ENHANCING SPECIFICITY AND STABILITY OF TARGETED LIPOSOMES BY COINCORPORATION OF SIALOGLYCOPROTEIN AND ANTIBODY ON LIPOSOMES

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Liposomes may act as *in vivo* transport vehicles for radionuclides or pharmaceutical agents. The linkage of specific antibodies to the surface of the liposome allows for site-specific targeting. Surface antibodies, however promote the sequestration of liposomes in the reticuloendothelial system, significantly reducing the effectiveness of selective localization. In this study, the binding of the sialoglycoprotein, fetuin, to the liposome surface by hydrophobic interaction significantly diminished hepatic concentration of IgG-substituted liposomes in mice. Macrophages also showed reduced phagocytosis of fetuin-coated liposomes *in vitro* in the presence of soluble fetuin.

INTRODUCTION

The potential application of liposomes as *in vivo* transport vehicles for pharmaceuticals has been recognized for a number of years [1-4]. More recently, targeting of liposomes has been accomplished by incorporation of either nonspecific immunoglobulins [5] or of specific antibodies [6-8]. We have achieved the specific targeting of liposomes for infarcted myocardium by glutaraldehyde coupling of immunospecifically purified cardiac myosin-specific antibodies to the liposome surface [8]. These tagged liposomes were shown to localize in experimental myocardial infarcts by gamma scintigraphic visualization of ^{111}InCl contained within the liposomes. While the liposomes significantly concentrated in the region of the myocardial infarct and not within normal cardiac tissue, the larger part of the radioactivity was localized in the liver. Similar hepatic sequestration has also been observed with other targeted liposome preparations [1, 6, 9]. Presumed phagocytosis by the liver's Kupfer cells has been a major impediment to the *in vivo* application of liposomes as vehicles for drug delivery. Attempts to overcome this problem have included pretreatment with empty liposomes [10] and/or carbon particles to saturate phagocytic cells [10, 11]. The implementation of this approach would be undesirable in a clinical application, and effectiveness has been variable.

Torchilin and co-workers have shown that phagocytosis of liposomes by macrophages *in vitro* may be reduced when excess protein is present in the culture medium [12]. It has also been shown previously that cells or organelles that possess surface sialoglycoproteins escape sequestration by the reticuloendothelial system, whereas asialation promoted uptake [13]. These observations led us to consider the use of a sialoglycoprotein coat on liposomes in order to inhibit phagocytosis.

We report studies that demonstrate targeting of liposomes that have reduced hepatic sequestration by virtue of surface binding of the sialoglycoprotein fetuin. Binding of fetuin to the liposome was effected either by covalent binding through glutaraldehyde [8, 14] or by noncovalent linkage utilizing fetuin that had previously been modified with a hydrophobic molecule [15, 16]. The resultant liposomes that had both fetuin and normal rabbit IgG substituted for antimyosin antibody on their surface showed reduced macrophage uptake *in vitro* as well as reduced liver sequestration *in vivo*, while in *in vitro* experiments with liposomes bearing both fetuin and myosin-specific antibody, the ability of the liposomes to bind myosin was retained.

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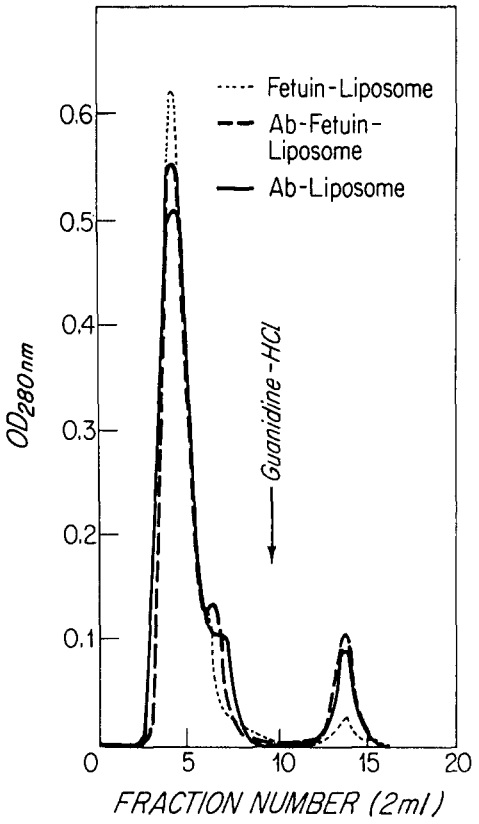

Fig. 1. Myosin-Sepharose affinity chromatographs of equiconcentrations of antimyosin-liposomes, antimyosin-fetuin-liposomes, and fetuin-liposomes. The column was developed with 0.3 M PBS, and bound liposomes were desorbed with 5 M guanidine-HCl. Elution profiles were determined by optical-density tracings at 280 nm employing a UV monitor-recorder unit (LKB).

