degree of target binding to the lung was 56.6% of the injected dose, 1.5-fold higher than that of Type A. The target binding of 34A-Type B is comparable with that of 34A-G_{M1}-immunoliposomes. Although long-circulating liposomes can also be obtained by incorporating PE-PEG derivatives in place of G_{M1}, inclusion of DSPE-PEG with an average molecular weight of 3000 reduced the target binding of 34A-Type B. This effect de-

Table 4. Characteristics of 34A-Immunoliposomes

	34A-Type		
	A	B	C
PEG content (mol% of total lipid)	0	6	6
Mean diameter (nm)	121	111	122
Initial antibody : lipid ratio (w/w)	1.6	1.6	1.5
Conjugation efficiency (%)	35.6	31.8	24.8
# Ab molec/liposome	35	30	30

The plain liposomes used for preparing Type A or Type C were composed of egg PC : Chol (2 : 1, m/m) with 6 mol% of NGPE or DSPE-PEG-COOH with average molecular weight of 2000, respectively. The plain liposomes for Type B were composed of egg PC : Chol (2 : 1, m/m) with 6 mol% of NGPE and DSPE-PEG with an average molecular weight of 2000. The average number of antibody molecules per liposome was estimated according to H. G. Enoch and P. Strittmatter (*Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194—4198 (1978)).

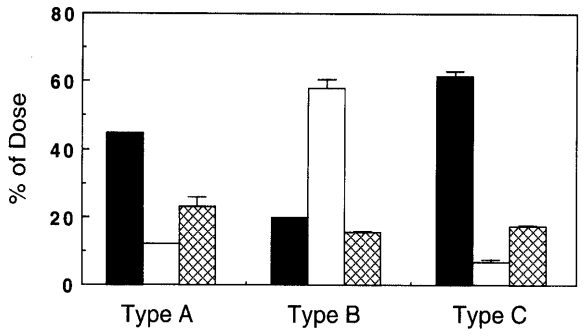

Fig. 5. Comparison of Target Binding to the Lung (■) and Biodistribution (□; Blood, ▨; Liver) among the Three Different Types of 34A-Immunoliposomes

34A-Type A, B, and C contained approximately 35, 30 and 30 antibody molecules per liposome, respectively. Biodistribution (% of dose) was measured 30 min after intravenous injection. For details, see Table 4.

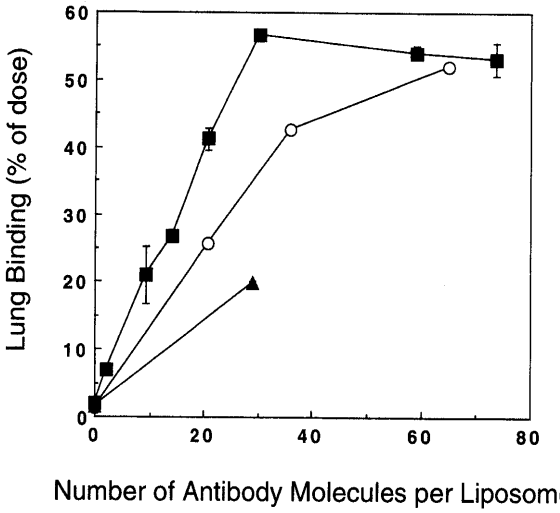

Fig. 6. Effect of Antibody Density on the Lung Targeting of 34A-Type A (○), B (▲), and C (■)

Lung binding (% of dose) was measured 30 min after injection.

pended on the chain length of PEG, suggesting that although PEG prolongs the circulation time of immunoliposomes, it sterically hinders the binding of immunoliposomes to the target sites.[29,30] Therefore, it was proposed that the use of longer-chain PEG with antibodies attached at the distal terminal of the PEG chain (Type C) would afford immunoliposomes with both prolonged circulation time and good target binding.[29,33]

The efficiency of 34A-Type C binding to the target was evaluated as a function of the antibody content. A series of 34A-Type C was prepared with various initial antibody-to-lipid ratios. The final number of antibody molecules per liposome varied from 0 to 74, but the average sizes of the immunoliposomes were approximately the same, 90—130 nm in diameter. It is clear from the data in Fig. 6 that the efficiency of lung targeting was dependent on the antibody content of the immunoliposomes. Liposomes containing small numbers of antibody molecules per liposome accumulated in the lung at low levels and were retained in the blood at high levels, whereas liposomes linking an average of 74 antibody molecules per liposome on the PEG terminals accumulated 53% of the injected dose in the lung and their blood resident amount was only 7% of the injected dose. Lung binding reached a plateau at about 30 antibody molecules per liposome and a further increase in antibody content only resulted in increased liver uptake. Thus, antibody density is an important factor for target binding even in the targeting of Type C immunoliposomes. At low numbers of antibody molecules per liposome, such as 2 and 9 molecules, free PEG favors evasion of RES uptake of the liposomes, resulting in high blood residence of 34A-Type C together with a low efficiency of target binding due to the low antibody content. Type C showed higher immunotargetability than Type A and B at low antibody content (less than 30 antibody molecules per vesicle). Thus, Type C is accumulated more effectively in the lung than the other immunoliposomes, in spite of the low antibody content.

