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The effects of polyethyleneglycol (PEG)-derived lipid on the activity of target-sensitive immunoliposome

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Abstract

In this study, serum stability and target-sensitivity of phosphatidylethanolamine (PE) immunoliposomes prepared with dioleoylphosphatidylethanolamine (DOPE), HYB-241 monoclonal antibody that targets *p*-glycoproteins, and various levels of polyethyleneglycol 2000 dioleoylphosphatidylethanolamine (PEG_{2000}–DOPE) were determined. Incubation of calcein-laden pegylated immunoliposomes prepared with different levels of PEG_{2000}–DOPE (0.3, 0.5 and 1.0 mol%) with *p*-glycoprotein rich bovine brain microvessel endothelial cells in 10% serum cell culture medium, all resulted in time-dependent release of calcein from the liposomes. The release of calcein was greatest for immunoliposomes prepared with 0.3 mol% PEG_{2000}–DOPE (66% in 1 h). Contrarily, the release of calcein from the other two immunoliposomes reached only $\sim$10–3% after same period of incubation. When serum-induced leakage of calcein was investigated for the above liposome preparations, liposomes prepared with 0.3 and 0.5 mol% PEG_{2000}–DOPE had the highest leakage level (10% in 1 h). Contrarily, the release of calcein from liposomes prepared with 1.0 mol% PEG_{2000}–DOPE reached only 3% after same period of incubation. Together, it would appear that release of calcein from the immunoliposomes prepared with 0.3 mol% PEG_{2000}–DOPE is a result of both serum-induced and target-induced destabilization of liposomes. The net release of calcein due to target-induced destabilization of liposomes is calculated to be at $\sim$56%. In contrast, there is no target-induced leakage of calcein from immunoliposomes prepared with either 0.5 or 1.0 mol% PEG_{2000}–DOPE.

Keywords: Immunoliposome; Blood–brain barrier; *p*-Glycoprotein; Target-sensitive; Liposome; Sterically stabilized

1. Introduction

Unsaturated phosphatidylethanolamine (PE) does not normally form stable lipid bilayers under normal physiological conditions. When dispersed in aqueous media, unsaturated PE assembles into nonbilayer hexagonal (H_{II}) structure (Cullis and

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de Kruijff, 1979). Formation of stable PE liposomes, however, can be formed if stabilizers are added to the PE. Several of these stabilizers have been studied in details (Connor et al., 1984; Ellens et al., 1984; Duzgunes et al., 1985; Ho et al., 1986). Generally, they are molecules that possess bulky polar headgroup and a small hydrophobic tail, giving these molecules an overall inverted cone shape (Connor et al., 1984; Ellens et al., 1984; Duzgunes et al., 1985; Ho et al., 1986). The unique shape of these molecules is believed to provide the necessary stabilizing effects on PE liposomes as it complements very nicely the cone-shaped unsaturated PE molecules in bilayer packing (Litzinger and Huang, 1992).

The nonbilayer preference of unsaturated PE and its ability to form stable liposomes in the presence of stabilizers has been exploited in many novel therapeutic and diagnostic procedures (Litzinger and Huang, 1992). One interesting application of unsaturated PE is the formation of a specific class of immunoliposomes called target-sensitive immunoliposomes (TSIL) (Ho et al., 1986, 1987a,b). These immunoliposomes require the antibody (conjugated to a lipid anchor) not only for specific target cell recognition but also as stabilizer of the otherwise unstable PE liposome (Ho et al., 1986, 1987a,b). In this design, target-specific binding of antibody-covered liposomes induces bilayer destabilization, resulting in a site-specific release of the liposome contents. Thus, TSIL should be an ideal carrier for site-specific delivery of both extracellular-acting and intracellular-acting drugs provided that (a) antibody that targets a specific antigen on a targeted cell is available and (b) cellular uptake of intracellular-acting drugs at the targeted site is not a particular concern.

