Biochimica et Biophysica Acta 812 (1985) 116–126
Elsevier

BBA 72336

Preparation and characterization of heat-sensitive immunoliposomes

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(Received May 21st, 1984)

Key words: Immunoliposome; Monoclonal antibody; Heat sensitivity; Liposome targeting

Immunoliposomes able to bind specifically to target cells and to release their encapsulated contents upon brief heating were prepared. Monoclonal anti-H2K^k was covalently derivatized with palmitic acid by the method of Huang, A. et al. (Huang, A., Tsao, Y.S., Kennel, S.J. and Huang, L. (1982) Biochim. Biophys. Acta 716, 140–150). The palmitoyl antibody was injected at a controlled rate into a suspension of fused unilamellar dipalmitoylphosphatidylcholine liposomes maintained at a constant temperature. The final protein-to-lipid ratio of the resultant liposomes with incorporated antibody (immunoliposomes) was dependent upon the rate of antibody injection and the lipid concentration. Injection of palmitoyl antibody into a liposome suspension containing 50 mM carboxyfluorescein at 41°C resulted in simultaneous antibody incorporation and entrapment of dye. Immunoliposomes were able to release the entrapped carboxyfluorescein upon heating. The release of dye at temperatures between the pre- and main-transition temperatures of DPPC was abolished by the addition of calf serum (5%). Furthermore, the presence of serum resulted in an increase in the temperature of the maximal release rate and also in the rate of release at that temperature. Retention of antigen-binding capacity was demonstrated by the ability of the immunoliposomes to bind specifically to the target cells. Rapid release of entrapped carboxyfluorescein from immunoliposomes bound to target cells at 4°C was achieved upon brief exposure (less than 3 min) at 41°C. These heat-sensitive immunoliposomes may be useful in enhancing drug delivery to target cells.

Introduction

Liposomes serve as an attractive drug-delivery system for several reasons. They are nontoxic, biodegradable, capable of encapsulating a wide variety of compounds, and the bilayer can be modified with biological recognition molecules which serve to target the liposomes to specific cells. Liposome targeting has been demonstrated through the use of lectins [2], noncovalently [3–5] and covalently [6,7] attached antibodies, and glycolipids [8,9]. The liposomes used in these systems deliver their entrapped contents via an endocytotic pathway [10,11]. Consequently, for effective delivery the target cells must actively endocytose, the drug must be stable in acidic environments and capable of escape at some point in the endocytotic pathway in an active form. Alternative approaches to increase the efficiency for cell delivery include the induction of endosome-liposome fusion [12–14] and liposome-plasma membrane fusion [15]. We have been exploring an alternative approach in which the liposome-entrapped contents are released at the target cell surface. Controlled release of the liposome contents has been achieved by using heat-sensitive liposomes [16,17]. These liposomes are capable of releasing their entrapped

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; T_m, phase transition temperature; NHSP, *N*-hydroxysuccinimide ester of palmitic acid; HDL, high-density lipoproteins.

content upon heating to a temperature at which liposomal lipid is phase-separated (T_m). These 'heat-sensitive' liposomes have been used to deliver entrapped methotrexate to heated tumors in vivo. The results show a significant enhancement in the uptake of the drug by the heated tumor in comparison to that of the unheated tumor [18]. Due to a lack of target-cell specificity by liposomes used in these studies, the drug was also absorbed by tissues other than the tumor. Consequently, the therapeutic index was increased only slightly over that obtained with free drug. Therefore, we have chosen to engineer 'heat-sensitive' immunoliposomes in order to achieve site-directed release of entrapped drugs at the tumor cell surface thereby improving the delivery efficiency of the encapsulated drug.

Ideally, monoclonal antibody against a tumor specific cell surface antigen is covalently attached to the liposomes with a thermal phase transition temperature (T_m) a few degrees higher than the body temperature. After the immunoliposomes specifically bind to the tumor cell surface, a brief heating of the tumor to the T_m causes the release of entrapped antitumor drugs. The therapeutic efficacy of the drug should be enhanced. To achieve this goal, we have used a monoclonal antibody to the mouse major histocompatibility antigen as a model system and prepared heat-sensitive immunoliposomes. We report here the procedure of the preparation and the characterization of the liposomes.