3. TARGETING TO A LESS ACCESSIBLE SITE (SOLID TUMOR)

a. The Influence of Prolonged Circulation Time of Liposomes on the Extravasation of Liposomes into Solid Tumor Tissue Generally, the capillary permeability of the endothelial barrier in newly vascularized tumors is significantly greater than that of normal tissues.[34] Normal tissues outside the RES are known generally to have continuous and nonfenestrated vascular endothelia, and extravasation of macromolecules or liposomes is greatly limited. The extravasation of circulating molecules from blood vessels to the tumor tissue is a function of both local blood flow and microvascular permeability.[35] In addition, since there is little or no lymphatic drainage in tumor tissues, macromolecules are, after accumulation, retained in the tumor interstitium for a prolonged period of time. Such a phenomenon, termed the enhanced permeability and retention (EPR) effect, has been shown to occur universally among tumors.[36,37] It is conceivable that long-circulating liposomes could take advantage of the EPR effect for efficient target binding in the tumor.

The extravasation of liposomes of different sizes into solid tumors was examined in various tumor models in mice.[21,38] As shown in Fig. 7, long-circulating liposomes composed of DSPC/Chol/DSPE-PEG (1 : 1 : 0.13, m/m) with an average diameter of 100—200 nm were accumulated efficiently in all tumor tissues examined. Clearly, liposome size is also an important factor for extravasation. Observation using fluorescence microscopy has shown that PEG-liposomes can indeed extravasate beyond the endothelial barrier, mainly in postcap-

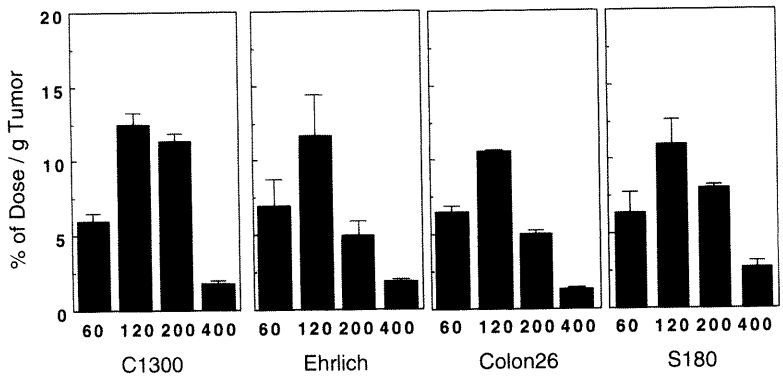

Fig. 7. Effect of Liposomal Diameter on the Accumulation of PEG-Liposomes into Various Implanted Tumors in Mice

PEG-liposomes composed of DSPC : Chol : DSPE-PEG (1 : 1 : 0.13, m/m) and containing ^{67}Ga-deferoxamine were intravenously injected into tumor-bearing mice. Tumor-bearing mice were prepared by inoculating the following cells (1×10^7 cells) into the hind foot and used when the tumor had reached a diameter of 8 mm: mouse C-1300 neuroblastoma to A/J Sic mouse, mouse colon 26 adenocarcinoma to BALB/c mouse, mouse Ehrlich (1×10^7 cells) to ddY mouse or mouse sarcoma 180 to ddY mouse. Biodistribution was estimated at 6 h after injection.

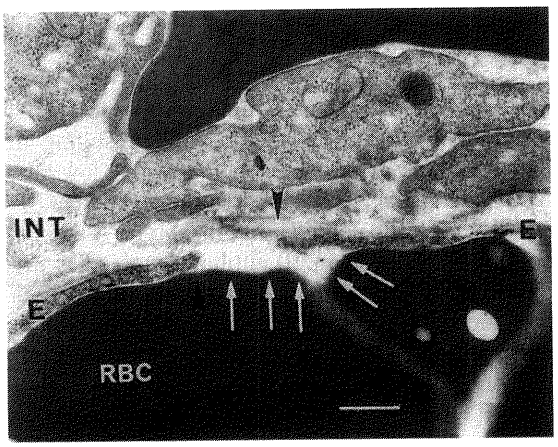

Fig. 8. Electron Micrographs Showing Extravasation and Localization of PEG-Liposomes (126±35 nm Mean Diameter) in Colon 26 Solid Tumor Tissue

RBC: red blood cell. E : endothelium. INT: interstitial space. Between arrowheads: the gap between adjacent endothelial cells, Arrows: liposomes across the gap. Bar= 500 nm.