Despite these potential therapeutic applications, the in vivo use of this and other immunoliposomes has been limited. This is because immunoliposomes, like all non-stealth liposomes, are rapidly cleared from the circulation by the reticuloendothelial system (RES) found in the liver and spleen (Aragnol and Leserman, 1986; Derksen et al., 1988). The reason for the efficient uptake of the injected liposomes by RES cells has been attributed to the opsonization of liposomes by plasma proteins, which then causes the liposomes to interact with the RES cells (Hoekstra and Scherphof, 1979). Avoidance of this obstacle, however, is possible when hydrophilic lipids, such as gangliosides (Allen and Chonn, 1987) or polyethyleneglycol (PEG)-derived lipids (Klibanov et al., 1990; Allen et al., 1991; Torchilin et al., 1992) are incorporated into the bilayer of conventional liposome to form the so-called sterically-stabilizied or stealth liposomes (Woodle and Lasic, 1992). The mechanism for the reduction in RES uptake of liposome is thought to be related to the ability of these hydrophilic lipids to provide a sufficiently hydrophilic surface to the liposome against any opsonization (Moghimi and Patel, 1992; Papahadjopoulos et al., 1991).

The advent of sterically stabilized liposomes suggests that preparation of TSILs that are also long circulating is probably feasible. Unfortunately, these hydrophilic lipids are also known stabilizers for PE liposomes. Therefore, one likely outcome of adding hydrophilic lipids to TSIL is the creation of a TSIL that is too stable to undergo target-induced destabilization. Therefore, the present study was instigated to delineate the effects of hydrophilic lipids on the stability and target sensitivity of dioleoylphosphatidylethanolamine (DOPE) immunoliposome. Utilizing an array of serum stability and target-sensitivity studies, our results demonstrate the modulating effects of hydrophilic lipids on the stability and target-sensitivity of a PEG-coated DOPE immunoliposome that targets *p*-glycoproteins expressed on a cell culture model of the blood–brain barrier (BBB).

2. Materials and methods

2.1. Materials

DOPE, PEG_{2000}–DOPE, dioleoylphosphatidic acid and *N*-glutarylphosphatidylethanolamine (NGPE) were purchased from Avanti Polar Lipids (Alabaster, AL). HYB-241 (MW 150 kDa), a mouse antihuman *p*-glycoprotein monoclonal IgG_1 antibody (Meyers et al., 1989), was kindly provided by Hybritech, Inc. (San Diego, CA). All other chemicals were from Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

2.2. Cell culture

Bovine brain microvessel endothelial cells (BBMEC) were isolated from cerebral gray matter of bovine brain as described previously (Audus et al., 1996; Ng and Schallenkemp, 1996). Briefly, brain gray matter was collected and minced to 1–2-mm cubes with razor blades before undergoing a 2.5-h dispase digestion (4 ml 12.5% dispase solution per 50 g of gray matter). The microvessels were then separated from the cell debris by centrifugation in 13% dextran. The isolated microvessels were further incubated on a per g basis with 3 ml of collagenase/dispase (at 1 mg/ml or 0.3 U collagenase and 4.12 U dispase/ml) for 4 h at 37°C. At the conclusion of this incubation, the microvessels were subjected to Percoll gradient centrifugation for final separation of microvessels from pericytes, cell debris and other contaminated cells. The purified microvessels were stored frozen at −180°C in freezing medium (36% minimum essential medium (MEM), 36% F-12 medium, 18% platelet poor horse serum, 10% DMSO, 50 U/ml penicillin, 50 mg/ml streptomycin, 125 mg/ml heparin) until used.

For target sensitivity studies, purified BBMECs were seeded at a density of 50 000 cells/cm^2 onto collagen coated and fibronectin-treated 96-well cell culture plate in BBMEC plating medium (45% MEM, 45% F-12 medium, 10% platelet poor horse serum, 50 U/ml penicillin, 50 mg/ml streptomycin, 2.5 mg/ml amphotericin B, 50 mg/ml polymixin B and 25 mg/ml endothelial cell growth supplement (ECGS)). The cells were grown in a 37°C incubator with 5% CO_2 and 95% humidity. The plating medium was replaced with BBMEC culture medium (plating medium less polymixin B and ECGS) on the third day after plating and every other day thereafter. BBMECs were allowed to grow to confluency prior to target sensitivity studies. Typically, formation of monolayers took about 6–8 days.