Materials and Methods

Reagents. Procedures for isolation of monoclonal anti-H2K^k and P3 IgG have been described previously [10]. The antibody was iodinated and acylated using the method of Huang et al. [1] employing an antibody to *N*-hydroxysuccinimide ester of palmitic acid (NHSP) ratio of 1 to 10. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Birmingham, AL) and stored in $CHCl_3$ under N_2 at $-20°C$. Carboxyfluorescein (Kodak, NY) was recrystallized by the method of Ralston et al. [19] with the following modification. Recrystallized carboxyfluorescein was dissolved in 95% EtOH and applied to an LH-20 column (1.5×90 cm) equilibrated and eluted with 50% EtOH. Fractions were analyzed by TLC using $CHCl_3/MeOH/H_2O$ (65 : 24 : 4). Those fractions lacking contaminants with an R_f value greater than 0.6 were pooled and used. The extinction coefficient of the purified carboxyfluorescein was $5.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$. Deoxycholate was purchased from Calbiochem, and recrystallized. Hexadecyl cholesteryl ether was synthesized according to Paltauf [21]. It was then radiolabelled with catalytic hydrogenation using tritium gas (ICN Radiochemicals). A trace amount was used as a nontransferable lipid marker [22]. Lipid purity was assessed by TLC using 100 μg lipid. Inorganic phosphate was determined by the method of Bartlett [26].

Liposome preparation. Fused unilamellar liposomes of DPPC were prepared according to Wong et al. [20] with the following modifications. Small unilamellar liposomes of DPPC were prepared in phosphate-buffered saline containing 1 mM EGTA and 0.02% NaN_3 (pH 7.4). The DPPC liposome suspension was incubated at 4°C for 7–21 days. Stable liposomes were separated from kinetic intermediates by a 30 ml 5–20% continuous sucrose gradient with a 3.5 ml 65% sucrose cushion. The suspension was heated at 43°C for 20 min before loading on to the gradient. The maximum loading capacity was 35 mg in 1 ml of buffer. The preparative sucrose gradients were centrifuged in a SW 27 rotor at 2.5×10^4 rpm for 16 h. The liposomes were collected from the buffer/5% sucrose interface and dialyzed against phosphate-buffered saline at pH 8.0 for 8 h.

Immunoliposome preparation. Palmitoyl antibody was concentrated to 15 mg/ml by ultrafiltration (Amicon) and dialyzed against 2 l 0.15% deoxycholate in phosphate-buffered saline (pH 8.0) for 16 h. A liposome suspension (2.5 mg/ml) was placed in a 4 ml, plastic vial (omnivial, Fisher) and then fitted into a water-jacketed chamber. The temperature was controlled by a circulating water bath (Neslab, NH). Concentrated palmitoyl antibody was injected into the liposome suspension with a 50 μl Hamilton syringe attached to an infusion pump. The syringe needle pierced a water jacket septum and the wall of the plastic vial. The injection rate of palmitoyl antibody into the liposome suspension was routinely 0.2 μl/min and the injection temperature was 41°C. The amount of

palmitoyl antibody injected was such that the lipid-to-deoxycholate molar ratio was greater than 60. For simultaneous antibody incorporation and dye entrapment, the dye was added to the liposome suspension prior to injection of palmitoyl antibody. After injection, the suspension was incubated at 44°C for 20 min followed by a slow cooling (30 min) to room temperature. Dialysis against 4 l phosphate-buffered saline containing 1 mM EGTA and 0.02% NaN_3 (pH 8.0) for 16 h was used to remove residual detergent and untrapped dye. Immunoliposomes were separated from unincorporated palmitoyl antibody by the preparative sucrose gradient procedure described above. Fractions containing palmitoyl antibody, lipid, and dye were pooled and dialyzed against 4 l phosphate-buffered saline (pH 7.4) for 12 h.

Release of carboxyfluorescein by heating. Carboxyfluorescein fluorescence was measured by exciting at 490 nm and the emission was monitored at 520 nm using a Perkin-Elmer LS5 spectrofluorimeter equipped with a water-jacketed cell holder. The rate of temperature increase was 0.3 deg. C/min using a programmable temperature controller (Neslabs, NH). For a rapid increase in temperature, the cuvette holder was equilibrated at 48°C. The cuvette and contents were equilibrated at 4°C. Placement of the cuvette into the cuvette holder resulted in an increase in temperature to 42°C within 3 min. A thermocouple was used to monitor the cuvette temperature directly. The total dye release was obtained by addition of 5% deoxycholate to a final concentration of 0.2% above the T_m for DPPC.

Cell binding. The attached cell lines L-929 and A-31 were grown in McCoy's 5A medium (Flow, MA) containing 10% donor calf serum (Gibco, NY). The RDM4 and P3 cells were grown in RMPI 1640 medium containing 10 mM pyruvate and 10% fetal calf serum. The liposomes were added in a volume of 1 ml to $2 \cdot 10^6$ cells in a 35 mm dish and incubated for 2 h at 4°C, washed three times with phosphate-buffered saline and solubilized with 1 ml 1% Triton X-100. For RDM4 and P3 cells, the sample volume was 0.5 ml and cell number was $2 \cdot 10^6$ cells. Unbound material was separated from the cells by centrifugation through 6% Ficoll (Pharmacia, NJ) in phosphate-buffered saline (pH 7.4) using an Eppendorf microfuge. The cell pellet was solubilized with 0.1 ml 1% Triton X-100 and counted for ^{125}I and ^{3}H.

