Report

Preparation of Asialofetuin-Labeled Liposomes with Encapsulated Human Interferon-γ and Their Uptake by Isolated Rat Hepatocytes

Hiroshi Ishihara,[1] Toshifumi Hara,[1] Yukihiko Aramaki,[1] Seishi Tsuchiya,[1,3] and Kaoru Hosoi[2]

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The selective delivery of human recombinant interferon (IFN)-γ to isolated rat hepatocytes was studied with asialofetuin (AF)-labeled liposomes. AF-liposomes containing buffer solution were initially prepared by the detergent removal method, and IFN-γ was subsequently encapsulated by the freeze-thawing method without loss of activity. Virtually no free [^{32}P]IFN-γ was internalized into isolated rat hepatocytes, whereas AF-liposomes containing [^{32}P]IFN-γ were taken up to a significant degree. Liposomal binding to the hepatocytes (estimated at 4°C) was one-fifth of the uptake (estimated at 37°C). Since the uptake was inhibited by the addition of free AF, AF-liposomes may be taken up by the action of galactose-binding protein on the hepatocytic cell surface. The liposome preparation method reported in this paper provides a useful means for the encapsulation of unstable macromolecules into AF-liposomes. AF-liposomes were found effectively to carry IFN-γ into hepatocytes *in vitro*.

KEY WORDS: liposome; targeting; asialofetuin; hepatocytes; interferon-gamma.

INTRODUCTION

As drug carriers, liposomes may improve the biological fate and action of drugs. Highly effective targeting of liposomes may be achieved through ligands coupled to the liposomal surface (1).

Asialofetuin (AF), a glycoprotein having triantennary galactose terminated sugar chains, is selectively recognized by the galactose-binding protein on the plasma membrane of mammalian hepatocytes (2,3). We have already reported the selective distribution and association of AF-liposomes to rat hepatocytes *in vivo* (4,5) and *in vitro* (6).

Interferon (IFN)-γ, a lymphokine produced by T lymphocytes in response to mitogenic and antigenic stimuli (7), may prove of value for its therapeutic effect on viral hepatitis (8) and IFN-γ-sensitive carcinomas (9,10). Systemic or local administration of IFN-γ has been used (11), but the dosage form characteristics necessary to enhance its therapeutic effects and to reduce adverse side effects remain to be clarified. The selective delivery of IFN-γ to the liver using hepatocyte-targeting liposomes may enhance its effectiveness against some hepatic diseases. However, IFN-γ is an unstable protein and prone to be inactivated by physicochemical treatment. It was therefore deemed important to develop techniques to encapsulate IFN-γ into AF-liposomes without loss of its activity.

In this study, unstable IFN-γ was successfully encapsulated into AF-liposomes by the combination of the detergent removal and the freeze-thawing methods. The interactions of AF-liposomes containing IFN-γ with isolated rat hepatocytes were investigated.

MATERIALS AND METHODS

Materials

Recombinant human IFN-γ (Meiji Seika Kaisha Ltd., Japan) with antiviral activity ranging from 1×10^7 to 2×10^7 U/mg as determined with FL cells and Sindbis virus (12) was dissolved (1 mg protein/ml) in 50 m*M* phosphate-buffered saline (pH 5.5) containing 2 m*M* dithiothreitol. Phosphatidylcholine from egg yoke (PC), cholesterol (Chol), and collagenase were purchased from Wako Pure Chemicals (Japan). L-α-Phosphatidic acid (PA), stearylamine (SA), trypsin inhibitor from soybean, and cAMP-dependent protein kinase catalytic subunits from bovine heart were from Sigma (St. Louis, MO). [γ-^{32}P]ATP (>6000 mCi/mmol) and Aquasol-II were from New England Nuclear (Boston, MA). AF was prepared from fetuin (type III, Sigma) according to the method of Spiro (13). *N*-Palmitoylhydroxysuccinimide (NPSI) was synthesized by the method of Lapidot *et al.* (14). All other chemicals were commercial reagent-grade products.

[1] Tokyo College of Pharmacy, 1432-I Horinouchi, Hachioji, Tokyo 192-03, Japan.
[2] Meiji Seika Kaisya Ltd., 760 Morooka-cho, Yokohama, Kanagawa 222, Japan.
[3] To whom correspondence should be addressed.