2.3. Derivatization of antibody with NGPE

HYB-241 monoclonal antibody was conjugated to NGPE using a method described previously (Pinnaduwage and Huang, 1992) with slight modification. Briefly, 0.029 mmol of NGPE was dissolved in 50 ml of *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes) buffer (0.01 M, 0.15 M NaCl, pH 7.5) containing 0.1 M *N*-octyl-D-gluco-pyranoside. To this solution, 10 ml each of 0.25 M 1-ethyl-3(3″(dimethylamino)propyl)carbodiimide and 0.1 M *N*-hydroxyl-sulfosuccinimide dissolved in Hepes (0.01 M, 0.15 M NaCl, pH 7.5) were added. The resulting mixture was incubated at room temperature for 5 min. To 70 ml of this mixture, 500 mg of a 0.857 mg/ml HYB-241 monolconal antibody solution was then added (approximately 8.5:1 molar ratio between NGPE and antibody) and the resulting mixture was incubated at 4°C for 8 h with frequent mixing. The resulting HYB-241-NGPE conjugate was then dialyzed overnight with Micro DispoDialyzer (Spectrum, Laguna Hills, CA) at 4°C against two changes of 1 l of 0.01 M Hepes buffer (0.15 M NaCl, pH 7.5) to remove *N*-octyl-D-gluco-pyranoside and other excess reagents.

2.4. Preparation of sterically stablized liposomes

PEG_{2000}–DOPE/DOPE liposomes were prepared by the sonification method (Papahadjopoulos and Miller, 1967). This method usually generates small unilamellar vesicles (SUV). Briefly, DOPE and PEG_{2000}–DOPE were mixed and dried under a stream of argon and vacuum desiccated for no less than 30 min. The dried lipid mixture was then hydrated with 1 ml of 0.01 M Hepes buffer (0.15 M NaCl, pH 7.5) containing 50 mM calcein at 4°C for 24 h. After the mixture was sonicated in a bath sonicator (Laboratory Supplies, Inc., Hicksville, NY) for 5 min, the mixture was incubated at 4°C for 8 h followed by another 5-min sonification. Liposomes were separated from unencapsulated calcein by gel chromatography with a Bio-Gel A-15M (Bio-Rad, Hercules, CA) column. The column was eluted with 0.01 M Hepes buffer (0.15 M NaCl, pH 7.5). Encapsulation of calcein was determined by fluorescence quenching measurement as described below. The phospholipid content of the liposomes was analyzed by the method of Bartlett (1959). The average liposome size prepared were deter-

mined to be $\sim 280 \pm 120$ Å (Nicomp 370 submicron particle sizer, Silver Spring, MD).

2.5. Preparation of sterically stabilized immunoliposomes

Immunoliposomes containing HYB-241 monoclonal antibody were prepared using a procedure described previously (Pinnaduwage and Huang, 1992) with slight modification. Briefly, DOPE and PEG_{2000}–DOPE were mixed and dried under a stream of argon and vacuum desiccated for no less than 2 h. The lipid mixture was then hydrated with 1 ml of 0.01 M Hepes buffer (0.15 M NaCl, pH 7.5) containing NGPE-conjugated HYB-241 monoclonal antibody and 50 mM calcein. The mixture was incubated at 4°C for 24 h with occasional mixing, followed by a brief period of sonification (5 min) in a bath sonicator. After an additional 8 h of incubation at 4°C, another sonification (5 min) was performed. Immunoliposomes was separated from unencapsulated calcein and unbound antibody by gel chromatography with a Bio-Gel A 15 M column (BioRad, Hercules, CA). The peak immunoliposomes fractions, eluted with isotonic Hepes buffer (0.15 M NaCl, pH 7.5), was then pooled and measured for their protein and lipid contents. Protein analysis was performed using BioRad microplate protein assay protocol. Lipid analysis was by the method of Bartlett (1959). The average size of the SUV prepared were determined to be $\sim 280 \pm 120$ Å (Nicomp 370 submicron particle sizer, Silver Spring, MD).

