COATING LIPOSOMES WITH PROTEIN DECREASES THEIR CAPTURE BY MACROPHAGES

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Received 2 January 1980

1. Introduction

In recent years liposomes have gained recognition as promising carriers for drug transport in vivo [1–3]. Unfortunately, the relatively fast clearance of liposomes from circulation practically independently of their phospholipid composition, charge, and size, which results from liposome capture by the cells of the reticuloendothelial system, primarily in the liver [2–4], still limits their everyday clinical use.

One can assume that the first step in liposome capture is the 'recognition' of liposomes and their consequential binding to the appropriate receptors or binding sites on the membrane of an endocytotic cell. Several attempts have been made to decrease the capture of 'therapeutic' liposomes by the preliminary saturation of their hypothetic binding sites with 'empty' liposomes [5,6].

On the other hand, we think one can expect the circulation time to increase for liposomes coated with natural macromolecules that are present in blood in high concentrations and certainly saturate the appropriate receptors of 'absorptive cells'; such 'disguised' liposomes would 'deceive' their own binding sites.

As the process of liposome capture by macrophages can to a certain extent be considered as a model of liposome removal from the circulation by the reticuloendothelial system, we have studied the interaction of mouse peritoneal macrophages with liposomes coated with a protein immobilized on their surface in the presence or in the absence of the same protein in the incubation mixture.

2. Materials and methods

2.1. *Materials*

Egg-yolk lecithin, cholesterol, phosphatidyl ethanolamine and α-chymotrypsin were purchased from Sigma; bovine serum albumin was a product of Merck; bovine serum γ-globulin (Cohn Fr. II) was a product of Serva; sodium caseinate and iodacetamide were obtained from Serva and Fluka, respectively. Medium 199 and Hank's solution were products of the Institute of Poliomyelitis and Virus Encephalitis (USSR). ^{131}I-labeled albumin was obtained from the 'Medradiopreparat' plant (USSR); cholesteryl-[1-^{14}C]oleate was a product of the Radiochemical Centre Amersham. NCS tissue solubilizer was purchased from Amersham/Searle. All the other reagents were analytical grade preparations. Corning tissue culture dishes (35 × 10) were used for macrophage incubation in vitro.

2.2. *Preparation of liposomes*

Liposomes were obtained by sonication by the method in [7] from a mixture of lecithin, cholesterol and phosphatidyl ethanolamine in 6:2:2 molar ratio, respectively, with the addition of trace amounts of cholesteryl-[1-^{14}C]oleate which is a non-exchangeable membrane marker [8]. Large liposomes were separated from small liposomes by Sepharose-4B chromatography. According to electron microscopy data [7], the small liposomes were vesicles with av. diam. <400 Å, while the large liposomes had av. diam. >1000 Å.

For protein coupling liposomes were activated by the action of glutaraldehyde, and the activated liposome suspension was added to a protein solution (albumin, γ-globulin, or α-chymotrypsin first inactivated by heating at 60°C for 40 min). The mixture was incubated overnight at 4°C. The excess protein was removed by Sepharose-4B chromatography (detailed in [7]). The quantity of immobilized albumin was determined in separate experiments observing the

^{131}I-radioactivity associated with liposomes when trace amounts of ^{131}I-labeled albumin were added to the incubation mixture of activated liposomes and albumin. According to the data obtained the av. no. protein molecules/1000 lipid molecules was ~0.2. (In case of large liposomes this means that 20–30 protein molecules were bound to the surface of a single liposome [9].)

2.3. *Incubation of macrophages with liposomes*

Three days before cell isolation $(CBA \times C_{57}Bl)F_1$ male mice were stimulated by intraperitoneal injection of 2 ml a 2% solution of sodium caseinate in water. Peritoneal macrophages were isolated as in [10]. The cells were used immediately after precipitation at 4°C and 240 × *g* and resuspended in medium 199. The content of macrophages in suspension was 75–80% according to the data on phagocytosis of latex particles and *Candida albicans* [11]. Cells (7×10^6) as an aliquot of cell suspension, were placed in a dish containing 2 ml medium 199 and incubated at 37°C for 60 min in an atmosphere of humid air and 5% vol. CO_2. When necessary, to the monolayer formed was added 0.1 ml 2.5×10^{-4} M iodacetamide (an endocytosis inhibitor [12]) in 0.145 M NaCl and the mixture incubated for an additional 40 min in the same conditions. Then the medium was changed, fresh medium in some cases contained certain concentrations of albumin or γ-globulin. (When the monolayer in the dish had already been treated with iodacetamide, the fresh medium contained the same concentration of the inhibitor.) The dishes were incubated for 15 min, the liposome suspension added, and incubation continued for an additional 60 min.