Release of entrapped carboxyfluorescein from immunoliposomes bound to RDM4 cells. Immunoliposomes were bound to RDM4 cells according to the cell-binding protocol. The cell pellet was resuspended in 100 μl phosphate-buffered saline and divided into two 50-μl aliquots. Each aliquot was added to 2 ml phosphate-buffered saline. One sample remained at room temperature while the other suspension was subjected to a rapid increase in temperature as described above with simultaneous monitoring of carboxyfluorescein fluorescence. Both samples were then layered over 1 ml of 6% Ficoll-phosphate-buffered saline and centrifuged at 3×10^3 rpm in a desk top centrifuge. ^{125}I cpm was counted in a cell pellet.

Negative stain electron microscopy. The liposome and immunoliposome suspensions (1 mg lipid/ml) were applied to 400 mesh Formvar-coated copper grids and allowed to settle for 1 min. Samples were stained with 0.5% phosphotungstic acid. An Hitachi 600 electron microscope was used to visualize the samples.

Results

Incorporation of palmitoyl antibody into liposomes

Palmitoyl anti-$H2K^k$ was injected into a temperature-regulated liposome suspension at a controlled rate of 4 μg palmitoyl antibody per min. The amount of palmitoyl antibody associated with the liposomes was determined by analytical sucrose density gradient centrifugation. The gradient profile for the immunoliposome is shown in panel A of Fig. 1. The incorporated palmitoyl antibody comigrated with the lipid peak, whereas the unincorporated palmitoyl antibody sedimented to the bottom of the gradient. The same result has also been previously observed in assessing palmitoyl antibody incorporation into detergent dialysis liposomes [5]. Although the palmitoyl antibody was bound to the liposomes, the protein-to-lipid ratios across the peak were not constant and those liposomes enriched with palmitoyl antibody sedimented further into the gradient. This result indicated that the palmitoyl antibody was somewhat heterogeneously distributed among the liposomes. The gradient profile for the unincorporated anti-

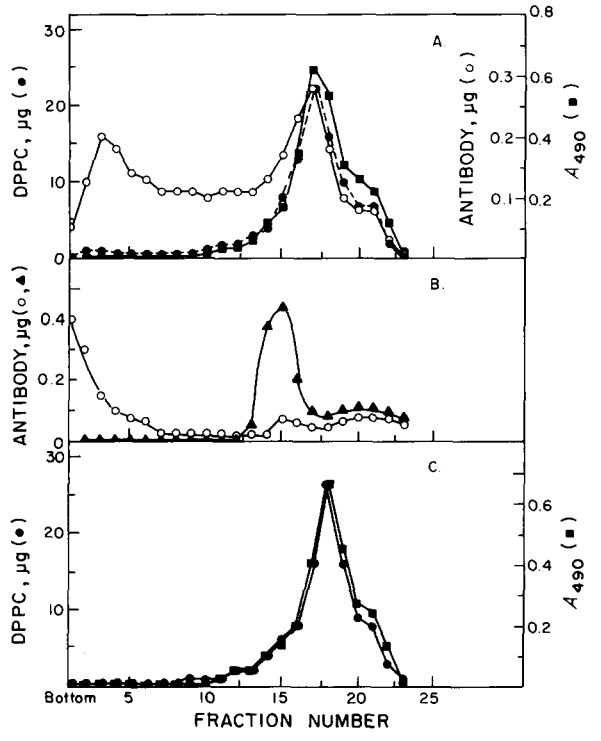

Fig. 1. Analysis of liposome-associated palmitoyl antibody by sucrose density centrifugation. The amount of DPPC (●), palmitoyl antibody (○), native antibody (▲) and the A_{490} (■) were determined. Gradient profiles are shown for immunoliposomes (A), palmitoyl and native anti-H2K^k (B), and bare liposomes (C).

body in panel A was similar to the profile for palmitoyl antibody alone shown in panel B in that the unincorporated antibody sedimented to the bottom of the gradient. However, the difference in sedimentation velocities and the high background between fraction 5–12 in panel A may be attributed to a difference in the protein aggregate size. Incorporation of antibody into liposomes absolutely required the derivatization with NHSP. Injection of native underivatized antibody into a liposome suspension under the identical conditions as palmitoyl antibody resulted in no antibody association with the liposomes.