2.6. Fluorescence measurments

Formation of liposomes was monitored by their ability to encapsulate a 50 mM self-quenching fluorescence dye, calcein, and to quench calcein fluorescence. At 50 mM concentration, calcein fluorescence was $\sim 70\%$ quenched (Ho et al., 1986). Encapsulation of calcein (expressed as mole ratio of calcein and lipid) was determined quantitatively by fluorescence spectrophotometry ($\lambda_{ex} =$ 490 nm and $\lambda_{em} = 520$ nm). Briefly, fluorescence of lysed liposomes after addition of 0.15% Triton X-100 was measured using a Shimadzu RF-1501 spectrofluorometer. The amount of calcein in each sample was determined from the fluorescence measurements by the construction of a calcein standard curve. Percent fluorescence quenching was calculated using the following formula (Ho et al., 1986):

$$\% \text{ quenching} = (1 - F_o/F_t) \times 100$$

where F_o and F_t are the fluorescence of the liposome samples before and after the addition of 0.15% Triton X-100, respectively.

2.7. Target-induced lysis of sterically stabilized HYB-241 immunoliposome

Confluent monolayers of BBMEC in 96-well cell culture plate were used in these experiments and each experiment was performed in duplicate at 37°C. Prior to the experiments, the growth medium of BBMEC was removed and the cells were washed with pH 7.4 serum free MEM/F12 cell culture medium. Then 70 ml of pH 7.4 MEM/F12 buffer (with 10% serum) containing calcein-encapsulated sterically stabilized HYB-241 immunoliposome (320 mM) was added to the targeted cells. After incubating the immunoliposomes with the cells for 60 minutes, the incubation mixture was removed from each well and diluted with 0.01 M pH 7.9 phosphate buffered saline (PBS) for fluorescence measurement using a Shimadzu RF-1501 spectrofluorometer ($\lambda_{ex} = 490$ nm and $\lambda_{em} = 520$ nm). The total calcein fluorescence in the incubation buffer (0.01 M pH 7.9 PBS) was measured after the addition of Triton X-100 to a final concentration of 0.15%. The percentage of calcein release was calculated using the following formula (Ho et al., 1986):

$$\% \text{ release} = (F - F_o)/(F_t - F_o) \times 100$$

where F_o and F are the calcein fluorescence before and after incubation of the immunoliposomes with the targeted cells, respectively, and F_t is the total fluorescence after lysis of liposomes with 0.15% Triton X-100.

2.8. Serum stability of liposomes

To determine the serum stability of the above immunoliposomes, calcein-encapsulated immunoliposomes or control liposomes (DOPE liposomes prepared with either 5 mol% PEG_{2000}–DOPE or 20 mol% DOPA) were added to the cell-free wells of a 96-well cell culture plate containing either 10 or 75% serum MEM/F12 cell culture media. After incubating the liposomes with the cell culture media for various time intervals, the incubation medium was removed and analyzed for the percentage release of calcein using the method as described above.

3. Results

3.1. Effect of PEG_{2000}–DOPE on DOPE liposome formation

The effects of the hydrophilic lipid, PEG_{2000}–DOPE, on DOPE liposome formation were studied. For these studies, formation of DOPE liposomes was monitored by their ability to encapsulate a 50 mM self-quenching fluorescence dye, calcein, and to quench calcein fluorescence. As shown in Table 1, increasing the PEG_{2000}–DOPE content in a DOPE liposome caused a direct increase in the levels of fluorescence quenching and calcein encapsulation. These results are in line with previous observations (Blume and Cevc, 1990; Senior et al., 1991; Needham et al., 1992) and confirm the stabilizing effect pegylated lipids exert on DOPE liposome formation (Woodle, 1993). Further, the optimal amount of PEG_{2000}–DOPE to enhance DOPE liposome formation appears to occur at 40:1 molar ratio of DOPE and PEG_{2000}–DOPE (2.5 mol% PEG_{2000}–DOPE). This is because further increasing the proportion of PEG_{2000}–DOPE in a DOPE liposome preparation did not result in additional enhancement in the fluorescence quenching and the amount of calcein encapsulated per mole of lipid. In contrast, the minimal amount of PEG_{2000}–DOPE required for induction of DOPE liposome formation occurs at ~ 0.5 mol%, as incorporation of PEG_{2000}–DOPE at less than 0.5 mol% resulted in negligible fluorescence quenching and calcein encapsulation.

