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Stability of antibody-bearing liposomes containing dideoxyinosine triphosphate

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Summary

Liposomes bearing surface-attached antibody were prepared to study the retention of dideoxyinosine triphosphate (ddITP). Liposomes of various lipid composition were prepared and conjugated with modified mouse monoclonal antibodies. The antibody (H-2-K^k) used in this study is for Fc-mediated targeting. Antibody specificity was measured by studying the binding of antibody-liposome conjugates to antimouse IgG-Sepharose. The binding of antibody-liposome conjugates (L-Ab) was maximum when negatively charged liposomes (DMPC:CHOL:DCP) were employed. Inclusion of cholesterol to DMPC liposomes increased the binding by 4%. The binding was least when the neutral phospholipid compositions were employed (DMPC, DPPC and DMPC:CHOL) to prepare liposomes. The retention of ddITP was measured in plain liposomes and antibody-bearing liposomes stored at 4, 25 and 37°C. The leakage was maximal in DMPC liposomes. Only 20% of ddITP was retained in DMPC liposomes stored at 4°C after a month. However, when samples were stored at 25 and 37°C the retention was 12% and 4% respectively. There was no leakage of ddITP at 4 and 25°C in liposomes prepared using DMPC:CHOL (1:1 mole ratio) and DMPC:CHOL:DCP (7:2:1 mole ratio). The retention of ddITP was significantly increased in DMPC and DPPC liposomes after conjugation with antibodies. The retention of ddITP in DMPC:CHOL and DMPC:CHOL:DCP liposomes conjugated with antibodies was comparable to plain liposomes. These results suggest that the lipid composition used in the preparation of liposomes affect the conjugation of antibodies to liposomes and also the retention of an encapsulated drug.

Introduction

Dideoxynucleosides have shown the ability to suppress the replication of human immunodeficiency virus (HIV) by inhibiting the reverse transcriptase of viral RNA into DNA. Among the nucleosides dideoxyinosine (ddI) has demonstrated significant clinical activity as measured by increase in CD4 cell count (Balis et al., 1992). The active form of ddI is dideoxyinosine triphosphate (ddITP) is unstable in vivo if administered in the free form. However, encapsulation of ddITP in liposomes can stabilize the drug from degradation and targeting can be achieved by attaching monoclonal antibodies on the surface. Targeting of ddI therapy is required because it is associated with severe toxicities including peripheral neuropathy, hepatitis and life threatening pancreatitis (Yarchoan et al., 1990). The delivery

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of dideoxycytidine triphosphtae (ddCTP) to human monocyte/macrophages was increased by encapsulating into antibody (H-2-K^k)-bearing liposomes (L-Ab) to human monocyte/macrophages which act as reservoir for HIV (Betageri et al., 1992). The delivery of ddCTP was acheived through Fc-receptor mediated targeting.

Liposome bilayer characteristics can be changed by varying the lipid composition and changing the method of preparation. It has been reported that increasing the length of the phospholipid fatty acyl chains produces a decrease in the permeability of the liposomes to dideoxyinosine triphosphate (Betageri et al., 1992). This is due to a decrease in fluidity of the membrane and increase in ordered structure of the bilayer. A decrease in permeability can also be produced by the addition of ionophores such as valinomycin. The addition of some proteins such as lysozyme, myelin basic proteins and immunoglobulins to a liposome suspension increases the leakage of anions and glucose from the liposomes, while general anesthetics, such as ether and chloroform, produce an increase in cation permeability of the bilayers but no increase in glucose permeability (Bangham et al., 1974). However, there are no reports in the literature examining the liposome structure in terms of size, charge and lipid composition on the stability of liposome antibody conjugation and retention of encapsulated material. The potential use of antibody-liposome conjugates for targeted drug delivery requires closer consideration of liposome structure on retention of encapsulated drug, conjugation with monoclonal antibodies, and stability of antibody-liposome conjugation. The objective of this paper is to study the effect of liposome structure in terms of lipid composition on retention of encapsulated drug and conjugation with monoclonal antibodies.