To show that the vesicular structure of the liposomes was maintained after antibody incorporation, 50 mM carboxyfluorescein was entrapped simultaneously with the incorporation of palmitoyl antibody. Coincident with the lipid and protein peaks was the absorbance of carboxyfluorescein at 490 nm. The carboxyfluorescein fluorescence of the peak fractions was self-quenched 80%. Also, the carboxyfluorescein-to-DPPC ratio for immunoliposomes was very similar to that obtained for liposomes without antibody (bare liposomes) shown in panel C. Determination of the trap volume per mole of phospholipid yielded results characteristic of unilamellar liposomes (data not shown).

Liposome integrity was further established by

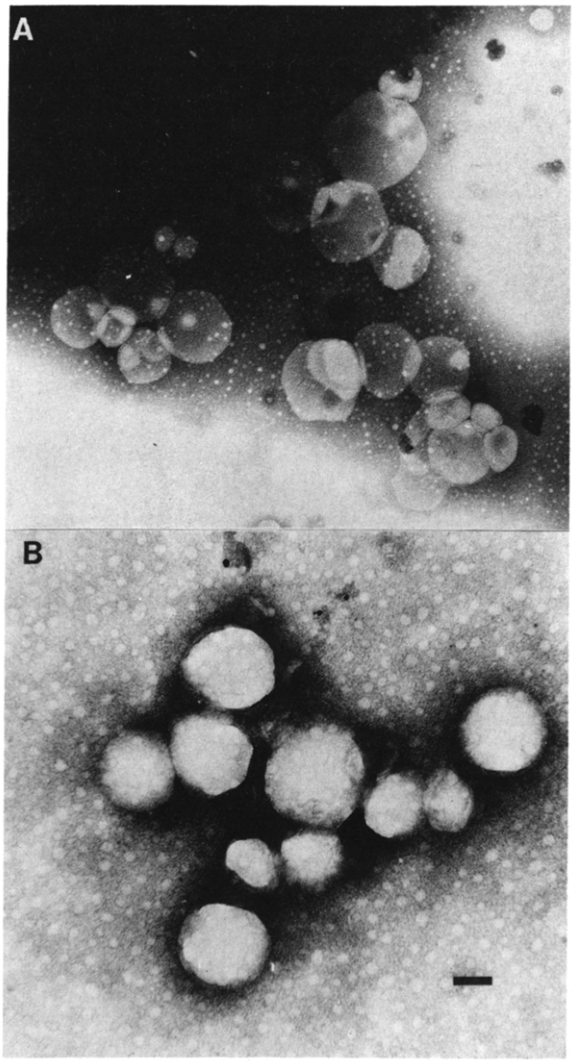

Fig. 2. Negative stain electron micrograph of bare liposomes (A) and immunoliposomes (B) with a protein-to-lipid ratio of $1.33 \cdot 10^{-4}$; bar = 0.1 μm.

electron microscopy. Fig. 2 shows negative stain electron micrographs of the immunoliposomes and bare liposomes. Each preparation displayed some size heterogeneity. The bare liposomes had an average diameter of approx. 180 ± 80 nm whereas the immunoliposomes had an average diameter of 190 ± 70 nm. These data taken together with that of the sucrose density centrifugation show the liposomes to be large and unilamellar.

These results indicate that a substantial amount of palmitoyl antibody can be incorporated into liposomes with simultaneous entrapment of carboxyfluorescein. The entrapment of carboxyfluorescein by immunoliposomes was stable for at least 2 days at 4°C.

Optimization of immunoliposome formation

Several conditions were examined for efficient antibody incorporation. These conditions were liposome concentration, rate of antibody injection, lipid-to-deoxycholate molar ratio, injection temperature and protein-to-lipid molar ratio. A summary of the results are shown in Table I. The condition which exerted the largest effect was the liposome concentration, with the optimal concentration being greater than or equal to 10 mg/ml. The percent of antibody incorporation was uncharged for the palmitoyl antibody injection rates tested. However, rapid addition of palmitoyl antibody by pipette to a liposome suspension resulted in no antibody incorporation regardless of other incubation conditions. The lipid-to-deoxycholate molar ratio had no effect on the percent of incorporated antibody. Consequently, this ratio was kept as large as possible with a minimum of 60 to minimize the potential residual detergent remaining after dialysis. A protein-to-lipid ratio less than or equal to $5 \cdot 10^{-4}$ had no effect on the percent of palmitoyl antibody incorporation. However, further increase in this ratio produced large protein-lipid aggregates.

