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Role of cholesterol in the stability of pH-sensitive, large unilamellar liposomes prepared by the detergent-dialysis method

Dexi Liu and Leaf Huang

Department of Biochemistry, University of Tennessee, Knoxville, TN (U.S.A.)

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Large unilamellar liposomes prepared by an octyl glucoside-dialysis method were examined for stability at 37°C in the presence or absence of human plasma, using the release of the entrapped calcein as a fluorescence marker. The liposomes were acid-sensitive as they were composed of dioleoylphosphatidylethanolamine, oleic acid and cholesterol. The stability of the liposomes in the absence of plasma was significantly enhanced with increasing cholesterol content. However, the maximal calcein release at pH 5 decreased linearly with increasing cholesterol content of the liposome, indicating that cholesterol had reduced the acid-sensitivity of the liposomes. In the presence of human plasma, calcein release exhibited a biphasic behavior with a fast (plasma-sensitive) and a slow (plasma-resistant) component. Inclusion of cholesterol in the liposomes resulted in an increased proportion of the plasma-resistant component. Liposomes pretreated with human plasma, after removal of excess plasma and the released calcein by gel-filtration, showed a remarkable stability both in the presence and absence of human plasma. The acid-sensitivity of the plasma-treated liposomes with 40% cholesterol was the same as that of the untreated liposomes. These results are discussed in terms of the mechanism by which these liposomes deliver their contents to the cytoplasm of the cells via the endocytic pathway, a known biological activity of the type of liposome described here.

Introduction

Liposomes have been widely used as a delivery vehicle for drugs, enzymes, antibodies and DNA (for recent reviews, see Ref. 1). One of the primary findings of the liposomal drug delivery is that the liposomes are largely endocytosed by the cells and the final cellular destination is the lysosomal compartment [2,3]. Many drugs, especially the macromolecular drugs, are hydrolysed in the lysosome, and/or cannot effectively escape from the lysosome, thus resulting in poor biological activities of the delivered drug [3].

In recent years, pH-sensitive liposomes have been developed by several investigators [4–7] for improved cytoplasmic delivery of the liposomal drugs. These liposomes are primarily composed of PE, a lipid with a high tendency of forming hexagonal phase [8]. The bilayer phase of PE is stabilized with a weakly acidic amphiphile which, when protonated at an acidic pH, triggers the destabilization of the PE liposomes. The cytoplasmic delivery activity of these liposomes is considerably enhanced as compared to the conventional pH-insensitive liposomes. Fluorescent dyes [5,9,10], anticancer drugs [11], toxins [12] and DNA [13,14] are efficiently delivered to the cells cultured in vitro. However, the in vivo delivery activity of these liposomes is severely limited by the instability of the liposome in the presence of plasma or serum. It has been shown by us that the large unilamellar liposomes prepared by the reverse-phase evaporation method composed of DOPE and oleic acid (8 : 2 molar ratio) rapidly aggregate and become leaky when exposed to serum [15]. It was concluded that this type of pH-sensitive liposome is not suitable for delivery of water-soluble drugs in vivo.

Nevertheless, an attempt has been made to deliver intact plasmid DNA to the target cells in mouse [13]. The pH-sensitive liposomes used in the study were prepared by a detergent-dialysis method, modified from the original procedure of Philippot et al. [16]. In addition, cholesterol was included in the lipid composition to increase the trapping efficiency of DNA [16]. The liposome entrapped (or associated) DNA was injected

Abbreviations: Chol, cholesterol; DOPE, dioleoylphosphatidylethanolamine; EGTA, ethyleneglycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; OA, oleic acid; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine.

Correspondence: L. Huang, Department of Biochemistry, University of Tennessee, Knoxville, TN 37996-0840, U.S.A.

into nude mice bearing the target lymphoma cells. Successful delivery and expression of the marker gene were detected in the target cells [13]. The results of these experiments strongly suggest that at least a portion of the liposome is stable under the in vivo conditions. It has thus prompted us to study the stability, both in the presence and absence of plasma, of the pH-sensitive liposomes prepared by the detergent-dialysis method. Furthermore, we have investigated the role of cholesterol in liposome stability.