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Preparation of [^{32}P]IFN-γ

The radiolabeling of IFN-γ with ^{32}P was performed by the method of Kung and Bekesi (15). IFN-γ (1 mg) was phosphorylated (37°C, 1 hr) by [γ-^{32}P]ATP (0.1 mCi) and cAMP-dependent protein kinase catalytic subunits (150 units), then purified on a CM-Sephadex C-50 (Pharmacia, Sweden) column with a stepwise gradient of 30–300 m*M* phosphate buffer, and dialyzed against 10 m*M* Tris–HCl-buffered saline (TBS; pH 7.4). The radioactivity was precipitated more than 95% by trichloroacetic acid solution (final concentration, 10%). The specific radioactivity of [^{32}P]IFN-γ was 30–60 μCi/mg of protein.

Encapsulation of IFN-γ into AF-Liposomes

AF (10 mg) was dissolved in 2 ml of TBS containing 2% sodium desoxycholate (DOC) and 50 μg/ml NPSI. The mixture was then incubated at 37°C for 12 hr to prepare palmitoyl-AF. A $CHCl_3$:EtOH (1:1) solution of lipids (PC:PA:Chol = 6:1:6, PC:Chol = 1:1, or PC:SA:Chol = 6:1:6, total lipids = 110 μmol) was evaporated at reduced pressure under N_2 gas into a thin lipid film. This film was suspended in TBS (4 ml) by vortexing for 3 min, and after adding a palmitoyl-AF/DOC mixture, vortexing was continued for another 5 min. DOC was dialyzed out against TBS (4°C, 48 hr) to obtain AF-liposomes with encapsulated TBS. This AF-liposome suspension was concentrated with polyethylene glycol (MW 20,000) to 1 ml. The concentrated AF-liposomes were mixed with IFN-γ (2×10^6 U) solution, frozen at −40°C for 24 hr, and thawed at room temperature. Control liposomes (N-liposomes) were prepared without palmitoyl-AF. The AF-liposomes were applied to a Sephadex G-100 column (1.2 × 30 cm) equilibrated with TBS. To each fraction (0.5 ml) collected in polypropylene tubes, Triton X-100 (final concentration, 0.1%) was added and the RIA activity of IFN-γ and phospholipid (PL) concentration were determined.

The AF- and N-liposomes were uniformalized by continuous extrusion under N_2 gas using Extruder (Lipex Biomembranes Inc., Canada) with polycarbonate membranes (pore size, 0.4, 0.2, and 0.1 μm; Nuclepore, USA) and by dialysis using the same membrane (0.1 μm) at 4°C for 3 days. The diameter of liposomes was measured by a NICOMP Model 370 submicron particle sizer (Pacific Scientific Co., Ltd., USA).

The amount of AF on the outer surface of the AF-liposomes was determined by hemoagglutination inhibition assay as described in a previous report (4). Capture volume was estimated by measuring 5,6-carboxyfluoresceine as an aqueous phase marker.

Interactions of AF-Liposomes with Rat Hepatocytes

Isolated rat hepatocytes were prepared by the collagenase perfusion method described previously (6). Hepatocytes (2×10^6 cells/ml), suspended in 30 ml of Kreb's Henseleit balanced buffer (KHBB) containing trypsin inhibitor (50 mg/l), were incubated with free [^{32}P]IFN-γ (5×10^5 cpm) or AF- or N-liposomes with encapsulated [^{32}P]IFN-γ (150 nmol of total lipids, 5×10^5 cpm) in the absence and then in the presence of free AF (1 mg) at 37°C under an O_2:CO_2 (95:5) atmosphere. In the binding experiment, liposomes were incubated in KHBB adjusted to pH 7.4 with HCl at 4°C under the air. The cells were kept in suspension by gently swirling by hand every 10 to 15 min. At various time intervals, triplicate samples (1 ml) were withdrawn and washed twice with ice-cooled KHBB (5 ml). After centrifugation (80*xg*, 2 min), the cell pellets were solubilized by 1% Triton X-100 (0.5 ml) at 60°C for 1 hr and measured for ^{32}P radioactivity.