MATERIALS AND METHODS

Purification of Anticanine Cardiac Myosin

Rabbit anticanine cardiac myosin antibody (Ab) was prepared by affinity chromatography of immune sera on a myosin-Sepharose column [17], which was prepared by cyanogen bromide coupling [18]. Nonimmune rabbit IgG was obtained from Miles Laboratories (Kanakee, Illinois).

Preparation of Liposomes

Liposomes were prepared according to a previously reported sonication method utilizing lecithin, cholesterol, and phosphatidylethanolamine at molar ratios of 6:2:2, respectively [8]. Cholesteryl-[1-^{14}C]oleate was used in trace amounts as nonexchangeable membrane radioactive marker [19]. Proteins such as antimyosin, normal rabbit IgG, and/or fetuin (Sigma Co.) were coupled to liposomes utilizing glutaraldehyde [8]. One mg of protein or sialoglycoprotein was added to 25 mg of glutaraldehyde-activated liposomes. The mixture was incubated overnight at 4°C and the unbound protein removed from liposomes by Sepharose 4B chromatography [8].

Preparation of Coupled Liposomes by Cholate Dialysis Method [20, 21]. To incorporate the desired protein into the liposome membrane, it first had to be rendered hydrophobic [15, 16]. To 5 ml of 2% cholate in 0.145 M NaCl, pH 7.5, 100 λ of 10% palmityl chloride in acetone were added and the mixture sonicated for 5 sec. The desired protein was then added to give a final concentration of 5×10^{-5} M. The suspension was maintained at 4°C, and the pH at about 8 by continual addition of 0.1 N NaOH. A precipitate that formed was removed by centrifugation at 1500 × g. The modified protein was in the supernatant.

TABLE 1. Macrophage Uptake of Lipsome Preparations

Type of liposome preparation	Macrophage uptake (d.p.m./culture)			
	without fetuin in medium		with fetuin in medium (2 mg/ml)	
	d.p.m.	%*	d.p.m	%*
Lip	1579 ± 123	100	1170 ± 39	74
Lip-IgG(g)	2118 ± 90	134	1620 ± 114	103
Lip-Fet(g)	1808 ± 55	115	1451 ± 100	92
Lip-Fet-IgG(g)	1640 ± 75	104	1536 ± 86	97
Lip-Fet(c)	1453 ± 72	92	851 ± 13	54

*Macrophage uptake of empty liposomes is considered as 100% for reference purposes.

To prepare liposomes, lecithin, cholesterol, phosphatidylethanolamine, and trace amounts of cholesteryl-[1-^{14}C]oleate (25 mg total) were suspended in 5 ml of 2% cholate solution. The modified protein was then added to a final concentration of 2.5×10^{-5} M. Small liposomes were then prepared by gel filtration on Sephadex G-75 (1 × 50 cm). Large liposomes were prepared by saline dialysis.

Liposome Binding to Myosin

Antigen binding of antibody-substituted liposomes was determined by their capacity to bind to myosin-Sepharose. A 2.5-ml column of myosin-Sepharose was equilibrated with 0.3 M phosphate and 0.15 M NaCl, pH 7.0. The liposome preparation was passed over this column, which was then washed with the developing buffer. Bound liposomes and antibody were desorbed using 5 M guanidine HCl. Antibody concentration eluted from the column was determined by adsorption at 280 nm and liposomes by ^{14}C counts as determined in a liquid scintillation counter (Mark III 6R80 Liquid Scintillation System).