Materials and Methods

Materials

Dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC) and maleimidophenyl butyrate phosphatidylethanolamine (MPB-PE) were obtained from Avanti polar lipids, Alabaster, AL. Cholesterol (CHOL), dicetyl phosphate (DCP), dithiothreitol (DTT), Protein A-Sepharose and dideoxyinosine triphosphate (ddITP) were purchased from Sigma Chemical Co., St. Louis, MO. [^{14}C]Cholesteryl oleate was obtained from Amersham. Succinimidyl pyridyl dithiopropionate (SPDP) was purchased from Pierce Chemical Co., IL. [^{3}H]ddITP was obtained from Moravek Biochemicals (Brea, CA). All other chemicals and solvents were reagent grade. Glass triple distilled water was used in the preparation of all aqueous solutions.

Methods

Preparation of liposomes

All liposome preparations had a total lipid concentration of 120 mg/ml. The concentration of ddITP was 40 mM + 1 μCi [^{3}H]ddITP.

Lipids were dissolved in chloroform and the chloroform was evaporated using a rotary evaporator to form a lipid film inside the flask. Aqueous phase containing ddITP was added to the flask containing the lipid film and vortex mixed until the lipid was dispersed. This produces multilamellar liposomes (MLVs). MLVs were subjected to freeze-thaw cycle 10 times using liquid nitrogen and warm water. This preparation was processed in Liposor (Lidex Technologies Inc., Bedford, MA) at room temperature with 0.1 μm pore size to produce homogeneous unilamellar liposomes. Encapsulation of ddITP in liposomes was calculated using the following equation

$$\% \text{ encapsulation} = (\text{cpm(liposomes)}) / (\text{cpm(stock solution)}) \times 100$$

Liposome size distribution, as measured by photon correlation spectroscopy (Coulter Electronics, Inc., Haleigh, FL., Model N4MD) was 115 ± 24 nm (SD) in a typical preparation. The inter-experimental mean and coefficient of variation were 107 nm and 5%, respectively ($n = 5$).

Separation of free ddITP

The liposome sample was placed on the Sepharose 4B column and the column eluted with

PBS (pH 7.4). Approx. 0.7 ml fractions were collected and the fractions containing liposomes were pooled for further study. The liposomes were eluted in the void volume.

Isolation of antibody

Mouse monoclonal IgG_{2a} antibody (H-2-K^k) was prepared and purified as described by Heath et al. (1983). Briefly, 2.0 ml of ascites were diluted with 2.0 ml of PBS (pH 7.4) and applied to a protein A column equilibrated with PBS (pH 7.4). The column was incubated at room temperature for 30 min and washed with PBS (pH 7.4) until the peak at 280 nm returned to baseline (Catty and Murphy, 1989). Bound antibody was eluted with 0.1 M sodium citrate/citric acid buffered saline (pH 4.0). Fractions were collected in 0.5 ml of 1 M Tris buffer (pH 9.0). The fractions eluted with sodium citrate/citric acid buffer (pH 4.0) were dialyzed against PBS (pH 7.4). The purified antibody was stored in PBS at −20°C. The antibody reacts only to K^k antigens without any detectable cross-reactions, thus suggesting reaction to a particular specificity of the K^k molecule.

Modification of antibody by heterobifunctional reagent

A 1.6 μl volume of 20 mM succinimidyl pyridyl dithiopropionate (SPDP) was added to 1 ml of PBS containing 1 mg of antibody (absorbance at 280 nm = 1.5 (1 mg/ml)) in a glass tube (Catty and Murphy, 1989). The reaction mixture was allowed to stand for 30 min at room temperature. The solution was applied onto a small Sephadex G-25 column equilibrated with acetate buffer (pH 4.5). Fractions were followed by measuring the absorbance at 280 nm. The sample was reduced by the addition of 50 mM dithiothreitol (DTT). After 20 min at room temperature, the antibody was separated from DTT by a second column passage using a column pre-equilibrated with 10 mM Hepes-buffered saline, pH 8.0.