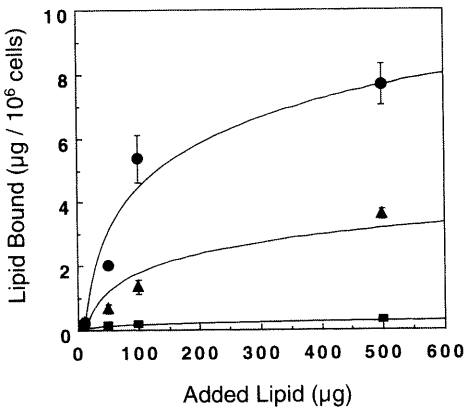

Fig. 9. *In Vitro* Cell Binding of 21B2-Type C and Fab′-Type C

The plain liposomes for preparing 21B2-Type C or Fab′-Type C were composed of DSPC : Chol (2 : 1, m/m) with 6 mol% of DSPE-PEG-COOH or DPPE-PEG-Mal with an average molecular weight of 3000, respectively. Binding of PEG-Mal liposomes (■), 21B2-Type C [70] (▲) or Fab′-Type C [517] (●) was plotted as a function of added lipid. A trace amount of ^{3}H-cholesteryl hexadecylether was contained as lipid marker. The average number of antibody molecules per liposome was estimated according to the report of H. G. Enoch and P. Strittmatter (*Proc. Natl. Acad. Sci. U.S.A.*, **75**, 4194—4198 (1978)). Liposomes were incubated at various lipid concentrations with MKN-45 cells for 90 min at 4 °C. The number in square brackets represents the average number of antibody or Fab′ molecules per liposome.

illary venules.[38,39] In morphological, physiological and pharmacological studies of microvascular permeability, leakage of particles or macromolecules was observed at postcapillary venules by electron microscopy (Fig. 8).[40] Due to the increased circulation time of liposomes containing PE-PEG and the leaky structure of the microvasculature in the solid tumor tissue, those liposomes accumulate preferentially in the tumor tissue. Thus, under physiological tumor conditions, only small liposomes ranging from 100—200 nm in diameter with a prolonged circulation half-life have a high probability of encountering the leaky vessels of the tumor tissue.

Recent studies have shown that PEG-liposomes encapsulating anthracyclines such as doxorubicin (DXR) exhibit improved therapeutic efficacy and reduced toxicity after i.v. injection into solid tumor-bearing mice.[21,41,42]

b. Monoclonal Antibody and Solid Tumor Targeting Model in Mice To study whether immunoliposomes injected intravenously can extravasate into the solid tumor and bind to tumor cells, we used the monoclonal antibody 21B2, specific for the human carcinoembryonic antigen (CEA), and mice bearing CEA-positive human gastric cancer strain MKN-45.[43] 21B2 was isolated from BALB/c mice after immunization with human CEA antigen purified from cells of a CEA-producing human gastric cancer line, MKN-45. Fab′ fragments of 21B2 were prepared by pepsin digestion of the antibody and 2-aminoethanethiol reduction of the $F(ab')_2$ fragments.[44] We have synthesized a dipalmitoyl phosphatidylethanolamine derivative of PEG with a terminal maleimidyl group for the preparation of Fab′-Type C immunoliposomes (Fig. 4).[45,46] Two million MKN-45 cells were inoculated into the back of female BALB/c nu/nu mice. When the estimated tumor weight (calculated as $1/2\times\text{length}\times\text{width}^2$) reached about 300 mg, the mice were used for experiments.

