Transmucosal Passage of Liposomally-Entrapped Drugs in Rat Small Intestine

Toshikiro Kimura[1,3], Kazutaka Higaki[2] and Hitoshi Sezaki[2]

Received: January 24, 1984; accepted: April 27, 1984.

Abstract: Intestinal absorption of liposomally-entrapped drugs was investigated for egg yolk phosphatidylcholine-cholesterol (2:1 by molar ratio) liposomes (EggPC liposome) and distearoylphosphatidylcholine-cholesterol (2:1) liposomes (DSPC liposome). The release of carboxyfluorescein, an aqueous phase marker, induced by the presence of everted rat intestine was 40 % and 6 % in one hour from DSPC liposomes and EggPC liposomes, respectively, and it is suggested that EggPC liposomes are more stable in the intestinal lumen. The transport of a liposomally-entrapped drug was examined with fluoresceinisothiocyanate-conjugated dextran (FITC-D) as a model drug that has a small mucosal-to-serosal clearance because of its high average molecular weight (64200). The clearance of FITC-D entrapped in DSPC liposomes was largely reduced and could be accounted for by the clearance of the extraliposomal FITC-D concentration in the preparation. On the other hand, the calculated clearance of EggPC liposome-associated FITC-D was similar to or even higher than that of free FITC-D. The serosal appearance of the EggPC liposome-associated drug was inhibited by colchicine, cytochalasin B, and iodoacetate, suggesting that the liposome was incorporated into the epithelial cells by endocytosis. However, the observation that a lipid phase marker, ^{14}C-dipalmitoylphosphatidylcholine, failed to be transported into the serosal fluid indicates the absence of the penetration by an intact liposomal form.

A marked reduction of blood glucose levels has been reported in experimental animals after oral administration of insulin entrapped in liposomes (1, 2). However, considerable controversy exists over the absorption mechanism of liposomally-entrapped insulin. Gregoriadis and coworkers (1) claimed that liposomes penetrate the intestinal mucosa in the intact form and enter the blood together with entrapped drugs. Ryman et al. (2) showed that liposomes protect insulin from gastric, and perhaps intestinal, proteolysis, while they doubted whether liposomes are absorbed intact into the bloodstream from the gastrointestinal tract. Furthermore, Richards and Gardner (3) suggested that non-intact liposomes, after being broken up by intestinal bile salts, are responsible for enhancing the uptake of insulin. While it had been shown that only a small or no detectable fraction of liposome components cross the intestinal wall (4, 5), Rowland and Woodley (6, 7) recently reported the serosal appearance of liposome-entrapped macromolecules, the majority of which were associated with the liposome.

In this study we investigated the transmucosal passage of liposomally-entrapped drugs in more detail by using the everted intestine from adult rats. Some of the longstanding questions on the mode of liposome absorption have now been resolved.

[1] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan.
[2] Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan.
[3] Correspondence to be adressed to Prof. T. Kimura, Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka, Okayama, 700, Japan.

Materials and Methods

Materials

Carboxyfluorescein (CF) was purchased from Eastman Kodak Co., Rochester, NY, and was homogeneous on TLC (with $CH_3Cl/CH_3OH/H_2O$, 65:25:4, v/v). Fluoresceinisothiocyanate-conjugated dextran (FITC-D, average molecular weight, 64 200) was purchased from Sigma Chemical Co., St. Louis, MO. L-α-[Dipalmitoyl-l-^{14}C]-phosphatidylcholine (^{14}C-DPPC) was obtained from New England Nuclear Co., Boston, MA. L-α-Distearoylphosphatidylcholine (DSPC) was kindly supplied from Nippon Shoji Co., Osaka, and its purity was excellent as determined by TLC, IR, NMR, and GLC. Egg yolk phosphatidylcholine (EggPC) was prepared from hen eggs according to the method of Rhodes and Lea (8). Cholesterol (Ch) and other reagents used in this study were of reagent grade and were used without further purification.