Analytical Methods

Unlabeled IFN-γ activity was determined using a standardized solid-phase radioimmunoassay (RIA) kit (IMRX INTERFERON-GAMMA RIA, Centcore, USA) having specificity for biologically active IFN-γ (16). Briefly, the samples (200 μl) were incubated (2 hr, room temperature) with mouse anti-IFN-γ monoclonal antibody immobilized on polystyrene beads. The beads were then washed and incubated with ^{125}I-labeled monoclonal antibody, and the bound radiolabeled antibody was measured with a gamma counter Aloka JDC751 (Aloka, Japan). RIA activity was expressed as units based on the NIH reference unit. ^{32}P radioactivity was measured by a liquid scintillation counter Aloka 903 (Aloka, Japan). PL concentration was measured as inorganic phosphorus by the method of Chen *et al.* (17). Protein concentration was determined by the method of Lowry *et al.* (18).

RESULTS

Characteristics of [^{32}P]IFN-γ

The characteristics of [^{32}P]IFN-γ are compared with IFN-γ and listed in Table I. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions indicated the mobility of ^{32}P radioactivity to be indistinguishable from that of unlabeled IFN-γ corresponding to 17,500 daltons. The isoelectric point of [^{32}P]IFN-γ was somewhat elevated. No significant loss in the RIA activity of IFN-γ could be observed following radiophosphorylation.

Effects of Physical and Chemical Treatment on the RIA Activity of IFN-γ

Mechanical and chemical treatments, requisite for the preparation of AF-liposomes by the detergent removal method, both affected the RIA activity of IFN-γ (Table II).

Table I. Characteristics of IFN-γ and [^{32}P]IFN-γ

	IFN-γ	[^{32}P]IFN-γ
Electrophoretic mobility[a]	0.56	0.57
Isoelectric point[b]	8.1	8.7
Activity (U/mg protein)[c]	1.02×10^7	0.94×10^7
Radioactivity (μCi/mg protein)		30–60

[a] SDS-PAGE using 12.5% gel was carried out at 4°C for 6 hr (6 mA/gel).

[b] pI was determined by isoelectrofocusing.

[c] Activities of IFN-γ and [^{32}P]IFN-γ were determined by RIA and the means of two experiments.

Table II. Stability of IFN-γ with Physical and Chemical Treatment

Treatment	RIA activity of IFN-γ (U/mg protein)[a]	
	Before	After
Vortexing (5 min)[b]	1.86×10^7	6.14×10^6
Dialysis (4°C, 24 hr)[c]		
Without DOC	1.94×10^7	1.90×10^7
With 2% DOC	1.93×10^7	1.34×10^5
Freeze-thawing[d]	1.73×10^7	1.39×10^7

[a] The mean of two experiments.

[b] BSA was added (0.5%) to IFN-γ solution (200 μg/ml) to prevent the adsorptive loss of IFN-γ on the wall of the round-bottom glass flask.

[c] IFN-γ (200 μg/4 ml) in visking tube was dialysis against TBS.

[d] IFN-γ (200 μg/ml) in polypropylene tube was frozen at −40°C for 24 hr and thawed at room temperature.

A remarkable loss in RIA activity was observed after vortexing. IFN-γ was dissolved in TBS or TBS containing 0.2% DOC, then dialyzed against TBS. A striking decrease in RIA activity occurred by dialysis with DOC. These results show IFN-γ to be highly susceptible to vortexing and dialysis with DOC. After freeze-thawing in TBS, IFN-γ retained about 80% of its initial RIA activity.

Preparation of AF-Liposomes with Encapsulated IFN-γ

IFN-γ was encapsulated into AF-liposomes by a combination of the detergent removal and freeze-thawing methods. Under the neutral conditions of a Sephadex G-100 column, negatively charged AF-liposomes (PC:PA:Chol = 6:1:6, Fig. 1A) gave only one peak of RIA activity in fractions 6–9. Neutral (PC:Chol = 1:1, Fig 1B) and positively charged (PC:SA:Chol = 6:1:6, Fig. 1C) AF-liposomes each gave two peaks of RIA activity. The first peak overlapped that of PL and was made up of encapsulated IFN-γ in AF-liposomes. RIA activity of the second peak (fractions 11–16) coincided with that of free IFN-γ (Fig. 1D). Recovery of IFN-γ activity from the column was about 20% of that applied for all AF-liposomes.