Materials and Methods

Materials. DOPE was purchased from Avanti Polar Lipids. Oleic acid, cholesterol, calcein and octyl glucoside were purchased from Sigma Chemical. SM-2 beads were purchased from Bio-Rad. Freshly collected human plasma was obtained from Fort Sanders Regional Medical Center (Knoxville, TN). [^{14}C]Oleic acid (57 mCi/mmol) was purchased from, New England Nuclear. [^{14}C]Octyl glucoside (314 mCi/mmol), a kind gift from Dr. Carol Cunningham, was originally obtained from New England Nuclear.

Liposome preparation. Large unilamellar vesicles were prepared according to the method of Philippot et al. [16] with modifications. 10 μmol of lipid was dried with a gentle stream of N_2 gas. The dry lipid was kept under vacuum for a minimum of 30 min and suspended in 900 μl of PBS buffer/50 mM calcein/0.55 mM EGTA, which was isotonic to human plasma. [^{3}H]Cholestanyl etner was included in the lipid mixture to monitor the lipids [17]. The lipid suspension was sonicated with a bath sonicator (Laboratory Supplies, Hicksville, NY) and the pH was adjusted to pH 8.0. 100 μl of octyl glucoside (100 μmol) were added. The mixture was vortexed and dialyzed at room temperature against 100 ml of the buffer used above containing 1 g of washed SM-2 beads for 48 h with slow stirring. The liposomes were separated from free calcein by using Bio-Gel A-1.5m column chromatography equilibrated with PBS (160 mM NaCl/3.2 mM KCl/1.8 mM KH_2PO_4/0.12 mM Na_2HPO_4/1.2 mM EGTA (pH 8.0)), which was isotonic to the calcein-containing buffer. The liposomes had an average diameter of 500 $\pm$ 200 nm as measured by dynamic laser light scattering using a Coulter NS4D instrument.

Dissociation of oleic acid and octyl glucoside during liposome preparation. Trace amounts of [^{14}C]oleic acid or [^{14}C]octyl glucoside were included in different preparations of liposomes. 20 μl of lipid mixture from the dialysis bag were collected at different times and counted for ^{14}C radioactivity in a scintillation counter.

Pretreatment of liposomes with plasma. 500 μl of liposomes were mixed with the same volume of plasma and incubated for 1 h at room temperature before passing through a Bio-Gel A-1.5m column.

pH-sensitivity of liposomes. Fluorescence was measured by Perkin Elmer LS5 spectrofluorometer with λ_{ex} = 490 nm and λ_{em} = 520 nm. Liposomes (10 μl) were added to 1.99 ml PBS at the desired pH and incubated at room temperature for 5 min. The pH of the mixture was adjusted back to 8.0 after incubation by adding an appropriate amount of NaOH solution. Deoxycholate (final concn. 0.25%) was then added to completely release calcein from the liposomes.

The percent leakage of liposomes was calculated from the dequenching of calcein fluorescence according to the following equation:

$$\% \text{ Release} = \frac{(F_t/F_t')F_f - F_0}{F_t - F_0} \times 100$$

where F_0 is the fluorescence intensity of liposomes in PBS at pH 8.0 before the incubation; F_f is the fluorescence intensity of the liposome after incubation; F_t is total fluorescence intensity of liposomes incubated at pH 8.0 after deoxycholate addition; F_t' is the total fluorescence intensity of liposomes incubated at different pH. F_t/F_t' was used to normalize the amount of liposomes used in the measurement.

Stability of liposomes in plasma and isotonic PBS at 37°C. 100 μl of liposomes containing 60 nmol of lipid were added to 900 μl of buffer (pH 7.4) or plasma prewarmed to 37°C and maintained at 37°C in a water bath. 10 μl of the mixture were used for the fluorescence measurement at different incubation time-points. The percent leakage of liposomal calcein was calculated as above, except F_0 is the fluorescence intensity in PBS at zero time and F_t is the total fluorescence intensity of liposome in PBS at zero time. The F_t and F_t' are the fluorescence intensity at a given time-point before and after deoxycholate-induced lysis, respectively.

The reproducibility of the leakage measurement was ascertained by measuring three or more different samples and each data point was obtained as the average of duplicate measurements of each sample. The standard deviation was less than 10% of the mean. Repeat measurements on the same sample were very similar to each other.