Conjugation of modified antibody to liposomes

The antibody modified by SPDP was mixed with liposomes containing ddITP. The reaction was allowed to proceed at room temperature for 24 h. The SPDP will couple to any unprotonated amino group on the surface of the antibody. Therefore, the conjugation of antibody molecules to liposomes could be in random orientations. Separation of antibody-liposome conjugates (L-Ab) was performed by metrizamide density gradient (Heath et al., 1981). Briefly, 2.5 ml of 40% w/v solution of metrizamide was placed over the reaction mixture in a centrifuge tube followed by 1.5 ml of 20% metrizamide solution and 0.5 ml of PBS on top. The gradient was centrifuged for 16 h at 143 000 × *g*.

Characterization of antibody-liposome conjugates

Antimouse IgG-Sepharose was packed in a small column and antibody-liposome conjugates were applied to the column and allowed to stand for 30 min at room temperature. The column was eluted with phosphate-buffered saline (pH 7.4) and the extent of binding was determined by measuring the radioactivity of radiolabelled liposome using the following equation.

$$\% \text{ binding} = (\text{cpm(applied)} - \text{cpm(eluted)}) / (\text{cpm(applied)}) \times 100$$

Measurement of ddITP leakage

To study the leakage of ddITP from L-Ab, the L-Ab samples were stored at 4, 25 and 37°C. Every 48 h, 100 μl samples from each temperature were transferred to ultrafilter microcentrifuge filter system and were centrifuged in microcentrifuge (10 000 rpm) for 10 min. Only the free ddITP passes through the filter retaining the liposomes above the filter. The concentration of ddITP in the filtrate was determined by measuring the radioactivity of [^{3}H]ddITP.

Results and Discussion

Encapsulation of ddITP in liposomes of various lipid composition is shown in Table 1. Encapsulation was maximum in liposomes prepared using DMPC:CHOL (1:1 mole ratio). Addition of DCP confers negative charge to liposomes and

TABLE 1

Phase transition temperature (T_c) and encapsulation of ddITP in liposomes of various lipid compositions

Lipid composition	T_c (°C) [a]	% encapsulation (S.D.)
DMPC	23	28.0 (±6.1)
DPPC	41	28.9 (±5.6)
DMPC:CHOL (1:1 mole ratio)	–	64.3 (±7.6)
DMPC:CHOL:DCP (7:2:1 mole ratio)	< 23	18.5 (±3.8)

[a] From Chapman et al. (1967).
DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; CHOL, cholesterol; DCP, dicetyl phosphate.

the encapsulation of ddITP in these liposomes was the least in extent. This could be due to repulsive forces between the drug and liposomes because of the presence of negative charge on both. Increasing the fatty acid chain length did not change the amount of ddITP encapsulation. This is due to association of ddITP (hydrophilic compound) with the aqueous phase entrapped within the unilamellar liposomes. Similar results have been reported for unilamellar liposomes in the literature (Taylor et al., 1990). Also, there was no correlation between encapsulation and phase transition temperature.