Negatively charged AF-liposomes were gel filtered under alkaline conditions (Fig. 2A). Since IFN-γ lost its RIA activity at alkaline pH (unpublished data), the radioactivity of [^{32}P]IFN-γ was traced. Two peaks of ^{32}P radioactivity appeared, one (fractions 6–9) coincided with that of PL, and the other (fractions 13–20) with free [^{32}P]IFN-γ (Fig. 2A). The chromatogram for negatively charged AF-liposomes dialyzed for 3 days with polycarbonate membranes gave only one radioactivity peak (Fig. 2B). Recovery of ^{32}P radioactivity from the column was about 90% of that applied for negatively charged AF-liposomes.

Characteristics of AF-Liposomes

The characteristics of AF-liposomes with encapsulated IFN-γ are summarized in Table III. Diameters, amounts of AF on the liposomal surface, and capture volumes of AF-liposomes varying in charge were essentially the same. Encapsulation efficiency, however, increased in the order positive < neutral < negative.

Interactions of AF-Liposomes Containing [^{32}P]IFN-γ with Hepatocytes

Figure 3 shows the time course of negatively charged AF-liposomal interactions with isolated rat hepatocytes. At

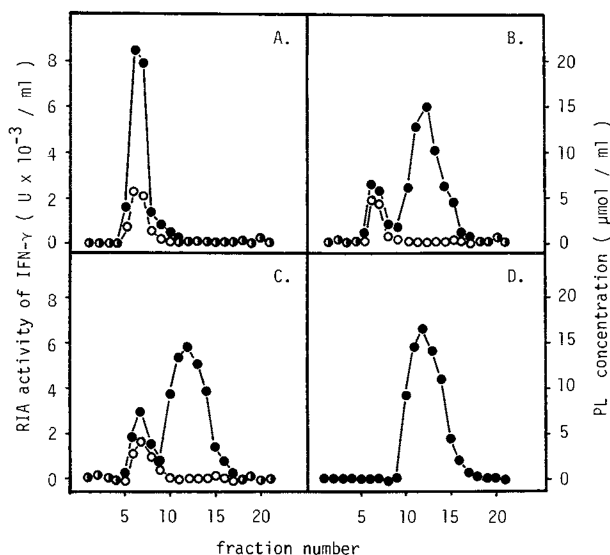

Fig. 1. Elution profiles of variously charged AF-liposomes and IFN-γ under neutral conditions. An aliquot of 0.5 ml AF-liposomes was loaded to a Sephadex G-100 column (1.2 × 30 cm) equilibrated with 10 m*M* Tris–HCl—buffered saline (pH 7.4). (A) Negative (PC:PA:Chol = 6:1:6); (B) neutral (PC:Chol = 1:1); (C) positive (PC:SA:Chol = 6:1:6); (D) free IFN-γ. Each fraction was assayed for IFN-γ activity (●) and PL concentration (○).

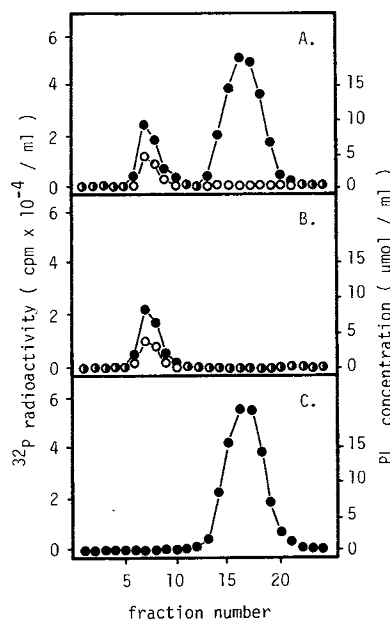

Fig. 2. Elution profiles of negatively charged AF-liposomes (PC:PA:Chol = 6:1:6) and [^{32}P]IFN-γ under alkaline conditions. A Sephadex G-100 column (1.2 × 30 cm) was equilibrated with 10 m*M* Na_2CO_3 containing 0.9% NaCl (pH 11.0). (A) Before dialysis; (B) after dialysis with a polycarbonate membrane (0.1 μm) against TBS at 4°C for 3 days; (C) free [^{32}P]IFN-γ. Each fraction was assayed for ^{32}P radioactivity (●) and PL concentration (○).