Results

It was important to ascertain that the stability of the liposomes prepared by the detergent-dialysis method was not affected by the residual detergent, octyl glucoside, left in the liposome preparation. As one can see in Fig. 1(B), there was an undetectable amount of [^{14}C]octyl glucoside left in the liposome preparation after 48 h dialysis against a buffer containing SM-2 beads. In a parallel experiment, [^{14}C]oleic acid was used to see if any oleic acid was lost during dialysis. Total retention of oleic acid was observed under the same dialysis

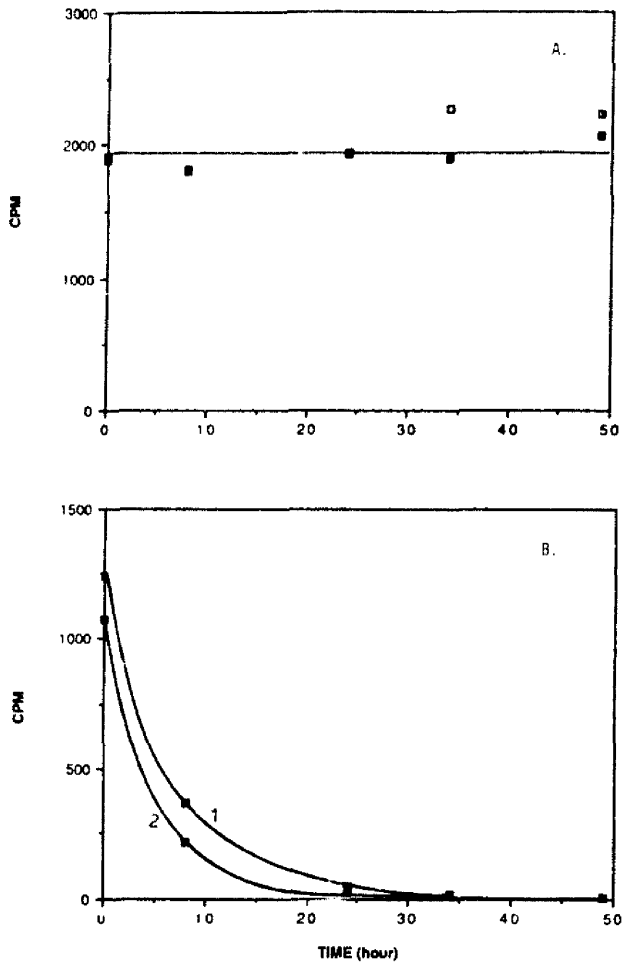

Fig. 1. Dissociation of oleic acid and octyl glucoside from liposomes during dialysis. A trace amount of [^{14}C]oleic acid or [^{14}C]octyl glucoside was included in separate liposome preparation either without or with 40% cholesterol. 20 μl of lipid mixture from the dialysis bag were collected at different times and counted for ^{14}C radioactivity. (A) Dissociation of oleic acid. (B) Dissociation of octyl glucoside. □, liposome without cholesterol (curve 1); ■, liposome with 40% cholesterol (curve 2).

condition. Inclusion of cholesterol in the lipid composition did not change the conclusions, as liposomes with or without cholesterol gave essentially the same results (Fig. 1).

In order to systematically investigate the role of chol in the stability of the pH-sensitive liposomes, liposomes containing 0–50% cholesterol were prepared. In all cases, the molar ratio of DOPE to oleic acid was kept at 2. For example, liposomes containing no cholesterol had a composition of DOPE/OA = 6.66 : 3.33, and those liposomes containing 40% cholesterol had a composition of DOPE/OA/Chol = 4 : 2 : 4. The acid-sensitivity of the liposomes was investigated by measuring calcein release after an acid treatment. The results shown in Fig. 2 clearly indicate that, although the pH value at which half-maximal calcein release took place remained at about pH 7, the maximal calcein release which took place at pH 4–5 decreased linearly with increasing cholesterol content of the liposome (Fig. 2 inset). Therefore, cholesterol in the liposome membrane caused the liposomes to become less acid-sensitive. For example, at 40% cholesterol content, only about 40% calcein could be released at pH 4. This number was approximately half of those of liposomes containing no cholesterol.

