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Influence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside GM_1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo

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A series of dioleoyl *N*-(monomethoxy polyethyleneglycol succinyl)phosphatidylethanolamine (PEG-PE) of different polymer chain length was used in this study. Both the activity of PEG-PE in prolonging the circulation time of liposomes and the relative steric barrier activity of amphipathic polymer, measured by a liposome agglutination assay, were found to be directly proportional to the chain length of PEG-PE (PEG5000-PE > PEG2000-PE > PEG750-PE). However, PEG5000-PE caused a reduced target binding of immunoliposomes in mice due to its overly strong steric barrier activity. The best PEG-PE species supporting the target binding of immunoliposomes was PEG2000-PE, the activity of which was compatible to that of ganglioside GM_1. However, GM_1 only showed a weak steric barrier activity, suggesting a different mechanism for this glycolipid.

Liposome; Polyethyleneglycol; Targeted drug delivery; Ganglioside GM_1

1. INTRODUCTION

A novel type of liposome with a reduced affinity to cells in the reticuloendothelial system (RES) and hence with a prolonged circulation time has been the focus of much recent research in liposome targeting. This interest has been fueled by the prospect that this novel liposome provides an efficient delivery system for various bioactive agents to the cells or tissues other than the RES. To construct such liposomes, a glycolipid such as ganglioside GM_1 (GM_1), hydrogenated phosphatidylinositol, or sulfatide is included in the lipid composition [1–4]. More recently, studies carried out in our laboratory have shown that the inclusion of amphipathic polyethyleneglycol (PEG5000) in the lipid composition also effectively reduces uptake by the RES and results in prolonged circulation time of liposomes [5,6]. Similar results have been reported by several other laboratories [7,8]. The precise mechanism of action of these additional amphiphiles remains obscure at present, but it has been postulated [5] that increased hydrophilicity of the liposomes may lead to an enhanced stability and a reduction of nonspecific uptake by the RES. In addition, the steric barrier [7] on the liposome surface provided by these amphiphiles may reduce the interaction with unidentified opsonin molecules [9], leading to a reduction of specific uptake of liposomes by the RES. In the present study, we have examined the importance of the steric barrier on the liposome surface, provided by an additional amphiphile, in prolonging the circulation time of liposomes, using a series of polyethyleneglycols, with different chain lengths, conjugated to phosphatidylethanolamine (PEG-PE) and GM_1. The effects of these amphiphiles on the target binding of immunoliposomes were also studied.

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Abbreviations: biotin-cap PE, biotinamidocaproyl-phosphatidyl ethanolamine; chol, cholesterol; DTPA-SA, diethylenetriamine pentaacetic acid distearylamide complex; GM1, 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

2. MATERIALS AND METHODS

Sources and grades of chol, GM_1, PC, and streptavidin have been described previously [6]. Synthesis of PEG-PEs with different polymer chain lengths (PEG750-PE, PEG2000-PE, PEG5000-PE) [5], biotin-cap-PE [10], DTPA-SA [11], and NGPE [12] have been previously described.

2.1. *Liposome agglutination assay*

Liposomes used in this study were prepared from PC and chol (1:1, mol/mol) additionally containing 2.5 mol% biotin-cap-PE and various amounts (2, 5 and 10%) of PEG-PE, using the extrusion method as described below. The agglutination was initiated by mixing the liposome suspension (60 μg phospholipid in 560 μl PBS, pH 7.5) with 10 μg streptavidin in a microcuvette and increase in turbidity was monitored by optical density at 440 nm.