Release of carboxyfluorescein from liposomes by heating

The effect of palmitoyl antibody incorporation on heat-sensitive release of entrapped content was

TABLE I

PARAMETERS REGULATING PALMITOYL ANTIBODY (pAb) INCORPORATION

Parameter varied	pAb/DPPC initial ($\times 10^5$)	pAb/DPPC final [e] ($\times 10^5$)	% pAb Incorporation
DPPC concentration [a] (mg/ml)			
40.1	8.85	4.74	42
20.2	8.33	4.18	43
10.2	8.77	3.70	42
5.2	7.35	2.61	30
2.7	7.81	1.95	27
Injection rate (μg pAb/min)			
2.7 [b]	13.89	5.88	44
5.4	10.64	5.35	44
10.8	10.64	5.13	41
5.4 [c]	11.24	6.71	52
10.8	12.05	6.58	52
Injection temp. [d] (°C)			
25	12.35	6.62	47
41	14.08	7.63	47
45	14.93	6.85	46

[a] DPPC/deoxycholate ratio was 100, injection rate was 3 μg/min.
[b] DPPC concentration was 20 mg/ml, DPPC/deoxycholate ratio was 200.
[c] DPPC concentration was 20 mg/ml, DPPC/deoxycholate ratio was 100.
[d] DPPC concentration was 20 mg/ml, DPPC/deoxycholate ratio was 100 and injection rate was 3 μg/min.
[e] Final protein-to-lipid ratio obtained after sucrose density gradient centrifugation.

investigated by measuring the release of carboxyfluorescein from the immunoliposomes as a function of temperature. Liposomes with entrapped carboxyfluorescein were heated from 25 to 45°C at a rate of 0.3deg. C/min and the increase in fluorescence was recorded. The heating scans are shown in Fig. 3. The percent of carboxyfluorescein release was calculated from the equation:

$$\% \text{ carboxyfluorescein release} = \frac{F_{\text{total}} - F_T}{F_{\text{total}} - F_0} \times 100$$

The fluorescence for the total dye release, F_{total}, was obtained by adding 5% deoxycholate to a final concentration of 0.2% above the T_m for DPPC; F_0 was the initial fluorescence at 25°C, and F_T was the fluorescence obtained at temperature T. No significant quenching of carboxyfluorescein fluorescence was observed by deoxycholate.

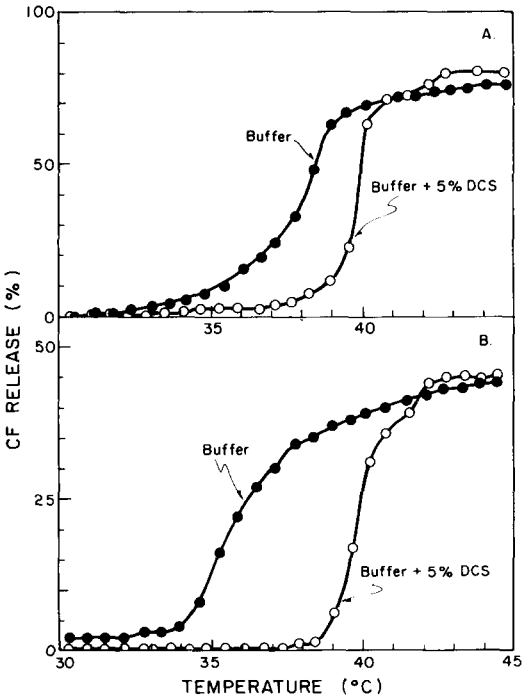

Fig. 3. Release of carboxyfluorescein (CF) by heating from bare liposomes (A) and immunoliposomes (B). Liposomes were suspended in phosphate-buffered saline containing 1.0 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 13 mM glucose (pH 7.4). Rate of heating was 0.3deg. C/min. Carboxyfluorescein release was monitored in the absence (●) and presence (○) of 5% donor calf serum (DCS).

The temperature scan for bare liposomes is shown in Fig. 3, panel A. In the absence of serum, the initial release of dye was observed at 33°C. The maximal rate of release was obtained between 38 and 39°C. In the presence of serum, the initial carboxyfluorescein release was not observed until 35°C and the temperature for the maximal rate of release was increased to 40°C. The presence of the serum components had also increased the rate of release at 40°C. No quenching of carboxyfluorescein fluorescence was observed in the presence of 5% donor calf serum. However, higher concentrations of serum produced a high degree of light scattering, thus obscuring the carboxyfluorescein fluorescence.

The temperature scan for immunoliposomes is shown in panel B. In the absence of serum, the initial dye release was observed at 34°C and the temperature for the maximum rate of release was between 35 and 36°C. In the presence of serum, the initial release was not observed until 39°C and the temperature for the maximum rate of release was increased to 40°C. The serum also increased the rate of release at this temperature.

The trapping protocol for carboxyfluorescein resulted in an 80 mosM hypoosmotic imbalance for the liposomes. Increase in the salt concentration of the external medium to a hyperosmotic condition in the absence of serum increased the maximal release temperature to 40°C and decreased the rate of release at that temperature (data not shown).