Cholesterol also affected the release kinetics of calcein from liposomes incubated in PBS at 37 °C (Fig. 3). The release of calcein at 37 °C followed first-order kinetics (data not shown), with the rate constant decreasing with elevated cholesterol content of the liposomes. Thus, inclusion of cholesterol in the liposome membrane significantly enhanced the stability of the pH-sensitive liposomes in a protein-free, PBS solution.

It is also important to investigate the stability of the liposomes in the presence of plasma. Liposomes were diluted 10-fold with human plasma and the release of calcein was measured at different times of incubation at 37 °C. As can be seen in Fig. 4, the kinetics of release was biphasic. The fast phase (plasma-sensitive phase) was so fast that it was difficult to measure with our

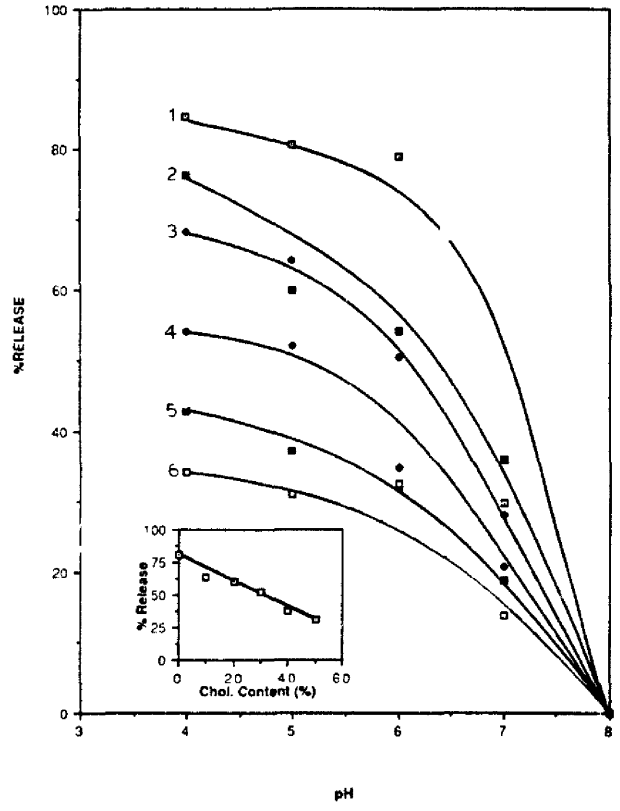

Fig. 2. Effect of cholesterol on the acid-sensitivity of pH-sensitive liposomes. Percent calcein release from liposomes is plotted against the pH of the incubation buffer. Curve 1, without cholesterol; curve 2, with 10% cholesterol; ◆, with 20% cholesterol (curve 3); ◇, with 30% cholesterol (curve 4); ■, with 40% cholesterol (curve 5); □ with 50% cholesterol (curve 6). Inset, the percent calcein release at pH 5 vs. the cholesterol content.

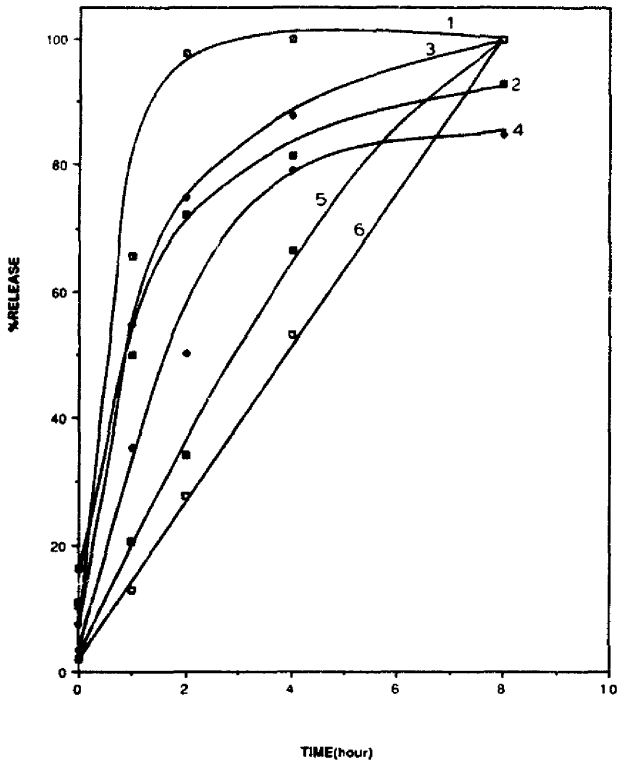

Fig. 3. Stability of liposomes in PBS (pH 7.4) at 37°C. Percent calcein release from liposomes is plotted against the time of incubation. Curve 1, without cholesterol; curve 2, with 10% cholesterol; ◆ with 20% cholesterol (curve 3); ◇, with 30% cholesterol (curve 4); ■, with 40% cholesterol (curve 5); □, with 50% cholesterol (curve 6).