We first tested whether these PEG-immunoliposomes specifically bound to the target cells *in vitro*. As shown in Fig. 9, PEG-immunoliposomes conjugated with either whole antibody (21B2) or Fab′ fragment of 21B2 readily bound with MKN-45 cells. The degree of target cell binding of immunoliposomes was greater than that of nontargeted PEG-Mal liposomes. The differences in cell binding between 21B2-Type C and Fab′-Type C were due to the variation of

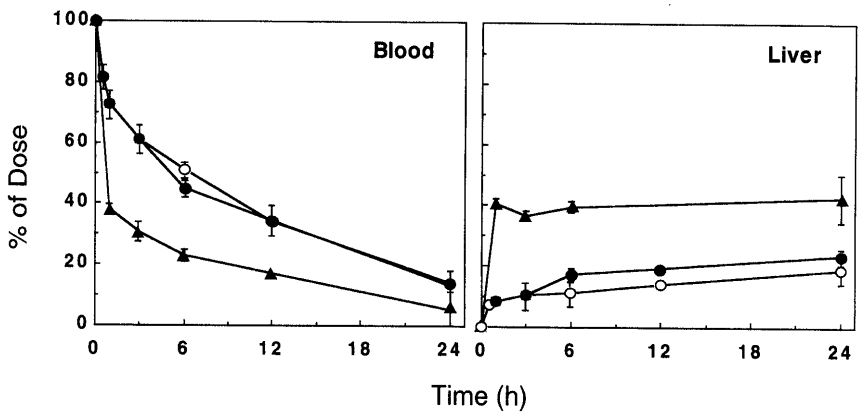

Fig. 10. Time Course of Blood Residence and Liver Uptake of PEG-Mal Liposomes (○), 21B2-Type C [51] (▲) or Fab′-Type C [517] (●) in MKN-45-Bearing BALB/c *nu/nu* Mice

Two million MKN-45 cells were inoculated into the back of female BALB/c *nu/nu* mice. The number in square brackets represents the average number of antibody or Fab′ molecules per liposome.

the coupled number of the antibody or Fab′ fragment. These results revealed that free PEG (not linked to the antibody) in liposomes does not interfere sterically with antigen-binding of the antibody or Fab′ fragment.

c. Characterization of *in Vivo* Immunoliposome Targeting to Solid Tumor We investigated whether the use of the Fab′ fragment instead of the whole antibody (21B2) would modify the pattern of tissue distribution of immunoliposomes. As shown in Fig. 10, PEG-Mal liposomes without the antibody showed prolonged residence in the circulation and low liver uptake, regardless of the presence of the terminal maleimidyl group. There were no marked differences in tissue distribution among liposomes containing DSPE-PEG, DSPE-PEG-COOH and DPPE-PEG-Mal, taking into account our previous results.[4,7,8] 21B2-Type C, bearing approximately 51 whole antibody molecules per liposome, was rapidly cleared from the blood and were found entirely in the liver. In contrast, Fab′-Type C, bearing approximately 517 Fab′ molecules per liposome, was retained longer in the circulation with a concomitant decrease in the liver uptake compared with 21B2-Type C. These results indicate that the linkage of whole 21B2 antibodies to the PEG terminal enhances RES uptake *via* the Fc receptor-mediated mechanism.[7,8] To overcome this problem, it is necessary to use the Fab′ fragment. In the case of Fab′-Type C, the absence of the Fc portion and the presence of free PEG-Mal (not linked to the Fab' fragment) may both play a role in the prolonged circulation of the liposomes. Thus, the Fab′ fragment is much better than whole IgG in terms of designing PEG-immunoliposomes with a prolonged circulation time. Further, the usage of the Fab′ fragment should greatly reduce the antigenicity.

The next question to arise was whether Fab′-Type C could extravasate into solid tumor tissue and bind to tumor cells. Figure 11 shows the accumulation of Fab′-Type C, 21B2-Type C and comparable PEG-liposomes, with an average diameter of 100—130 nm, in MKN-45 solid tumor in mice at 24 h after injection. A relatively high accumulation was obtained with PEG-COOH liposomes, PEG-Mal liposomes and Fab′-Type C immunoliposomes. These results were clearly correlated to the prolonged circulation time (Fig. 9). The accumulation rate of Fab′-Type C was 2-fold higher than that of 21B2-Type C or bare liposomes, and equal to that of PEG-Mal or PEG-COOH liposomes.

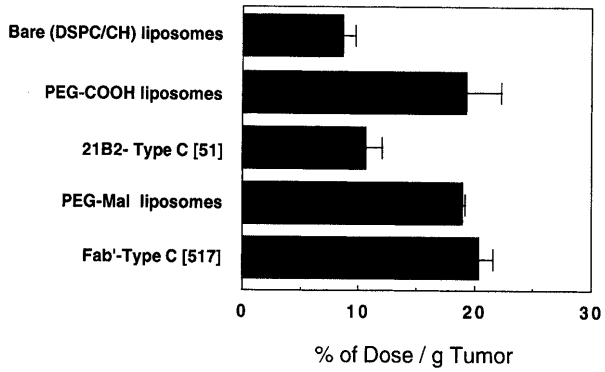

Fig. 11. Accumulation of Immunoliposomes in Solid Tumor in MKN-45-Bearing BALB/c *nu/nu* Mice at 24 h after Injection

Two million MKN-45 cells were inoculated into the back of female BALB/c *nu/nu* mice. The number in square brackets represents the average number of antibody or Fab′ molecules per liposome.