In Vitro Macrophage Uptake Studies

Macrophage cultures were prepared from peritoneal exudate of (CBA × C_{57}Bl) F_1 male mice by the method previously described [12]. Aliquots of macrophages (7×10^6) obtained in cell suspension were incubated at 37°C in 2 ml of medium 199 for 24 h. Experiments were performed in sets of five dishes each. Fifteen minutes before the experiment, medium was changed and fresh medium with and without fetuin (2 mg/ml) was added. To each set of culture plates, the following were added: 1) empty liposomes (Lip), 2) liposomes substituted with nonimmune IgG (NIgG-Lip), 3) liposomes substituted with fetuin (Fet-Lip), 4) liposomes substituted both with nonimmune IgG and fetuin (NIgG-Fet-Lip), and 5) liposomes to which palmitate-substituted fetuin had been added (Cd). Two sets comprising all five varieties of liposomes were tested, one containing 2 mg/ml fetuin in the culture medium and the other without added fetuin. The culture dishes were incubated for 60 min at 37°C. Following incubation, the culture medium was removed and dishes washed 3 times with Hank's solution. Macrophages were harvested from the culture plates with a soft brush in three 1-ml portions of Hank's solution, then centrifuged at 0°C at 240 × g for 15 min as described previously [12]. Prior to liquid scintillation counting, the cells were mixed with NCS tissue solubilizer (Amersham) and broken up with a glass ball. After shaking at 50°C for 40 min, the cell suspension was transferred to scintillation vials containing 10 ml of a toluene-base scintillation fluid. Counting was performed in a Mark III 6R80 Liquid Scintillation System Counter.

In Vivo Biodistribution

A liposome suspension containing 200,000 cpm of cholesteryl-[1-^{14}C]oleate was injected intravenously into groups of five 10- to 20-g female CBWA mice. The following sets were

TABLE 2. Biodistribution of Modified Liposomes in CBWA Mice (mean = SD)

Sample	Type of liposome preparation (activity as % of injected dose/organ)				
	Lip	Lip-Fet(c)*	Lip-IgG(g)†	Lip-IgG-Fet(g)	Lip-Fet(g)
Blood	41.8 ± 5.2	54.7 ± 11.0	6.5 ± 1.2	9.1 ± 1.9	10.6 ± 3.9
Liver	25.1 ± 3.6	25.2 ± 4.5	42.7 ± 12.6	30.6 ± 4.7	34.2 ± 5.6
Spleen	3.6 ± 0.7	2.3 ± 0.7	2.2 ± 0.6	2.1 ± 0.4	2.1 ± 0.5
Lung	2.3 ± 0.4	2.3 ± 1.2	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.2
Heart	1.0 ± 0.3	2.1 ± 0.9	1.7 ± 0.4	1.6 ± 0.4	1.6 ± 0.2
Kidneys	1.1 ± 0.2	1.0 ± 0.4	1.0 ± 0.2	1.2 ± 0.4	1.0 ± 0.2

*Coupling by cholate dialysis method.
†Coupling by glutaraldehyde method.

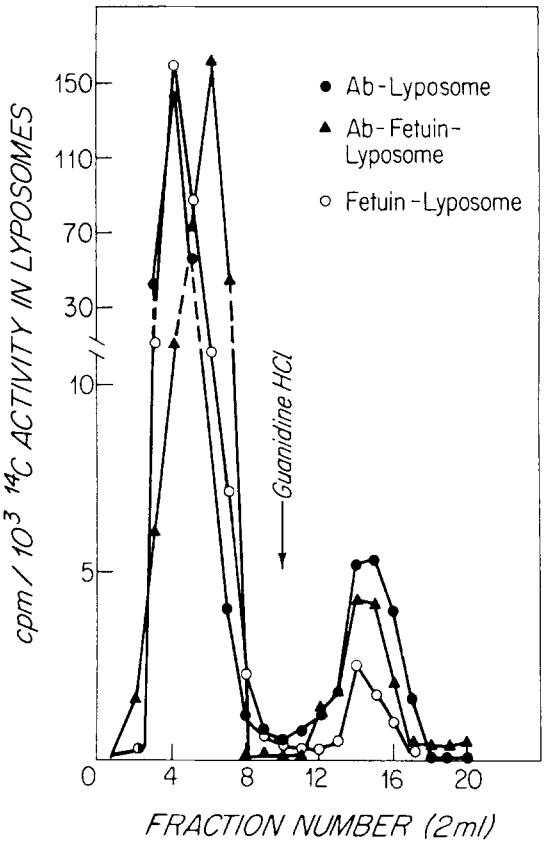

Fig. 2. Myosin-Sepharose affinity chromatographs of equiconcentrations of Ab-Lip, Ab-Fet-Lip, and Fet-Lip of Fig. 1, but the elution profiles were determined by ^{14}C activity. Bound activity was eluted by 5 M guanidine-HCl gradient.