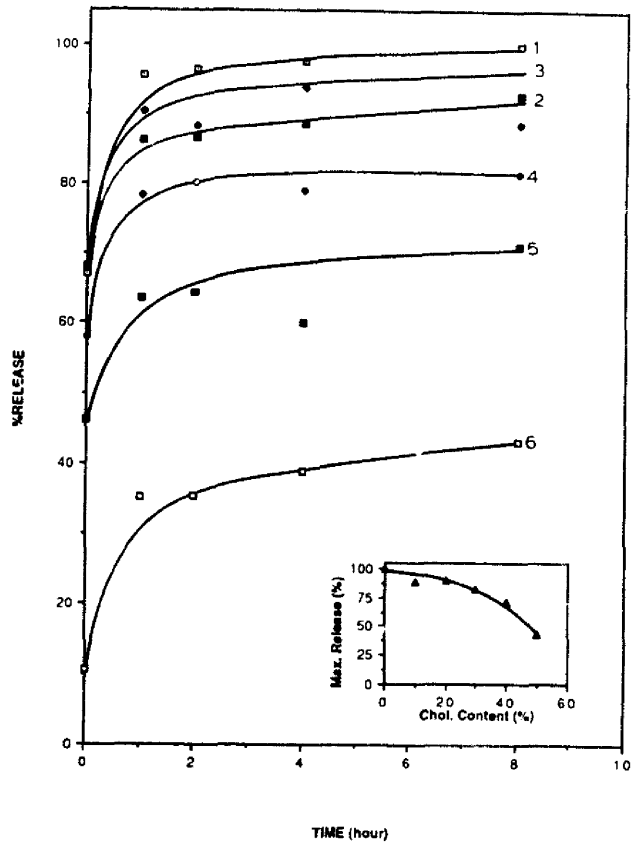

Fig. 4. Effect of cholesterol on the stability of pH-sensitive liposomes in human plasma at 37°C. Percent calcein release from liposomes is plotted against the time of incubation. Curve 1, without cholesterol; curve 2, with 10% cholesterol; ◆, with 20% cholesterol (curve 3); ◇, with 30% cholesterol (curve 4); ■, with 40% cholesterol (curve 5); □, with 50% cholesterol (curve 6). Inset, maximal calcein release vs. cholesterol content of the liposome.

method. The slow phase was very slow; essentially no significant calcein leakage was detected up to 8 h incubation (Fig. 4). Interestingly, the component of the slow, or plasma-resistant, phase was dependent on the cholesterol content of the liposome. As the cholesterol content in the liposome increased, the resistant phase component became larger (Fig. 4, inset). For example, liposomes with 40% cholesterol only released about 60% calcein after 8 h of incubation, whereas the liposomes without cholesterol released 100% in the same time-period. The kinetics of the calcein release from liposomes was essentially the same when the plasma concentration was reduced from 90% in the routine experiments to 50 and 25% in some experiments. Below 25% of plasma, calcein release was slower and less complete (data not shown).

It was interesting to see if the plasma-resistant component of the cholesterol-containing liposomes is sensitive to acid. Liposomes containing 40% cholesterol were exposed to 50% plasma for 1 h at room temperature, effecting the release of calcein from the plasma-sensitive component. The liposomes were then chromatographed on a Bio-Gel A-1.5m column to remove free calcein and most, if not all, of the plasma proteins. The resulting liposomes were then tested for the acid-induced release of calcein. As shown in Fig. 5, the acid-sensitivity of the plasma-treated liposomes containing 40% cholesterol was indistinguishable from one without the plasma treatment. Thus, the acid-sensitivity of the plasma-re-

TABLE 1

Transfer of oleic acid from liposome to plasma component(s)

Liposomes containing [^{14}C]oleic acid, with or without plasma treatment, were chromatographed on Bio-Gel A-1.5m to separate liposome (void volume) from the plasma component(s) (included volume). Data are average of the two experiments. Plasma treatment was the same as described in Materials and Methods.