The permeability of the tumor vasculature is generally increased as compared to normal tissue, so Fab′-Type C with both smaller size and a prolonged circulation time could extravasate through the leaky endothelium by passive convective transport. The ligand-directed targeting by Fab′-recognition is rather weak. On the other hand, 21B2-Type C showed a short circulation time due to a high liver uptake, so that they do not exhibit sufficient concentration and residence time for extravasation through the leaky endothelium. Though there were no differences in accumulation into solid tumor between Fab′-PEG immunoliposomes and PEG-liposomes without antibodies, only Fab′-PEG immunoliposomes bind to the surface of MKN-45 cells. This is important for extravasated Fab′-Type C, because Fab′-Type C can bind readily with MKN-45 cells in *in vitro* binding assay as shown in Fig. 8. This delivery system should be particularly valuable for endocytotic internalization of many bioactive materials.

4. CONCLUSION

The accumulation of immunoliposomes in the target site is determined by two kinetically competing processes: binding to the target site and uptake by the RES. Consequently, general requirements for efficient *in vivo* targeting of immunoli-

posomes are a sufficient antibody density and a reduced affinity of the RES. We have designed a new type of long-circulating immunoliposome (Type C) which can be extravasated to targeted solid tumors *in vivo*.

The ability to target Type C to specific tissues located either in an intravascular site or in an extravascular area was shown by using antibody recognizing mouse lung endothelial cells or antibody recognizing implanted mouse tumors, respectively. Type C was prepared by the use of newly synthesized DSPE-PEG-COOH and DPPC-PEG-Mal to couple antibodies (for targeting) directly to the distal terminal of PEG chains. In particular, the Fab′-Type C was newly designed to gain a sufficient long-circulating character to allow extravasation to the targeted solid tumor *in vivo*. It is noteworthy that Type C showed much higher targetability than ordinary immunoliposomes (Type A) to both lung endothelial cells and solid tumor tissue. This is because the free PEG chains effectively block the RES uptake of the liposomes, resulting in elevated blood concentration of the liposomes. Thus, the presence of free PEG-COOH (not linked to the antibody) on the Type C surface does not interfere with the binding of the terminally linked antibody to the antigen. Instead, the free PEG-COOH enhances the target binding of immunoliposomes, owing to its prolonging effect on the circulation time of the liposomes. Therefore, they can escape from the gaps between adjacent endothelial cells and openings at the vessel termini during tumor angiogenesis by passive convective transport rather than ligand-directed targeting.

Once the liposomes bind to the target cells, the entrapped drugs must be released to the appropriate intracellular compartment. The further development of such immunoliposomes showed aim to make them able to release their contents at low pH following endocytosis or during a temperature increase produced by local hyperthermia or external irradiation. Drugs released from the immunoliposomes can reach the target cells by free diffusion over a relatively short distance, thereby producing the localized high concentration needed for treatment. Even though this strategy may not prevent the side effects, a relatively high dose of the drug in the target area can be achieved with this strategy. Indeed, recent studies by Allen's group have shown that the targeting of Type C immunoliposomes, containing entrapped doxorubicin, resulted in superior therapeutic effects in the treatment of solid tumors.[47,48]

The potential advantage of using Type C immunoliposomes for site-specific delivery has been shown in this review. A future possibility is to conjugate other ligands to the free PEG terminals of Fab′-Type C, in order to direct the liposomes to surface receptors known to be internalized at a high rate by cells.[49,50] The ultimate goal is the incorporation of a fusogenic molecules that would induce fusion of Type C immunoliposomes following their binding to the target cells, or their internalization by endocytosis. Such liposomal formulations should be useful for endocytotic internalization of plasmid DNA and other bioactive materials.

I hope that this review provides a realistic perspective on where the field is going. Ideally, all administered immunoliposomes should bind to their target sites, and all encapsulated drugs should become therapeutically available upon binding of immunoliposomes to the target sites. To date, all targeted systems, including immunoliposomes, fall short of meeting both criteria. However, recent advances have improved the prospects for immunoliposomes applications. Immunoliposomes still have a long way to go, but the strong need for more effective chemotherapeutics will continue to motivate studies on immunoliposomes.

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