2.2. *Liposome preparation*

Normally, liposomes composed of PC and chol (10:5, mol/mol)

additionally containing 6.3 mol% of either PEG-PE or GM_1 were prepared by the extrusion method as described previously [6]. Briefly, the solvent free lipid mixture containing 1 mol% ^{111}In-labeled DTPA-SA as a lipid marker [11] was hydrated with PBS (pH 7.5) overnight, and the liposome suspension was extruded several times through stacked 0.4 and 0.2 μm Nucleopore membranes. Liposome size was determined by dynamic laser light scattering using a Coulter N4SD instrument (Hialeah, FL). For the target binding study of immunoliposomes, monoclonal antibody 34A (34A) bearing liposomes composed of PC and chol (1:1, mol/mol) additionally containing 7.0 mol% of various PEG-PEs or GM_1 were prepared by the detergent dialysis method as described previously [13]. Briefly, the lipid mixture containing 1 mol% ^{111}In-labeled DTPA-SA solubilized with octylglucoside was mixed with 34A conjugated to NGPE, followed by the removal of detergent by dialysis. The resulting 34A-liposomes were extruded, and the unbound antibody was then removed by column chromatography on BioGel A1.5M. The antibody-to-lipid weight ratio of 34A-liposomes was determined by the specific radioactivities of ^{111}In for lipids and ^{125}I for the antibody.

2.3. *Biodistribution studies*

^{111}In-labeled liposomes (400 μg lipid) were injected i.v. into male Balb/c mice. At various time intervals, % injected dose of liposomes in blood and major organs was examined by ^{111}In radioactivity counting, determined using a Beckman gamma-counter. % injected dose in blood was determined by assuming that the blood volume of mouse is 7.3% of the body weight.

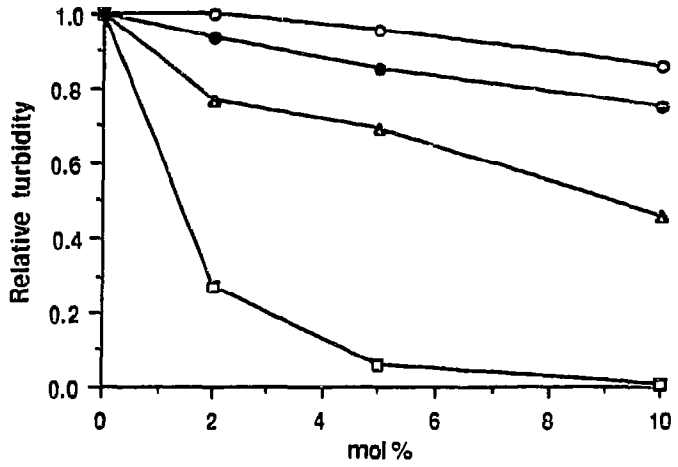

Fig. 1. Effect of increasing concentrations of PEG-PEs and GM_1 on streptavidin-induced agglutination of liposomes containing biotin-cap-PE. Increase of turbidity (o.d. 440 nm) was measured 12 min after incubation for liposomes composed of PC and chol (1:1, mol/mol) additionally containing PEG750-PE (○), PEG2000-PE (△), PEG5000-PE (□), and GM_1 (●). Liposome agglutination was normalized with respect to the control liposomes (PC-chol) and was plotted as a function of concentration of PEG-PE and GM_1.

3. RESULTS AND DISCUSSION

We first examined the steric barrier activities of these PEG-PEs using a liposome agglutination assay. 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 [14], and thus the decrease in turbidity compared to control liposomes directly reflects the degree of steric barrier produced on the liposome surface. Data were expressed as a relative turbidity ratio of the test liposomes to liposomes composed of PC and chol at 12 min after the incubation. At this time, the turbidity increase showed a plateau and was plotted as a function of the amphiphile concentration (Fig. 1). In all PEG-PEs tested here, the relative turbidity decreased with increasing concentration of the polymer. Also, the longer the chain length of PEG, the greater the decrease in the relative turbidity. At 10 mol%, liposome agglutination was inhibited by 14%, 55%, 100% for PEG750-PE, PEG2000-PE, PEG5000-PE containing liposomes, respectively. Thus, steric barrier activity is directly correlated to the chain length of PEG. Also shown in Fig. 1 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.