Cholesterol content in liposome [a] (%)	Plasma treatment	% [^{14}C]oleic acid eluted in	
		void volume	included volume
0	no	100	0
	yes	41	59
40	no	100	0
	yes	22	78

[a] Lipid composition was DOPE/OA (2:1) with 0 or 40% cholesterol.

sistant component was the same as that of the original liposome population.

One explanation of the above data was that the plasma-liposome interaction was reversible such that the plasma-treated liposomes reverted back to untreated state after the removal of plasma proteins. This possibility was investigated by measuring the calcein release at 37 °C in the presence and absence of human plasma for the plasma-treated liposomes (Fig. 6). The plasma-treated liposomes showed striking stability both in the presence or absence of plasma; only 20–30% of calcein release was observed after 8 h of incubation. This is to be compared with the untreated liposomes which were much more unstable either in PBS or in 90% plasma (Fig. 6). Thus, the plasma treatment of the liposomes was not reversible; the plasma-resistant component of the liposome stayed resistant after the excess plasma was removed.

To examine whether oleic acid in the liposome was transferred to the plasma component(s), liposomes containing trace [^{14}C]oleic acid were pretreated with plasma, then chromatographed on Bio-Gel A-1.5m gel-filtration column to separate liposome (void volume) and the plasma component(s) (included volume). The results presented in Table I clearly show that most (60% or greater) of the liposomal oleic acid had transferred to the plasma component(s). Furthermore, liposomes containing 40% cholesterol showed a greater decrease in oleic acid than those containing no cholesterol.

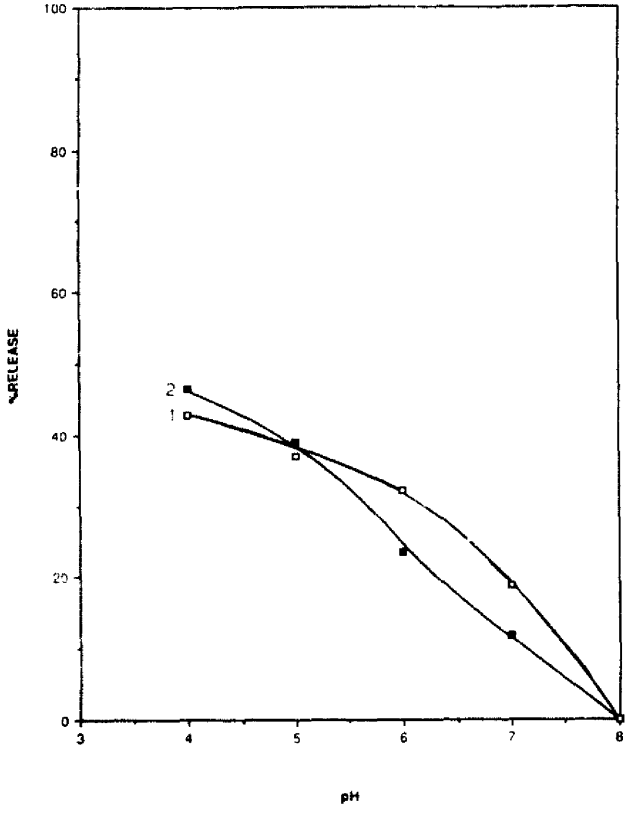

Fig. 5. Acid-sensitivity of plasma-treated liposomes. Percent calcein release of liposome containing 40% cholesterol was measured at different pH. □, plasma untreated (curve 1); ■, plasma-treated (curve 2).