3.2. Effect of NGPE-conjugated antibody on DOPE/PEG_{2000}–DOPE liposome formation

As indicated in Table 1, when used at 2.5 mol% or above, PEG_{2000}–DOPE tends to provide the most optimal effects in stabilizing DOPE liposome formation. This result implies that at less than 2.5 mol%, PEG_{2000}–DOPE might not provide optimal condition for DOPE liposome formation and incorporation of other known bilayer stabilizers in addition to PEG_{2000}–DOPE should further enhance DOPE liposome formation. Because antibody conjugated to a lipid is a known stabilizer (Ho et al., 1986) of DOPE lipid, it should supplement the stabilizing effects of PEG_{2000}–DOPE (when used at < 2.5 mol%) in DOPE liposome formation. Further, if one could incorporate the antibody to PEG_{2000}–DOPE/DOPE liposome at an optimal ratio, one might be able to prepare a sterically stabilized immunoliposome that exhibits both the property of a conven-

Table 1
Effect of PEG_{2000}–DOPE on DOPE liposome formation[a]

Molar ratio (DOPE:PEG_{2000}–DOPE)	20:1	40:1	80:1	160:1	320:1	640:1
Mol% PEG_{2000}–DOPE	~ 5	~ 2.5	~ 1.25	~ 0.6	~ 0.3	~ 0.15
% Quenching	77.3 ± 1.1	73.2 ± 0.1	$65.8 \pm .0.4$	58.9 ± 2.5	26 ± 0.7	6.2 ± 1.9
Molar ratio (calcein:lipid)	4.82	4.53	3.25	1.33	0.07	0.019

[a] Percent fluorescence quenching for three samples ($n = 3$) was calculated using the following formula: % quenching $= (1 - F_o/F_t) \times 100$, where F_o and F_t are the fluorescence of the liposome samples before and after the addition of 0.15% Triton X-100, respectively. Each data point represents the mean $\pm$ S.D.

Table 2
Effect of HYB-241 antibody on DOPE/PEG_{2000}–DOPE liposome formation [a]

Molar ratio (DOPE:PEG_{2000}–DOPE)	100:1	200:1	320:1
Mol% PEG_{2000}–DOPE	~1.00	~0.5	~0.3
% Quenching (without antibody)	~65.8 ± 0.45	~58.9 ± 2.5	~26.1 ± 1.9
% Quenching (with antibody)	~77.5 ± 0.5	~67.9 ± 2.6	~59.9 ± 0.7
Molar ratio (lipid:antibody) [b]	3631.3 ± 31.6	3903.5 ± 274.5	4481.3 ± 37.5

[a] Percent fluorescence quenching for three samples ($n = 3$) was calculated using the following formula: % quenching $= (1 - F_o/F_t) \times 100$, where F_o and F_t are the fluorescence of the liposome samples before and after the addition of 0.15% Triton X-100, respectively.
[b] Molar antibody to lipid ratios were approximately the same for all liposome formulations and were around 1:4000. Each data point represents the mean ± S.D.

tional TSIL and that of a stealth liposome. The objective of this study was to determine if formation of stable PEG_{2000}–DOPE/DOPE liposome could be enhanced by antibody-derived lipid and, if so, whether these immunoliposomes would demonstrate target sensitivity and stability against opsonization (as reflected by their serum stability) (Huang, 1992).