From the data shown in Fig. 3, it can be concluded that the interaction of palmitoyl antibody with the liposomes alters the heat-sensitive release of the entrapped carboxyfluorescein. The temperature for the maximal rate of carboxyfluorescein release was lowered, and the amount of total released carboxyfluorescein decreased from 80 to 45%. A time-course of carboxyfluorescein release by the immunoliposomes incubated at 41°C in the presence of serum, showed a 40% release of carboxyfluorescein after 2 min. A slower release rate followed, which produced a further release of 8% after 10 min of additional incubation (data not shown). These results suggest that the initial burst of carboxyfluorescein may have been the result of an osmotic shock. Once the osmotic imbalance was relieved a much slower rate of equilibration

between the internal aqueous compartment and the external medium resulted.

The presence of serum stabilized both bare liposomes and immunoliposomes at temperatures below the maximal release temperature. No leakage was observed prior to the onset of the maximal release temperature range. The serum components also increased the maximal release temperature and the rate of release at this temperature. Upon repeated cooling and heating scans, further release of carboxyfluorescein could be obtained at 40°C. These results indicate no destruction of the vesicular structure as the result of the interaction of serum components with the immunoliposomes.

Binding of immunoliposomes to target cells

Immunoliposomes were incubated with L-929 cells ($H2K^k$) or A-31 cells ($H2K^d$) at 4°C for 2 h and the association of both ^{125}I-labeled antibody and ^3H-labeled lipid were determined. The degree of binding was also determined for ^{125}I-labeled underivatized antibody, bare liposomes, and immunoliposomes in the presence of a 50-fold excess of underivatized antibody. A summary of the results is shown in Table II.

Incubation of cells with immunoliposomes resulted in 4.7% of the antibody added and 2.1% of the lipid added bound to the L-929 cells whereas 0.2% of the antibody added and 0.6% of the lipid added bound to the A-31 cells. Immunoliposomes bound to L-929 cells had a higher protein-to-lipid ratio than that of the original immunoliposomes, indicating that only those liposomes containing sufficient antibody could bind to the target cells. The protein-to-lipid ratio of the immunoliposomes bound to A-31 cells, on the other hand, was significantly lower than that of the original liposomes, indicating that only those lipids containing little or no antibody nonspecifically bound to the control cells. Bare liposomes yielded approximately the same percent bound to the L-929 and A-31 cells as was observed for immunoliposome binding to the A-31 cells. Also, underivatized antibody incubated with the A-31 cells showed the same degree of binding that was observed for the immunoliposomes. These results clearly illustrate the binding specificity for the L-929 cells by the immunoliposomes.

To show the immunoliposome-binding specificity was mediated by antigen-antibody binding, a 50-fold excess of the native underivatized antibody was coincubated with the immunoliposomes. This resulted in an 89% inhibition of antibody binding and a 74% inhibition of lipid binding. Substitution

TABLE II

BINDING OF IMMUNOLIPOSOME TO TARGET CELLS

Cells ((1–2)·10^6 cells/well) were incubated at 4°C for 2 h in growth medium containing immunoliposomes (1 μg Ab/ml, 34 μg DPPC/ml), ^{125}I-labeled anti-$H2K^k$ (1 μg Ab/ml), or bare liposomes (34 μg DPPC/ml). Protein-to-lipid ratio was $1.33 \cdot 10^{-4}$. Ab, antibody; pAb, palmitoyl antibody.

Condition	Cell type	Bound		
		μg Ab	μg DPPC	pAb/DPPC [c]
Immunoliposome	L-929	0.045	0.74	$3.02 \cdot 10^{-4}$
Immunoliposome + anti-$H2K^k$ [a]	L-929	0.005	0.19	$1.29 \cdot 10^{-4}$
Immunoliposome + P3 IgG [b]	L-929	0.046	0.83	$2.71 \cdot 10^{-4}$
Immunoliposome	A-31	0.002	0.20	$4.89 \cdot 10^{-5}$
^{125}I-labeled anti-$H2K^k$	L-929	0.097		
^{125}I-labeled anti-$H2K^k$ + anti-$H2K^k$	L-929	0.008		
^{125}I-labeled anti-$H2K^k$ + P3 IgG	L-929	0.103		
^{125}I-labeled anti-$H2K^k$	A-31	0.002		
Bare liposome	L-929		0.26	
Bare liposome	A-31		0.16	

[a] 50 μg anti-$H2K^k$ added.

[b] 50 μg IgG secreted by P3 cells added.