Binding capacities of antibody-liposome conjugates were measured using antimouse IgG-sepharose. The results are listed in Table 2. The binding of antibody-liposome conjugates was maximum when negatively charged liposomes (DMPC : CHOL : DCP) were employed. Liposomes containing DCP, which has an unshielded phosphate group and which is often used to provide a negative charge in liposomes, has also shown excellent fusion properties when included in the liposome preparation (Haywood and Boyer, 1984). The increase in fusion may be due to increased binding capacity of liposomes. Inclusion of cholesterol in DMPC liposomes increased the binding only by 4%. This indicates that changes in liposome fluidity (DMPC : CHOL liposomes) do not affect binding significantly. However, cholesterol when present at 30–50 mol% has been shown to stabilize liposomes (Finkelstein and Weissmann, 1979; Kirby et al., 1980; Damen et al., 1981). The extent of binding was least when the neutral phospholipid compositions were employed (DMPC, DPPC and DMPC: CHOL) to prepare liposomes. These liposomes are electrically neutral and do not possess any surface charge. This could be the reason for the lower conjugation with antibodies which also leads to decreased binding of such liposomes to antigens. The effect of different lipid components for covalent attachment of antibodies on liposomal integrity during the conjugation reaction remains to be determined. Furthermore, different procedures for the preparation of large unilamellar liposomes may have an effect on the stability of the resulting liposomes. For the preparation of liposomes used in this study, we followed a procedure which avoids the use of detergents and sonication. Therefore, this method offers a significant advantage over other protocols, since it does not expose the molecules to be entrapped to harsh conditions and allows liposomal entrapment of labile compounds such as proteins (Gaber et al., 1983; Thompson and Gaber, 1985).

TABLE 2

Binding of antibody-bearing liposomes of various lipid composition to antimouse-IgG Sepharose

Lipid composition	% binding (S.D.)
DMPC	57.3 (±6.6)
DPPC	55.9 (±7.1)
DMPC:CHOL (1:1 mole ratio)	61.4 (±5.8)
DMPC:CHOL:DCP (7:2:1 mole ratio)	98.8 (±7.9)

The retention of ddITP was measured in liposomes and antibody-bearing liposomes in various lipid compositions at 4, 25 and 37°C. The retention of ddITP in plain liposomes is shown in Fig. 1. The leakage of ddITP was maximum in liposomes prepared using DMPC. Only 20% of ddITP was retained in DMPC liposomes stored at 4°C after 30 days. However, when samples were stored at 25 and 37°C the retention was 12 and 4%, respectively. Since DMPC has a T_c of 23°C the liposome samples stored at 25 and 37°C were in the fluid state, and thus highly permeable. This

indicates that the leakage of encapsulated drug increases with a rise in temperature above the T_c.

The retention of ddITP was greater in liposomes prepared using DPPC compared to DMPC. This could be due to the fraction of the bilayer in the gel state being larger in DPPC liposomes than DMPC liposomes since it is further from its T_c (41°C). Therefore, the samples stored at all three temperatures were below the T_c. The lipid bilayers exist in gel state below T_c and liquid crystalline or fluid state above T_c. The permeability is lower below T_c. The leakage of ddITP did not increase significantly when samples were stored at 25° compared to those stored at 4°C. The retention of ddITP decreased from 67% at 4°C to 61% at 25°C. However, the retention was 48% when samples were stored at 37°C. This decrease in retention at 37°C may be due to storage of samples closer to the T_c.

The inclusion of cholesterol and/or dicetyl phosphate reduced the leakage of ddITP significantly. There was no leakage of ddITP at 4 and 25°C in liposomes prepared using DMPC:CHOL (1:1 mole ratio) and DMPC:CHOL:DCP (7:2:1 mole ratio). The retention was 93 and 91% at 37°C in liposomes prepared using DMPC:CHOL and DMPC:CHOL:DCP, respectively. Addition of cholesterol reduces the permeability into the bilayer by a factor of 2 for 35 mol% cholesterol (Marsh and Watts, 1981). Diffusion of a variety of solutes through such bilayers is greatly reduced by incorporation of cholesterol (Papahadjopoulos et al., 1973; Inoue, 1974; Blok et al., 1975). It is therefore not surprising that incorporation of cholesterol into the bilayers of DMPC liposomes rendered them less permeable to encapsulated ddITP.