To investigate if formation of stable PEG_{2000}–DOPE/DOPE liposomes could be enhanced by antibody-derived lipid, calcein-loaded DOPE liposomes were prepared using a mixture of NGPE-conjugated HYB-241 monoclonal IgG antibody (500 mg) and variable levels of PEG_{2000}–DOPE (0.3, 0.5 and 1.0 mol%). Table 2 shows the fluorescence quenching values for these three groups of immunoliposomes. As indicated in Table 2, incorporating NGPE-conjugated IgG antibody in the liposomal preparation caused the fluorescence quenching to increase in all three groups of liposomes. These results are in line with our contention that antibody-derived lipid would provide extra stabilization in the formation of PEG_{2000}–DOPE/DOPE liposomes when PEG_{2000}–DOPE was used at less than 2.5 mol%. It is also interesting to note that as the mole% contribution of the PEG_{2000}–DOPE was increased gradually in the final liposome preparation, a noticeable decrease (from 4481 to 3631) in the molar antibody to lipid ratio was observed for all three liposomes. These results are expected as the presence of other bilayer stabilizer should decrease the incorporation NGPE-conjugated antibody into DOPE liposomes.

3.3. Target-induced lysis and serum stability of immunoliposomes

The target sensitivity of the above immunoliposomes was examined in a cell culture model of the BBB, BBMEC. This cell culture has previously

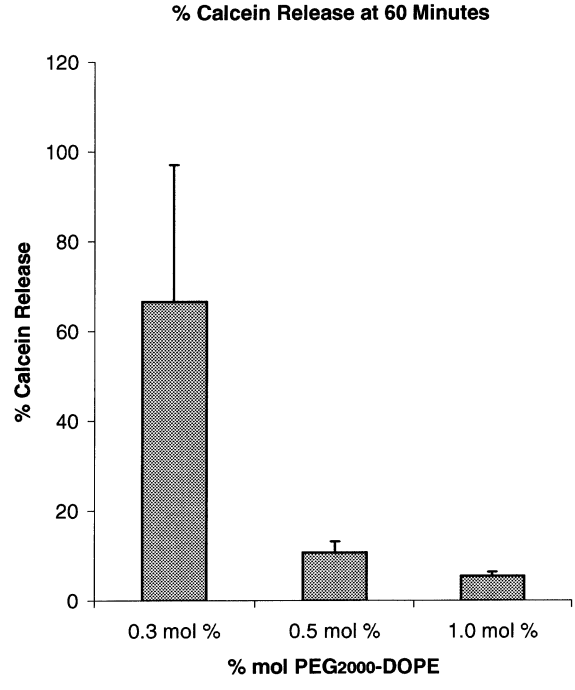

Fig. 1. Target-induced lysis of HYB-241 DOPE immunoliposomes prepared with various levels of PEG_{2000}–DOPE. Immunoliposomes were prepared as described in Section 2. The final ratio of lipid:antibody in the pooled HYB-241 DOPE immunoliposomes were 4481:1, 3903:1, and 3631:1, respectively for the liposomes prepared with 0.3, 0.5 and 1.0 mol% PEG_{2000}–DOPE. Each bar represents the mean ± S.D. for two separate experiments.