Preliminary experiments showed that the increase of the incubation time did not change the capture of liposomes at a given cell-liposome ratio and the membrane marker used (cholesteryl-[1-^{14}C]oleate) did not exchange between liposome membrane and cell membrane during the time of the experiment (A.A.B., V. S. Goldmacher, unpublished). After the incubation the medium was removed from the dishes, and the dishes were washed 3 times with Hank's solution. Cells were separated from the dishes with a soft brush in three 1 ml portions of Hank's solution and then centrifuged at 0°C and 240 × *g* for 15 min in siliconized tubes. To the precipitate was added 1 ml NCS and a glass ball; the tubes were shaken at 50°C for 40 min and their content was transferred into scintillation vials each containing 10 ml of toluene scintillator. Radioactivity was measured on a Mark III 6880 Liquid Scintillator System.

A set of 5 dishes was used for each experiment.

3. Results and discussion

Inasmuch as protein coupling to liposomes can change their surface properties thus affecting the process of macrophage–liposome interaction, we have preliminarily studied the effect of coating liposomes with proteins on this interaction. The data presented in table 1 show that coating liposomes with albumin or inactivated α-chymotrypsin does not affect the capture of liposomes by macrophages. Coating liposomes with γ-globulin caused a moderate increase in the capture, probably because of a more specific interaction between cells and γ-globulin, as observed in [13].

It also follows from table 1, that introduction of albumin or γ-globulin into the incubation medium does not affect the interaction of 'pure', non-coated liposomes with macrophages. One can assume that in these conditions liposomes are bound to their 'binding sites' and the proteins are bound to their own receptors on the cell membrane.

What happens if an excess of free protein (albumin or γ-globulin) and liposomes coated with the same protein are simultaneously present in the incubation mixture? Performing this experiment we separately studied the properties of small and large liposomes. Large particles (liposomes, particularly) are captured by macrophages by endocytosis and interaction of particles with the binding sites is the key step in this process [14]. At the same time small particles interact with cells by micropinocytosis, which does not require preliminary binding of such particles to binding sites or receptors. In other words, the preliminary saturation of binding sites may affect the capture of large liposomes and should not affect the capture of small liposomes. The experiment has confirmed our assumption. The data presented in table 2 show a noticeable decrease (almost 20%) in the capture of albumin-coated large liposomes (in comparison with protein free medium) if the medium contains 2 mg albumin/ml. The same or even a more pronounced phenomenon (a 25% decrease in the capture) is observed for γ-globulin-coated large liposomes in an excess of free γ-globulin.

For the system albumin-coated liposomes (free

Table 1
Interaction of liposomes with macrophages

Liposome preparation	Incubation medium	Capture of liposomes by macrophages (according to cell-associated radioactivity) (mean ± SEM) (dpm)	(%)
'Pure', non-modified liposomes	Protein-free	2905 ± 44	100.0 ± 1.5*
Albumin coated liposomes	Protein-free	2883 ± 52	99.2 ± 1.8
γ-Globulin-coated liposomes	Protein-free	3396 ± 104	117.0 ± 3.1
α-Chymotrypsin-coated liposomes	Protein-free	2964 ± 59	102.0 ± 2.0
'Pure' non-modified liposomes	Medium contains 2 mg albumin/ml (~3 × 10^{-5} M)	2956 ± 91	101.8 ± 3.1
'Pure', non-modified liposomes	Medium contains 5 mg γ-globulin/ml (~3 × 10^{-5} M)	2912 ± 63	100.2 ± 2.2

[a] Capture of non-modified liposomes was taken as 100%

Table 2
Capture of protein coated liposomes by macrophages

Liposome preparation		Incubation medium	Capture (%) (mean ± SEM)	p
Albumin-coated liposomes	Small	Protein-free	100.0 ± 2.7[a]	n.s.
		2 mg albumin/ml (~3 × 10^{-5} M)	107.0 ± 3.8	
	Large	Protein-free	100.0 ± 3.1[a]	$p < 0.01$
		2 mg albumin/ml (~3 × 10^{-5} M)	82.9 ± 3.0	
γ-Globulin-coated large liposomes		Protein-free	100.0 ± 3.9[a]	$p < 0.01$
		5 mg γ-globulin/ml (~3 × 10^{-5} M)	74.6 ± 4.1	
α-Chymotrypsin-coated large liposomes		Protein-free	100.0 ± 3.2[a]	n.s.
		2 mg albumin/ml (~3 × 10^{-5} M)	110.9 ± 4.3	

[a] In all the cases the capture of liposomes in protein-free medium was taken as 100%