Table III. Characteristics of AF-Liposomes with Encapsulated IFN-γ

	Lipid composition (molar ratio)		
	Negative (PC:PA:Chol = 6:1:6)	Neutral (PC:Chol = 1:1)	Positive (PC:SA:Chol = 6:1:6)
Diameter (nm, mean ± SD)[a]	117.3 ± 32.4	134.7 ± 59.3	142.7 ± 39.1
AF on liposomal surface (g/mol of lipid)[b]	84–169	72–144	79–158
Capture volume (L/mol of lipid)[c]	0.99 ± 0.34	1.24 ± 0.61	1.64 ± 0.52
Encapsulation efficiency of [^{32}P]IFN-γ (%)[d]	23.3 ± 7.9	13.7 ± 4.1	8.6 ± 5.2

[a] Measured by NICOMP Model 370 submicron particle sizer.
[b] Estimated by hemoagglutination inhibition assay.
[c] Determined by 5,6-carboxyfluorescein as an aqueous phase marker.
[d] Encapsulation efficiency (%) = 100 × EA/TA, where EA was ^{32}P radioactivity of liposome suspension and TA was total ^{32}P radioactivity used for preparation.

4°C, 3.8% of the total [^{32}P]IFN-γ encapsulated in AF-liposomes became bound to the hepatocytes within 90 min; this value decreased to 1.4% on adding free AF (Fig. 3B). At 37°C, ^{32}P accumulation (90 min) in the hepatocytes from AF-liposomes was about 20% of the total, and that from N-liposomes was about 7% (Fig. 3A). This accelerated uptake was inhibited to the level of N-liposomes on adding free AF, but N-liposome uptake underwent no significant change. Virtually no free [^{32}P]IFN-γ was taken up by the hepatocytes. The accumulation of ^{32}P in hepatocytes at 37°C varied significantly according to liposomal membrane charge (Fig. 4).

DISCUSSION

To target IFN-γ to hepatocytes using AF-liposomes, IFN-γ must be encapsulated without loss of biological activity. Since drug encapsulation and AF-labeling are possible by a simple one-step procedure, the detergent removal method is ideal for preparing AF-liposomes. IFN-γ, however, was found to be an unstable macromolecule toward vortexing and DOC treatment in the detergent removal method. However, the freeze-thawing method has been successfully used to encapsulate macromolecules, such as L-asparaginase (19) and the glucose transporter (20), without loss of activities.

In this study, detergent removal and freeze-thawing were used in combination, the former for AF-labeling and the latter for IFN-γ encapsulation. Since IFN-γ would not ordinarily encounter harsh treatment, the use of these methods together was considered an effective means by which AF-liposomes could encapsulate IFN-γ without loss of RIA activity. The amount of AF on the surface of AF-liposomes prepared in this manner was equivalent to that prepared by the detergent removal method (6). It is thus evident that AF on the liposomal surface is stable during freeze-thawing.

Neutral or positively charged AF-liposomes entrapped IFN-γ by freeze-thawing, and non-encapsulated IFN-γ was separated by gel filtration under neutral conditions (Figs. 1B and C). But in the elution profile of negatively charged AF-liposomes with encapsulated IFN-γ (Fig. 1A), all IFN-γ

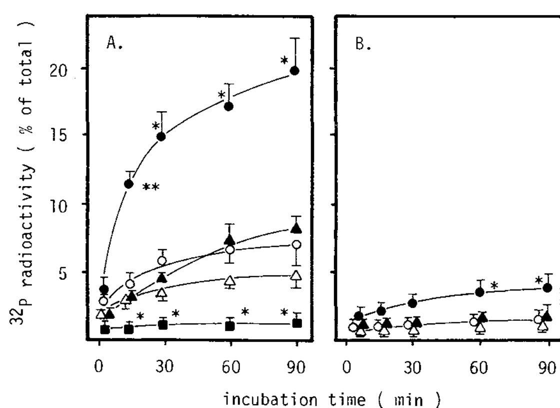

Fig. 3. Interaction between negatively charged AF-liposomes (PC:PA:Chol = 6:1:6) with encapsulated [^{32}P]IFN-γ and isolated rat hepatocytes. (●, ○) AF-liposomes with encapsulated [^{32}P]IFN-γ; (▲, △) N-liposomes with encapsulated [^{32}P]IFN-γ; (■) free [^{32}P]IFN-γ. Each sample (5 × 10^5 cpm) was incubated with isolated rat hepatocytes in the absence (filled symbol) and then in the presence (open) of free AF (1 mg) at 37°C (A) or 4°C (B). Each point presents the mean ± SD (n = 3). Significant differences (Student's t test) from N-liposomes (▲): (*) $P < 0.01$; (**) $P < 0.001$.