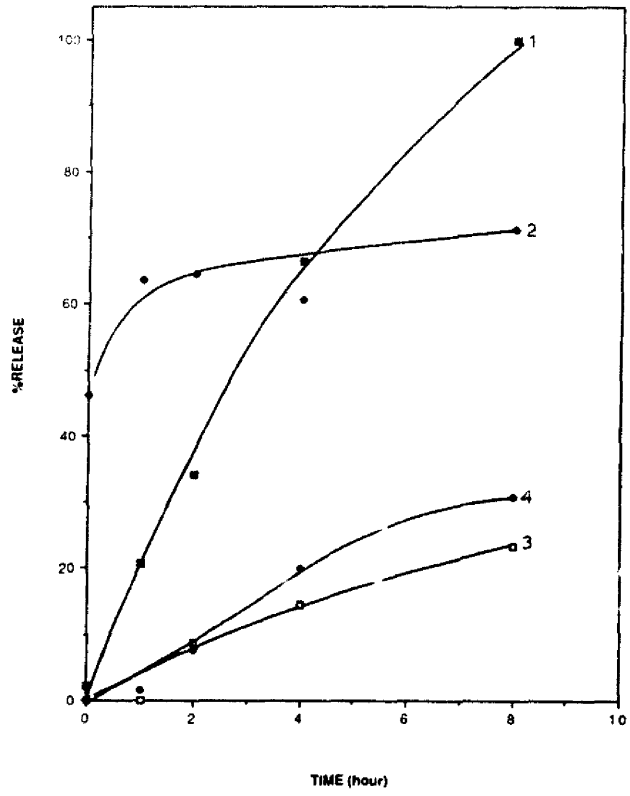

Fig. 6. Comparison of the stability of plasma-treated and untreated liposome containing 40% cholesterol in plasma (90%) and PBS (pH 7.4) at 37 °C. Percent calcein release from liposomes is plotted against the time of incubation. ■, untreated liposomes in PBS; (curve 1) ◆, untreated liposome in plasma (curve 2); □, plasma-treated liposome in PBS (curve 3); ◇, plasma-treated liposome in plasma (curve 4).

Discussion

In the absence of cholesterol, the stability of the pH-sensitive liposomes composed of DOPE and oleic acid prepared by the detergent-dialysis method is very similar to that of the liposomes of the same composition prepared by the reverse-phase evaporation as previously reported [15]. These liposomes, although relatively acid-sensitive, are unstable at 37 °C, especially in the presence of plasma. However, the presence of cholesterol significantly enhances the stability of the liposomes. For example, the first-order rate constant of the calcein release decreased from 1.8% · min^{-1} to 0.2% · min^{-1} when the liposome's cholesterol content increased from 0 to 50%. Such a remarkable effect of cholesterol must be related to the effect of cholesterol on the phase-transition behavior of PE as previously reported by Epand and Bottega [18]. Cholesterol lowers the bilayer-to-hexagonal phase-transition temperature of dielaidoyl

PE up to the mole fraction of about 0.1. At mole fractions above about 0.3, the effect of cholesterol is to stabilize the bilayer phase. In the current study, the cholesterol content ranged from 10 to 50%. Thus, only the bilayer-stabilization effect of cholesterol was seen. Epand and Bottega have attributed the bilayer-stabilization effect of chol to the poor fit of the cholesterol structure (e.g., single hydrocarbon chain) into the inverted hexagonal packing of PE. Furthermore, cholesterol is known to form a weak complex with phospholipids in the bilayer phase via H-bonding with the carbonyl oxygen of the neighboring phospholipids [19]. While the conformation of the phospholipid in the hexagonal phase packing is not known, cholesterol might not be able to form H-bonds with the neighboring PE, thus raising the free-energy level of the hexagonal phase.