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. The liposomes used in this study had an average diameter range from 189 nm to 244 nm since it has been shown that the liposomes larger than 300 nm in diameter have an increased tendency to accumulate in the spleen ([6] and Liu, D. et al., submitted), whereas the small liposomes, less than 80 nm in diameter, accumulate in the liver very efficiently (Liu, D. et al., in preparation). Data were expressed as % injected dose in blood and the RES (liver and spleen) and were plotted as a function of time after injection (Fig. 2). At 3 h after injection, liposomes composed of PC and chol were almost completely cleared from the circulation and accumulated exclusively in the liver and spleen. Inclusion of PEG-PE or GM_1 in the lipid composition resulted in slower clearance rate from the circulation to a varying degree. The estimated half-life for liposome blood clearance increased from 0.5 h to 0.7 h, 1.7 h, 6.2 h and 3.4 h by the inclusion of PEG750-PE, PEG2000-PE, PEG5000-PE and GM_1 in the lipid composition, respectively. In these mouse experiments, an increase of PEG750-PE concentration up to 10 mol% in liposomes resulted in a linear increase of liposomes in the circulation and a concurrent decrease in the RES, whereas the activities of PEG2000-PE and PEG5000-PE showed plateau at a concentration of 5 mol% (data not shown). These results clearly indicate that, among the various PEG-PEs tested in this study, 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 (Fig. 1).

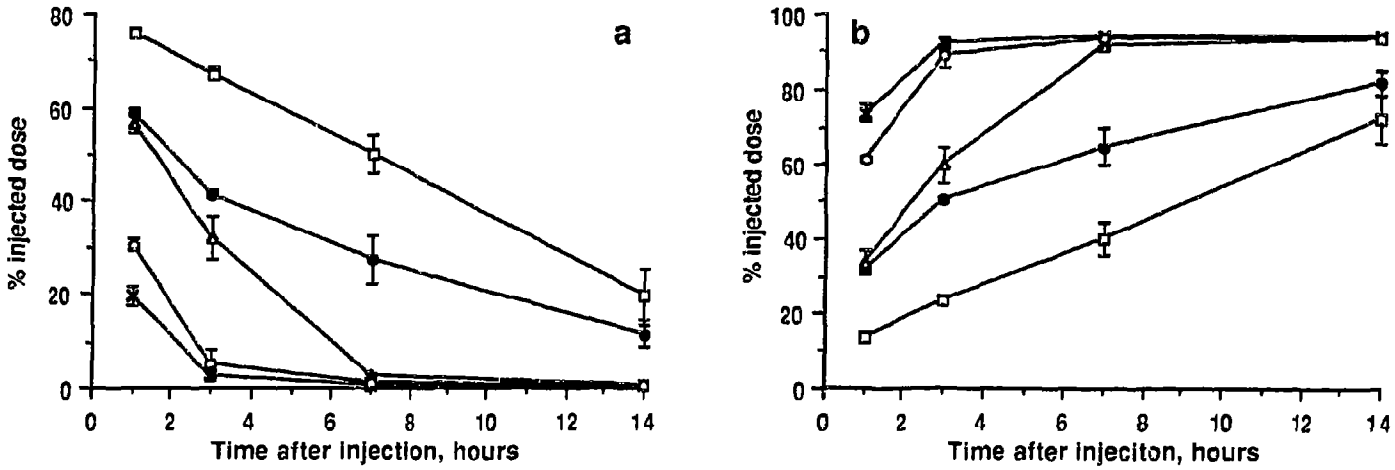

Fig. 2. Effect of various PEG-PEs and GM_1 on (a) blood clearance and (b) the RES (liver and spleen) uptake of i.v. injected ^{111}In-labeled liposomes. Bars represent S.D. ($n = 3$). Lipid composition and the average diameter of liposomes were: (×) PC/chol (10:5), 201 nm; (○) PC/chol/PEG750-PE (10:5:1), 244 nm; (△) PC/chol/PEG2000-PE (10:5:1), 189 nm; (□) PC/chol/PEG5000-PE (10:5:1), 194 nm; and (•) PC/chol/GM_1 (10:5:1), 193 nm.

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 the liposome surface appear to be in a partially extended conformation as studied by X-ray analysis [15]. In addition, the steric barrier provided by the amphipathic PEG1900 was estimated to be 6 nm thick, compared to 2.5 nm for GM_1 (calculated values using the data from [15]). In other words, GM_1 is a weak steric barrier compared with PEG1900 in agreement with our data from the liposome agglutination assay (Fig. 1). The results of this agglutination assay and the biodistribution study of liposomes containing various PEG-PEs clearly indicated that the activity of PEG-PE in prolonging the circulation time of liposomes directly correlates with the steric barrier activity of the amphipathic polymer. These data support the 'steric barrier hypothesis' [7] that amphipathic PEG prolongs the circulation time of liposomes by presenting a steric barrier which reduces the specific and/or nonspecific interaction of liposomes with the RES.