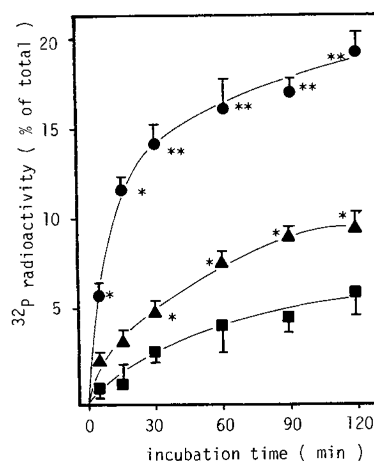

Fig. 4. Effects of charge of AF-liposomes with encapsulated [^{32}P]IFN-γ on uptake by isolated rat hepatocytes. (●) Negative (PC:PA:Chol = 6:1:6); (▲) neutral (PC:Chol = 1:1); (■) positive (PC:SA:Chol = 6:1:6). Each point presents the mean ± SD (n = 3). Significant differences (Student's t test) from PC/SA/Chol (■): (*) $P < 0.01$; (**) $P < 0.001$.

eluted in the fractions of void volume (coinciding with that of PL). When negatively charged AF-liposomes eluted under alkaline conditions, non-encapsulated [^{32}P]IFN-γ was separated (Fig. 2A). Since positively charged IFN-γ binds to negatively charged PL under neutral conditions but not under alkaline conditions (to be published elsewhere), it would appear that nonencapsulated IFN-γ should be able to bind to the outer surfaces of negatively charged AF-liposomes under neutral conditions. To assess the ability of AF-liposomes as carriers of IFN-γ, nonencapsulated IFN-γ must be removed. Exhaustive dialysis under neutral conditions was effective for removing this surface-bound IFN-γ (Fig. 2B) and this fact demonstrates the validity of that presumption. The greater encapsulation efficiency of [^{32}P]IFN-γ in negatively charged AF-liposomes (Table III) also indicates the possibility of ionic interactions between IFN-γ and the inner membrane of AF-liposomes.

The species-specific receptor for IFN-γ is present to lymphokine-sensitive cells (21,22), and the binding of IFN-γ to this receptor is generally accepted as a requirement for the expression of its effect (23). Eppstein *et al.* reported that IFN-γ encapsulated into liposomes did leak out and bind to the receptor (24). However, it has recently been demonstrated that human IFN-γ accumulating in murine transformed L cells with human IFN-γ cDNA is capable of increasing the antiviral activity of the cells (25). Fidler *et al.* found human and murine macrophages to be activated by IFN-γ encapsulated in liposomes without species specificity (26). If IFN-γ does not leak out from liposomes and its internalization into target cells can be accelerated, internalized IFN-γ may express its action irrespective of its binding to the receptor on the cellular surface. Incubation at a low temperature makes it impossible for cells to undergo endocytosis (27), thus permitting an examination of liposomal binding as a process separate and distinct from liposomal interactions. The uptake of [^{32}P]IFN-γ encapsulated in AF-liposomes (37°C) was five times the extent of their binding (4°C), while the corresponding values for free [^{32}P]IFN-γ were negligible (Fig. 3). It thus follows that the ^{32}P radioactivity detected in hepatocytes at 37°C originates from [^{32}P]IFN-γ encapsulated in AF-liposomes which are endocytosed, and IFN-γ in an aqueous space may be internalized into target cells independently of IFN-γ receptor. Negatively charged liposomes have a high affinity for the liver (28), as confirmed by the results of the present study (Fig. 4). In Fig. 3A, the portion of AF-liposome uptake (two-thirds of the total uptake) inhibited by free AF corresponds to the specific interaction of AF-liposomes with the galactose-binding protein, and the remaining portion (one third of the total uptake), to the charge interaction of AF-liposomes and hepatocytes. Both the specific binding of AF to the galactose-binding protein and the charge of liposomes probably affect the interaction of AF-liposomes with their target cells.

In conclusion, through application of the detergent removal and the freeze-thawing methods in combination, it is possible for IFN-γ, an unstable macromolecule, to be encapsulated into AF-liposomes without loss of RIA activity. There is also the possibility that AF-liposomes are effective carriers of IFN-γ to hepatocytes.

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