pH-sensitive liposomes without cholesterol rapidly released calcein when exposed to the human plasma. This behavior has previously been observed with liposomes prepared by reverse-phase evaporation method with the same lipid composition [15]. Inclusion of cholesterol in the lipid composition dramatically changed the stability of the liposomes in the plasma-containing medium. Apparently, there were two components in the cholesterol-containing liposomes: a component which was rapidly lysed by the action of the plasma and a component which was essentially resistant to plasma (Fig. 4). The proportion of the two components depended on the cholesterol content; liposomes with higher cholesterol content showed a larger plasma-resistant component and vice versa (Fig. 4 inset). This result can be understood by assuming that there are two opposing processes occurring when the liposome encounters plasma: a lytic reaction and a stabilization reaction. The stabilization reaction is apparently missing or the rate of the reaction is negligibly slow when the liposome does not contain cholesterol, because only rapid lysis of the liposome was observed in this case. The rate of stabilization must increase with increasing cholesterol content in the liposomes, since an increasing amount of the stable liposome was found at the steady-state. The lytic reaction is so fast a process that the initial rate of the reaction could not be measured with our method (Fig. 4). This process may be directly or indirectly related to the transfer of oleic acid from liposome to some plasma component(s) (Table I), most likely the albumin, leaving the liposomes with a low level of oleic acid which is insufficient to stabilize the bilayer phase of the liposomes. Insertion into the bilayer of apolipoproteins such as those in the HDL is known to cause liposome lysis (for a review, see Ref. 20). Small unilamellar liposomes with a high radius of curvature interact more extensively with lipoproteins or apolipoproteins than do the large multilamellar liposomes of the same composition. Bilayers at phase-transition temperature are most sensitive to the insertion of the apolipoproteins. Both the extraction of oleic acid and the interaction with the lipoproteins may be operating for the lytic reaction of the liposome. The molecular nature of the proposed stabilization reaction is not clear at the present time. DOPE is a lipid molecule with a weakly hydrated headgroup [21] and with bulky acyl chains, which are the reasons for its high tendency to form the inverted hexagonal phase at the physiological conditions [22]. The bilayer phase of lipid molecules such as DOPE can be stabilized by adding amphiphiles of the opposite molecular characteristics, i.e., molecules with strongly hydrated headgroup and relatively non-bulky acyl chain [22,23]. A number of plasma components, both proteins and lipids, can be the candidates for the stabilizer, e.g., lysophospholipids, gangliosides and apolipoproteins. Identification of the stabilization reaction and the stabilizer will have to await further investigations.

An alternative explanation differing from the above kinetic considerations is also plausible. The liposome population could simply be heterogeneous in the lipid composition. For example, the cholesterol distribution among the liposomes could be uneven, such that the cholesterol-rich liposomes are resistant to lysis by plasma and the cholesterol-poor liposomes are sensitive to lysis [24]. Increasing the average cholesterol content in the liposomes would increase the cholesterol-rich proportion in the liposome population, resulting in more resistant liposomes. Heterogeneity of the liposomes prepared by the detergent-dialysis method has been reported before. Liposomes containing reconstituted HLA antigen [25] and palmitoyl antibody [26] show heterogeneous distribution of the protein among the liposomes. The heterogeneity might have its origin in the uneven distribution of various lipids in the mixed micelles from which the liposomes are derived [27]. Heterogeneous distribution of cholesterol is, however, difficult to demonstrate experimentally because no significant difference in liposome size, charge or density is expected. Although the resistant liposomes can be isolated from the excess plasma proteins after the plasma treatment, they are unsuitable for the lipid composition analysis. This is because the plasma-sensitive liposomes, although losing all the entrapped calcein during the incubation with plasma, co-purify with the plasma-resistant liposomes under the chromatographic conditions used in the present study (data not shown).

While the hypothesis of the liposome heterogeneity remains speculative, it provides a theoretical basis for the observed acid-sensitivity of the cholesterol-containing liposomes. The data in Fig. 2 show that there was a subpopulation in the cholesterol-containing liposomes which was acid-stable. This subpopulation increased with increasing average cholesterol content of the liposome. It has been shown that the inclusion of cholesterol in the pH-sensitive liposomes reduces the sensitivity of

the liposome to acid treatment [4]. The PE bilayer-stabilization activity of cholesterol discussed above may become important when the oleic acid is protonated at acidic pH. Thus, liposomes enriched with cholesterol were still stable even when the oleic acid was protonated. Liposomes poor in cholesterol would rapidly lyse at the same pH because of the lack of the stabilizing chol.

These observations are important in view of our previous work using the detergent-dialysis liposomes composed of DOPE/OA/Chol (4:2:4) to deliver the plasmid DNA to the target lymphoma cells in mouse [13]. According to the data presented here, a portion of the liposomes might be resistant to and/or stabilized with plasma component(s), and may have delivered the DNA to the target cells. The delivery mechanism most likely involved an acid-induced destabilization mechanism because the plasma-treated liposomes are still partially acid-sensitive (Fig. 5). Thus, the pH-sensitive, cholesterol-containing liposomes prepared by the detergent-dialysis method show potential as target-specific, cytoplasmic delivery vehicles for the water-soluble drugs, such as DNA.

Acknowledgements

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