[c] Molar ratios.

of P3 IgG for anti-$H2^k$ did not inhibit immunoliposome binding to the L-929, ruling out the possibility of F_C receptor-mediated binding. These data clearly illustrate the binding specificity of the immunoliposomes for L-929 was mediated by cell surface antigen-antibody binding. RDM4 lymphoma cells grown in suspension also express $H2K^k$ antigen. Immunoliposomes exhibited the same degree of binding specificity for these cells as was observed for L-929 cells (data not shown).

Release of entrapped carboxyfluorescein from immunoliposomes bound to target cells

Carboxyfluorescein release from immunoliposomes bound to RDM4 cells was brought about by heating the cell suspension from 20 to 45°C within 3 min. The purpose for the rapid heating rate was to minimize endocytosis of the immunoliposomes by the target cells. The effect of this rapid heating rate on carboxyfluorescein release from the immunoliposomes alone was also examined. The results are shown in Fig. 4. The temperature for maximal release for immunoliposomes in the absence of serum was at 39°C. The rapid heating had increased the maximal release temperature by 4°C compared to that obtained for the 0.3deg. C/min heating rate shown in Fig. 3. In the presence of serum, the maximal release temperature was unchanged. This shows the rate of carboxyfluorescein release in the absence of serum was a kinetic product of the temperature-scanning rate whereas in the presence of serum this was not observed. The maximal release temperature for carboxyfluorescein release from immunoliposomes bound to target cells was very similar to that obtained for unbound immunoliposomes in the presence of serum. However, the bound immunoliposomes released their entrapped content to a lesser extent than the free immunoliposomes. This may be the result of the immunoliposome-cell interaction. The same percent quenching of carboxyfluorescein fluorescence was observed for bound and free immunoliposomes, indicating no premature loss of marker as the result of cell binding. Centrifugation through 1 ml 6% Ficoll-phosphate buffered saline for the heated sample and the sample maintained at room temperature showed the same degree of cell-associated antibody. This suggests heating of the immunoliposomes bound to target cells did not induce dissociation of the bound liposomes. These results illustrate that immunoliposomes bound to target cells retain their ability to release entrapped carboxyfluorescein upon heating.

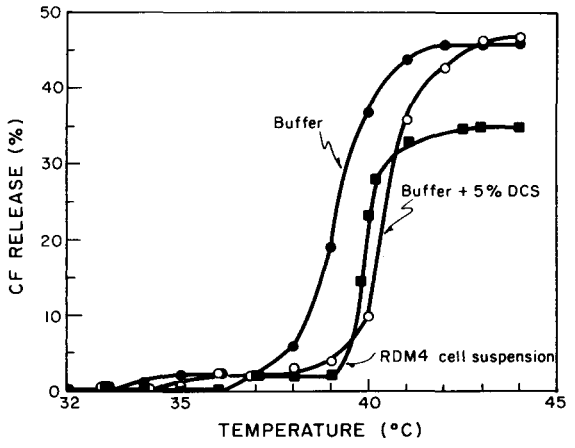

Fig. 4. Release of carboxyfluorescein (CF) by heating from free immunoliposomes (● and ○) and from immunoliposomes bound to RDM4 cells (■). Temperature was increased from 4 to 45°C within 3 min. Carboxyfluorescein release from free immunoliposomes was measured in the absence (●) and presence (○) of 5% donor calf serum (DCS).

Discussion

It has been well documented that near the gel-to-liquid phase transition temperature, the permeability of the bilayer for small, water-soluble, compounds is increased with the highest degree of permeability occurring at the phase transition temperature [16,25]. Thus, the rate of efflux of entrapped material from heat-sensitive liposomes can be controlled by temperature. We have developed a procedure for the preparation of heat-sensitive immunoliposomes. This procedure combines the incorporation of palmitoyl antibody into preformed heat-sensitive liposomes with the entrapment of an internal aqueous marker. Furthermore, the results shown in Figs. 3 and 4 show the ability of the immunoliposomes to release entrapped carboxyfluorescein upon heating. Comparison of the maximal release temperature for bare liposomes and immunoliposomes showed the immunoliposomes to release at a lower temperature. The trapping of carboxyfluorescein produced a hypoosmotic imbalance for the liposomes. Conse-

quently, the palmitoyl antibody-DPPC interaction may have sensitized the bilayer in a localized fashion to the osmotic pressure imbalance which resulted in release at a lower temperature. Alternatively, the palmitoyl antibody may alter the thermotropic behavior of the bulk lipid in such a manner as to broaden the phase transition and/or decrease the T_m. Acylated proteins and many other membrane proteins have been shown to alter the phase behavior of lipid bilayers in this manner [29,30], but at much higher protein-to-lipid molar ratios than that of our immunoliposomes. For this reason, this latter mechanism is less favored. Future investigations designed to examine the effect of palmitoyl antibody upon the thermotropic properties of DPPC will more clearly define the nature of the palmitoyl antibody-phospholipid interaction.