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. Its steric barrier activity is weaker than that of PEG2000-PE (Fig. 1), yet its activity in prolonging the circulation time of liposomes is greater than that of PEG2000-PE (Fig. 2). In other words, the activity of GM_1 in prolonging the circulation time of liposomes does not correlate with its steric barrier acticity. Moreover, it has been shown that many higher order gangliosides such as GD_{1a} and GT_{1b} have little activity in prolonging the circulation time of liposomes [1], although these gangliosides contain larger oligosaccharide headgroups than GM_1 and would be expected to be better steric barriers for liposomes. It is therefore unlikely that GM_1 prolongs the circulation time of liposomes solely by presenting a steric barrier on the liposome surface.

One of the important uses of liposomes with prolonged circulation time is ligand directed liposomal targeting. Previous studies have shown that optimal target binding of immunoliposomes is achieved only if GM_1 is included in the lipid composition to reduce uptake of immunoliposomes by the RES [16]. Furthermore, although the activity of PEG5000-PE in prolonging the circulation time of liposomes is greater than that of GM_1, the inclusion of PEG5000-PE in immunoliposomes resulted in a lower level of target binding presumably due to its strong steric barrier activity which prevents the antibody/antigen interaction [6]. The results of the liposome agglutination assay indicated that PEG750-PE and PEG2000-PE provide much weaker steric barriers than PEG5000-PE (Fig. 1), although their activities in prolonging the circulation time are also lower than that of PEG5000-PE (Fig. 2). We have thus studied the effects of these PEG-PEs on the target binding of immunoliposomes. As a model system, the monoclonal antibody 34A (34A) system was used in this study. 34A is a rat IgG_{2a} antibody which binds specifically with a glycoprotein antigen, gp112, expressed in high concentrations on the lumenal surface of the capillary endothelial cells of mouse lung [17,18]. The i.v. injected 34A-liposomes gain direct access to the target and bind efficiently to the lung [19,20]. The lung binding of 34A-liposomes containing PEG-PE or GM_1 was examined 1 h after i.v. injection and was expressed as % injected dose (Table I). The antibody-to-lipid weight ratio of 34A-liposomes used in this study ranged from 1:13.9 to 1:18.1. This range of weight ratio did not cause a significant change in lung binding as shown in the control 34A-liposomes with the 34A-to-lipid ratio of 1:13.9 and 1:18.1 (43% and 44%, respectively). Inclusion of PEG2000-PE in the liposomes resulted in

Table I

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 compositions were i.v. injected (0.4 mg lipid) and % injected dose was measured in lung, RES (liver and spleen), and blood 1 h 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, $P < 0.02$.

enhanced lung binding (53%) which was the same level as found in GM_1-containing 34A-liposomes (53%), whereas inclusion of PEG750-PE resulted in only a slightly enhanced binding (47%). Thus, the presence of PEG750-PE or PEG2000-PE on the liposome surface does not interfere with the binding of antibody to antigen and, instead, enhances the target binding of immunoliposomes according to their activities in prolonging the circulation time of liposomes. On the contrary, the steric barrier activity of PEG5000-PE is apparently too strong, lessening the antigen/antibody interaction of this stystem, resulting in a decreased lung binding (22%). Thus, among the PEG-PEs tested, PEG2000-PE seems to be the optimal one for target binding of immunoliposomes. It should be noted, however, that the target antigen for 34A-liposomes is located at a readily accessible site, i.e. the vascular endothelial cell surface. For a much less accessible target, such as the tumor cell surface antigens in a solid tumor, only liposomes with a strong steric barrier activity would be expected to circulate long enough to reach the target. It is thus of interest to test the PEGs of higher molecular weights in targeting system where the antigen is in less accessible sites.

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