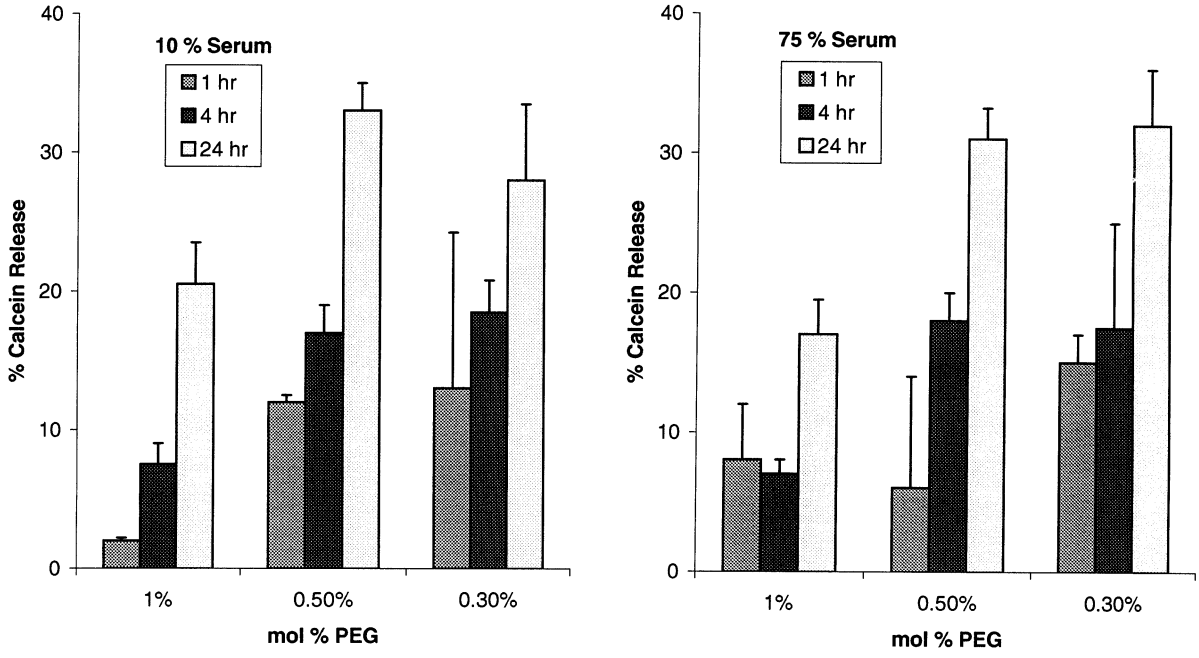

Fig. 2. Stability of PEG_{2000}–DOPE:DOPE HYB-241 immunoliposome in 10 and 75% serum. Liposome-serum incubation studies were performed at 37°C for various time points (1, 4 and 24 h). The percentage of calcein release was calculated using the following formula: % release $= (F - F_o)/(F_t - F_o) \times 100$, where F_o and F are the calcein fluorescence before and after incubation of the immunoliposomes with serum, respectively, and F_t is the total fluorescence after lysis of liposomes with 0.15% Triton X-100. The final ratio of lipid:antibody in the pooled HYB-241 immunoliposomes were 4481:1, 3903:1, and 3631:1, respectively, for the liposomes prepared with 0.3, 0.5 and 1.0 mol% PEG_{2000}–DOPE. Each bar represents the mean ± S.D. for three separate experiments.

been shown to express very high level of *p*-glycoproteins (Fontaine et al., 1996) and should, therefore, serve as a good model for the above study. As shown in Fig. 1, incubation of HYB-241 pegylated immunoliposomes prepared with three different levels of PEG_{2000}–DOPE (0.3, 0.5 and 1.0 mol%) with BBMEC monolayers in 10% serum cell culture medium for 60 min, all resulted in release of calcein from the liposomes. Release of calcein was greatest for immunoliposomes prepared with 0.3 mol% PEG_{2000}–DOPE, reaching a level of ~66% after 60 min of incubation. In contrast, release of calcein from immunoliposomes prepared with either 0.5 or 1.0 mol% PEG_{2000}–DOPE reached only ~10–3% after same period of incubation.

To investigate if release of calcein from these immunoliposomes was the direct result of serum induced leakage, liposomes were added to cell culture plates containing only 10 or 75% serum cell culture medium. As indicated in Fig. 2, serum produces a time-dependent release of calcein for all liposomes. Leakage of calcein from all three liposomes is independent of the final serum concentration as incubation of the liposomes in either of the media produces about the same leakage of calcein. At both level of serum used (10 and 75%), incorporation of higher percentages of PEG_{2000}–DOPE into the final immunoliposome preparation appears to reduce the serum-induced leakage of liposomal contents. Release is highest for the liposomes prepared with 0.3 and 0.5 mol% PEG, reaching a level of ~10% after 60 min of incubation. Contrarily, release of calcein from the liposomes prepared with 1.0 mol% reached only ~3% after same period of incubation.