examined: 1) Lip, 2) Cd, 3) NIgG-Lip, 4) NIgG-Fet-Lip, and 5) Fet-Lip. Fifteen minutes after injection, the animals were exanguinated and blood, liver, spleen, lung, heart, and kidneys were collected, weighed, and then treated with 2 ml of NCS tissue solubilizer for 24 h. The solubilized samples were then added to 6 ml of toluene scintillation fluid and counted for biodistribution studies. The total blood volume of each mouse was assumed to be 2 ml. Biodistribution of the isotope was computed for each organ and for the entire blood volume as a percent of the injected dose.

RESULTS AND DISCUSSION

Figure 1 shows a plot of absorbance at 280 nm of the eluate from the affinity chromatography columns, and Fig. 2 the ^{14}C counts of each fraction. The first peak represents the material that did not bind to the column. The liposomes were applied in considerable excess

of the antigen's binding capacity. The second peak represents the bound fraction as reflected by the material that was eluted with guanidine-HCl. It is apparent that both Ab-Lip and Ab-Fet-Lip bound significantly to the myosin-Sepharose, though the fraction of Ab-Fet-Lip that bound was somewhat diminished. Fet-Lip bound far less, though there appears to be a disparity between absorbance and ^{14}C counts. This might be explained by the nonspecific adsorption of ^{14}C-liposomal lipids not associated with Fet-Lip to the affinity adsorbent resulting in a falsely elevated radioactivity whereas absorbance at 280 nm reflects the concentration of the sialoglycoprotein associated with Fet-Lip following elution in 5 M guanidine HCl. It is of interest that the presence of fetuin on the surface of the liposome did not affect antibody binding to the immobilized antigen significantly.

The presence of fetuin in the medium clearly modifies liposome uptake by macrophages, as shown in Table 1. Liposomes subjected to different surface treatments are not significantlydifferent from naked liposomes in their uptake by macrophages in the absence of fetuin in the medium. However, when fetuin is present in the medium, additional reduction in uptake is effected by hydrophobic bonding of additional fetuin to the liposome surface. The covalent bonding of fetuin with glutaraldehyde appears to be ineffective in achieving this result. It is of some note that in the absence of fetuin, surface immunoglobulin on liposomes appears to enhance phagocytosis. These observations are in agreement with prior results [12].

Table 2 shows the results of the *in vivo* experiment. At 15 min following intravenous injection, 41.8% of naked liposomes remained in blood, whereas only 6.5% of liposomes coupled to IgG were found in this compartment. This is consonant with our observation *in vitro* detailed above and with the observations of others [6-8]. Similarly, liposomes covalently substituted both with fetuin alone or IgG and fetuin are rapidly removed from the vascular compartment. It is of great interest, however, that liposomes to which fetuin had been bound hydrophobically are more slowly cleared from blood (54.7% remaining in blood as opposed to 9.1% and 10.6% for covalent IgG fetuin and fetuin substitutions). Both naked liposomes and fetuin-substituted liposomes by the hydrophobic method showed minimal liver uptake, appropriately reflecting the observations made in the vascular compartment and confirming observations made *in vitro*. The reason for the difference between the two forms of fetuin-substituted liposomes is not apparent, but could be related to reaction of important sites on the protein's surface by glutaraldehyde.

This study has not yet demonstrated a method for diminishing hepatic uptake of IgG-substituted liposomes, though it has demonstrated the effectiveness of the sialoglycoprotein fetuin both in solution *in vitro* and noncovalently bound to the liposome surface *in vivo* in inhibiting phagocytosis. One might speculate that the negative surface charges imparted upon liposomes by fetuin might increase their resemblance to normal cells with respect to phagocyte recognition.

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