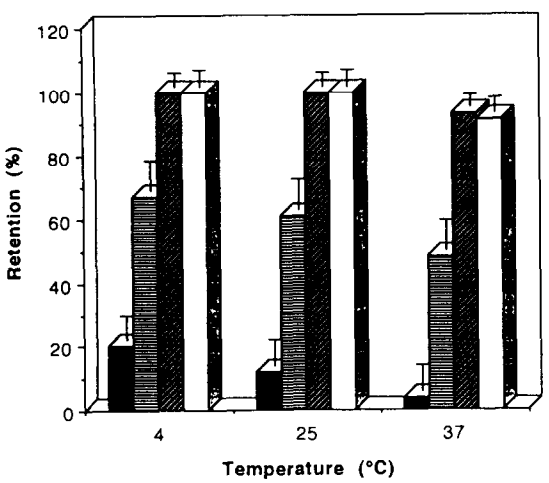

Fig. 1. Retention of ddITP in DMPC (filled bars), DPPC (horizontally hatched bars), DMPC:CHOL (1:1 mole ratio) (diagonally hatched bars) and DMPC:CHOL:DCP (7:2:1 mole ratio) (empty bars) liposomes stored at 4, 25 and 37°C after 30 days.

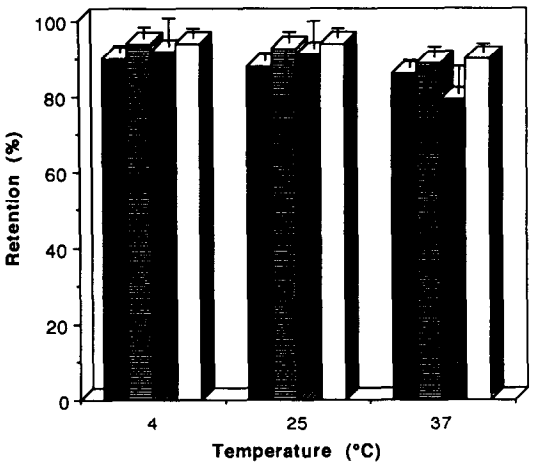

Fig. 2. Retention of ddITP in DMPC (filled bars), DPPC (horizontally hatched bars), DMPC:CHOL (1:1 mole ratio) (diagonally hatched bars) and DMPC:CHOL:DCP (7:2:1 mole ratio) (empty bars) antibody-bearing liposomes stored at 4, 25 and 37°C after 30 days.

The retention of ddITP at 4, 25 and 37°C in antibody-bearing liposomes is shown in Fig. 2. The retention of ddITP was significantly increased in DMPC and DPPC liposomes conjugated with antibodies compared to plain liposomes. The retention of ddITP was 86% in DMPC liposomes and 89% in DPPC liposomes stored at 37°C. The retention was greater at 4 and 25°C. The increased retention of ddITP in antibody-bearing liposomes could be explained as a result of reduced permeability of liposomes by closer binding of head groups due to the presence of surface-attached antibodies. The retention of ddITP in DMPC:CHOL and DMPC:CHOL:DCP liposomes conjugated to antibodies was comparable to that of plain liposomes. The retention of ddITP in DMPC:CHOL liposomes was

79% at 37°C. However, retention was 90% in DMPC:CHOL:DCP liposomes at 37°C. These results indicate that conjugation of antibodies to DMPC and DPPC liposomes increases the retention of an encapsulated drug. There was a slight reduction of retention in DMPC : CHOL liposomes stored at 37°C and no significant effect at 4 and 25°C in DMPC:CHOL and DMPC : CHOL : DCP liposomes.

The results obtained in this study clearly indicate that the lipid composition used in the preparation affects the conjugation of antibodies to liposomes and in turn binding to antimouse-IgG Sepharose. The lipid composition also affects the retention of an encapsulated drug. However, after conjugation with antibodies the lipid composition does not play a significant role in terms of retention of encapsulated drug. The results of this study also suggest that liposomes prepared with cholesterol and dicetyl phosphate are superior to those prepared with neutral lipid composition with respect to binding to antimouse-IgG Sepharose as well as retention of ddITP. The fluidity of the liposomes does not affect binding, however, it does affect retention of ddITP.

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