In the absence of serum, we are able to manipulate the release properties of the liposomes to a high degree. An alteration in the osmotic pressure or in the heating rate produced a shift in the maximal release temperature, and also changed the rate of release. However, in the presence of serum, the release properties were less dependent on the experimental conditions. Serum components (e.g., lipoproteins) have been shown to induce the release of liposome-entrapped solutes [35,36], possibly by transferring liposomal lipids to the serum lipoproteins such as the HDL. This process is particularly pronounced with small unilamellar liposomes [23]. The interaction between larger liposomes and HDL is much weaker except at the thermal phase transition temperature [24]. The interaction of HDL with large DPPC liposomes at the T_m dramatically increases the carboxyfluorescein release from the liposomes. Furthermore, other lipoprotein fractions and also, a nonlipoprotein fraction of plasma can promote the increase in carboxyfluorescein release at the T_m for DPPC [31]. The increase in the rate of carboxyfluorescein release from the bare liposomes in the presence of serum was most likely due to a destabilization of the bilayer as a result of a serum protein-phospholipid interaction at the phase transition temperature. In the present study, we found that the heat-release properties of the immunoliposomes in the presence of serum were similar to those of the bare liposomes. These results further suggest that the presence of palmitoyl antibody does not significantly effect the interaction of the serum proteins with the lipid bilayer.

Although serum components modify the release properties of the immunoliposomes, they do not affect the ability of the immunoliposomes to distinguish between target and nontarget cells (Table II). Comparison of the antibody-to-lipid ratios for immunoliposomes bound to L-929 and A-31 cells showed that those bound to L-929 cells were enriched with antibody whereas those bound to A-31 cells contained little antibody. These results indicate that immunoliposomes with a high protein-to-lipid ratio were specifically bound to L-929 cells by antigen-antibody complexes. The immunoliposomes with a low protein-to-lipid ratio were nonspecifically adsorbed to the cell surface. The same degree of nonspecific binding was also observed for bare liposomes bound to either cell type. Strong nonspecific adsorption of small gel-state liposomes to a variety of cell types has been reported, but the degree of binding was much greater than that observed for the bare liposomes in the present study [32–34]. Therefore, the nonspecific adsorption of large liposomes used in this work is not as severe as that of the smaller liposomes. In any case, it did not interfere with the specific binding of the immunoliposomes with target cells which is a necessary requirement for target-specific drug delivery.

Release of entrapped compounds from immunoliposomes bound to target cells will result in a transiently high local concentration of the compound at the target cell surface. For the efficient transport of this compound into the target cell, the transport system must operate at a high rate. Release of carboxyfluorescein from the immunoliposomes bound to the target cells was demonstrated by the results shown in Fig. 4. However, it is not likely that any significant amount of the released carboxyfluorescein is taken up by the target cells due to its slow transport rate. There are drugs which are transported at a much higher rate than carboxyfluorescein. We expect in these cases an increase in cellular uptake and in the therapeutic index for the immunoliposome-encapsulated drugs released by heating. Alternatively, drugs which bind to cell-surface receptors may also be suitable for the heat-sensitive immunoliposomes. In this

case, the drugs need not enter the cells to express a biological effect. Furthermore, the rate-limiting step for the biological response of this class of drug is the diffusion of the drug to its cell-surface receptor [27,28]. A local high concentration of the drug released from the immunoliposomes may bypass this rate-limiting step, thereby increasing the efficiency of delivery to the target cells.

Successful application of the heat-sensitive immunoliposomes as an antitumor drug carrier in vivo will depend upon the ability of the liposome to reach its target. Intravenous administration requires the immunoliposome to exit the circulatory system into the extravascular space of the target organ. However, bare liposomes administered intravenously have been shown to be excluded from the extravascular spaces [37,38]. Furthermore, accumulation of these liposomes in the liver, spleen and bone marrow results from endocytosis by resident macrophages [39]. From the available information, it is reasonable to assume the heat-sensitive immunoliposomes will behave in a similar manner. For these reasons, we envision a local administration of these immunoliposomes to be more effective than a systemic administration. This type of administration will also facilitate the localized heating of the target tissue. Other potential applications would be the treatment of target cells with the heat-sensitive immunoliposomes outside the body, such as the treatment of acute leukemia by autologus transplantation after immunotherapy in vitro.

Acknowledgements

We thank Drs. M. Wong and E. Freire for their helpful discussions concerning the properties of the fused liposomes. We also thank Dr. S. Kennel for providing the P3 IgG. A special thanks to Ms. Linda Pilcher for the preparation of the manuscript. This work was supported by NIH Grant CA 24553. L. Huang is a Research Career Development Awardee (CA 00718).

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