Taken the results from the target sensitivity and serum stability studies, it would appear that re-

lease of calcein from the immunoliposomes prepared with 0.3 mol% PEG_{2000}–DOPE is a result of both serum induced and target-induced destabilization of liposomes. The net release of calcein due to target-induced destabilization of liposomes is calculated to be at around 56% (difference of the 66 and 10% seen in the target sensitivity and serum stability studies, respectively). Contrarily, there is no target-induced leakage of calcein from immunoliposomes prepared with either 0.5 or 1.0 mol% PEG_{2000}–DOPE, as leakage of calcein from both liposome preparations in the presence or absence of targeted cells was about the same. These results imply that target-induced destabilization of immunoliposomes is only possible with the immunoliposomes prepared with 0.3 mol% PEG_{2000}–DOPE, but not higher levels.

4. Discussion

In a conventional TSIL, the antibody (conjugated to a lipid anchor) is required both for its target cell recognition ability and stabilizing activity (Ho et al., 1986, 1987a,b). Thus, it is expected if one could replace the stabilizing portion of the antibody with hydrophilic lipids, it would lead to the generation of a sterically stabilized TSIL or stealth TSIL. To test whether this premise is true, we examined the serum stability and target sensitivity of immunoliposomes prepared with different levels of PEG-derived lipids. In all of our cases, PEG_{2000}–DOPE was found to exert an opposite concentration-dependent modulating effect on the serum stability and target sensitivity of TSIL. Based on the known effect of hydrophilic lipids in stabilizing lipid bilayers (Woodle, 1993), these results were rather expected. What is most surprising, however, is the rather low level of PEG_{2000}–DOPE (0.5 mol%) needed to nullify the activity of a TSIL.

There are several mechanisms by which PEG-derived lipids could contribute to reduction in target sensitivity of TSIL. Because of their rather large size, PEG-derived lipids might modulate the activity of TSIL by interfering with the antibody in binding to its targeted antigen (Kilbanov et al., 1991; Torchilin, 1994) or the attainment of close bilayer contact between adhered liposomes implied for initiation of liposome destabilization (Pinnaduwage and Huang, 1992). Likewise, it is also possible that a certain level of antibody per liposome might be required to initiate the target-induced destabilization of TSIL (Ho et al., 1986) such that a slight reduction in the antibody amount might trigger a large change in the level of target-induced destabilization seen. As indicated by our data (Table 1), incorporation of PEG_{2000}–DOPE did indeed cause a slight reduction in the amount of antibody incorporated per liposome, thus pointing to the significance of such possibility.

Regardless of the mechanism responsible for the desensitization of PEG-coated TSIL, a most interesting finding of our study is the observation that preparation of a PEG-coated immunoliposome that may still be target-sensitive is possible at 0.3 mol% PEG_{2000}–DOPE. Although serum-induced leakage for this immunoliposome is high (10% in 60 min), it is within comparable range to conventional stealth liposomes prepared with 5 mol% PEG (10:0.5 DOPE:PEG_{2000}–DOPE; 3% in 60 min, data not shown). Consequently, these data are suggestive that sterically stabilized TSIL can be prepared with 0.3 mol% PEG_{2000}–DOPE and further experiments should be conducted to confirm or refute this possibility.

In conclusion, our results indicate that when sufficient PEG-derived lipids are added to a conventional TSIL to slow down serum lysis to levels that approach conventional 'stealth liposomes', the target sensitivity of the immunoliposomes is lost. Our results also indicate immunoliposomes having 0.3 mol% PEG-derived lipids release their contents when cultured with cells bearing a target antigen, and may offer some serum stability, albeit not at conventional levels, to the liposomes. Further experiments must be performed to confirm whether preparation of sterically stabilized TSIL is possible; however, the data presented here certainly indicate that additional investigation into this possibility is reasonable. Such a confirmation would represent an exciting and surprising advacne in therapeutic drug delivery. Such immunoliposomes may have huge potentials in delivering therapeutics to targeted sites

of action provided that (1) antibody that targets a specific antigen on a targeted cell is available and (2) cellular uptake of intracellular-acting drugs at the targeted site is not a particular concern. Currently, we are investigating the possible use of this carrier system to deliver vascular permeability factors for transient permeabilization of the blood–brain barrier for enhanced drug transport into the central nervous system.

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