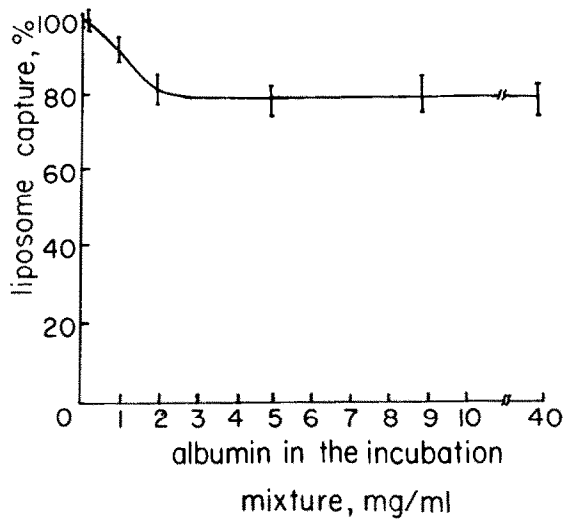

Fig.1. The dependence of capture of albumin-coated liposomes by macrophages on the free albumin concentration in the incubation mixture (for details see section 2).

albumin) macrophages we have studied the dependence of capture decrease on the free protein concentration and have found this effect to be concentration-dependent (see fig.1). It is evident that the effect is most expressed at a more or less physiological concentration of albumin.

Now one can suppose, that the free protein really blocks up the potential binding sites for liposomes coated with the same protein. Thus one can expect a new increase in liposome capture after their coating with some other protein noticeably differing in its properties from the free protein in the medium, because here the capture should be preceded by liposome binding on some other free receptors or binding sites. As follows from table 2, coating liposomes with denatured α-chymotrypsin instead of albumin again increases the capture of liposomes.

From general considerations it is evident that the capture of liposomes by macrophages consists of several more or less independent processes: non-specific adsorption of liposomes on the surface of the cell membrane, fusion of liposomes with membrane and endocytosis. In order to show that it is the endocytotic component that is blocked in our experiments, we have studied the process of capture of protein-coated large liposomes by macrophages in the presence of iodacetamide (an inhibitor of glycolysis [12]). It was shown (see table 3) that in case of albumin-coated liposomes the value characterizing inhibition of endocytosis in the protein free medium is almost 3 times higher than for the medium containing 2 mg albumin/ml. In case of γ-globulin-coated liposomes the difference in the endocytosis inhibition in γ-globulin-free and in γ-globulin-containing medium is even greater. At the same time the total capture in the presence of iodacetamide is similar both for protein-containing and protein-free media. This points to the decisive role of endocytosis as the mechanism of capture which is regulated by the state of receptors.

The real effect of coating liposomes with proteins may be much higher for liposomes (or cells) whose non-specific mutual adsorption or fusion will be lower than for the system under investigation.

Table 3
The effect of iodacetamide on the capture of protein coated large liposomes by macrophages

Liposome preparation	Incubation medium	$\frac{Capture_0 - Capture_{inhib}}{Capture_0}$ [a] × 100%
Albumin-coated liposomes	Protein-free medium contains	29.6
	2 mg albumin/ml (~3 × 10^{-5} M)	11.8
γ-globulin-coated liposomes	Protein-free medium contains	25.4
	5 mg γ-globulin/ml (~3 × 10^{-5} M)	No significant difference between $capture_0$ and $capture_{inhib}$

[a] $Capture_0$ = the capture of liposomes in the absence of iodacetamide
$Capture_{inhib}$ = the capture of liposomes in the presence of iodacetamide

For experimental details see section 2

References

[1] Papahadjopoulos, D. ed (1978) Ann. NY Acad Sci 308.
[2] Finkelstein, M. and Weissmann, G. (1978) J. Lipid Res. 19, 289–303.
[3] Gregoriadis, G. (1978) Nature 275, 695.
[4] De Barsy, T., Devos, P. and Van Hoof, F. (1976) Lab. Invest. 34, 273–282.
[5] Gregoriadis, G. and Neerunjun, D. E. (1974) Eur. J. Biochem. 47, 179–185.
[6] Caride, V. T., Taylor, W., Cramer, T. A. and Gottschalk, A. (1976) J. Nucl. Med. 17, 1067–1072.
[7] Torchilin, V. P., Goldmacher, V. S. and Smirnov, V. N. (1978) Biochem. Biophys. Res. Commun. 85, 983–990.
[8] Kamp, H. H., Wirtz, K. W. A. and Van Deenen, L. L. M. (1973) Biochim. Biophys. Acta 318, 313–325.
[9] Enoch, H. G. and Strittmatter, P. (1979) Proc. Natl. Acad. Sci. USA 76, 145–149.
[10] Boyum, A. (1976) in: Lymphocytes (Natvig, T. B. et al. eds) pp. 9–15, University Park Press, MD.
[11] Patterson-Delafield, T. and Lehrer, R. T. (1977) J. Immunol. Methods 18, 377–379.
[12] Webb, T. L. (1966) Enzyme and Metabolic Inhibitors, vol. 3, Academic Press, London, New York.
[13] Weissman, G., Bloomgarden, D., Kaplan, R., Cohen, C., Hoffstein, S., Collins, T., Gottlieb, A. and Nagle, D. (1975) Proc. Natl. Acad. Sci. USA 72, 88–92.
[14] Carr, T. (1973) The macrophage: A review of ultrastructure and function, Academic Press, London, New York.