Liposome Preparation

Multilamellar liposomes were prepared in pH 6.5 isotonic sodium phosphate buffer containing CF (200 mM) or FITC-D (10 mg/ml) with the lipid composition of EggPC-Ch (2:1 by molar ratio) (EggPC liposome) and DSPC-Ch (2:1) (DSPC liposome), according to the method of Bangham et al. (9). Briefly, the lipid mixture (64 mg phosphatidylcholine and 31 mg cholesterol) dissolved in chloroform was pipetted into a 25 ml round-bottomed flask. The chloroform was removed under nitrogen using a rotary evaporator. To the thin dry lipid film, 5 ml of drug solution were added. Mechanical shaking with a Vortex mixer for 10 min caused complete dispersion of the lipids. The evaporation and vortexing were carried out at room temperature and 60°C for EggPC liposomes and DSPC liposomes, respectively. The suspension was then sonicated with a probe (Ohtake Sonicator-5202, Japan; 20 KHz, 100W) under nitrogen for 2.5 min in ice-water and at 60°C for EggPC liposomes and DSPC liposomes, respectively. After standing overnight in a refrigerator, drug-containing liposomes were separated from non-encapsulated drugs by gel filtration (Sephadex G-25) and ultracentrifugation for CF and FITC-D, respectively. In the case of FITC-D, the liposomal suspension was centrifuged for 10 min at 105 000 g. The pellet was resuspended in the fresh buffer and centrifuged similarly. This washing step was repeated thrice. Particle sizes of the liposomes were not examined. The concentration of phosphatidylcholine in the mucosal solution was adjusted to be approximately 1 mg/ml.

Transport Experiments

The *in vitro* absorption kinetics for liposomally-entrapped drugs were followed with the use of a cannulated everted sac procedure modified slightly from that described by Jorgensen et al. (10). Five cm of the mid-jejunum from male Wistar rats (180–220 g) were used. Both ends of the sac were cannulated,

and serosal samples were withdrawn from the lower cannula, while the contents were replenished by introducing fresh solution through the upper cannula. Since the physiological pH of the small intestinal lumen seems to be 6.5 (11), transport experiments in this study was carried out at pH 6.5. The volumes of mucosal and serosal solutions were 15 ml and 1 ml, respectively. The mucosal solution was gassed with 95 % O_2/ 5 % CO_2 from 30 min before the start of experiments. Since serosal analyte concentrations were always negligible compared to the concentration of the mucosal solution, it can be assumed that the backward flow of drug from serosal solution to mucosal solution is negligible during the time of the experiment. In addition, because of the high concentration and the large volume of the mucosal solutions, it is safe to assume that mucosal concentration is a constant throughout the absorption experiment. Thus, mucosal-to-serosal clearances of drugs can be calculated by dividing the rate of appearance in serosal solution by the initial mucosal concentration. The clearance of sulfaguanidine, a poorly absorbable drug, was constant from 45 min to 120 min (data not shown), suggesting the structure of the intestinal mucosa had remained relatively intact for at least 2 h.

Stability of Liposomes in the Mucosal Solution

The effect of an everted intestine on liposome integrity was investigated by measuring the release of liposomally-entrapped CF in the apparatus for transport experiments. The composition of the bathing solution was pH 6.5 isotonic sodium phosphate buffer as in the transport experiments. The extraliposomal CF was separated by Micropartition System MPS-1 (Amicon, Danvers, MA).

Analytical Methods

The concentrations of CF and FITC-D were determined spectrofluorometrically. Fluorescence measurements were made with a Hitachi 512 spectrofluorometer at an excitation wavelength of 485 nm and emission wavelengths of 515 nm and 517 nm for CF and FITC-D, respectively. For ^{14}C-DPPC, radioactivities were measured with a Beckman LS-232 liquid scintillation counter.

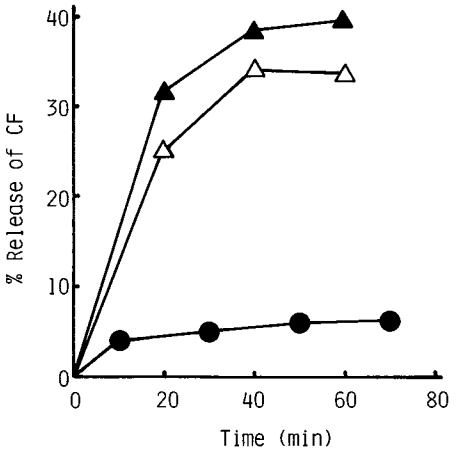

Fig. 1 Stability of liposome integrity in the presence of intestinal everted sacs. Each point represents the mean of duplicate measurements. Lipid composition of liposomes: ▲ DSPC-Ch (2:1); △ DSPC-Ch (7:2); ● EggPC-Ch (2:1).

Results and Discussion

Changes in Liposome Integrity by the Presence of Intestinal Everted Sacs

Before performing the transport experiments, the effect of intestinal everted sacs on the structural integrity of liposomes was examined. All the liposomes tested were relatively stable at 37°C in the absence of the intestine. As shown in Fig. 1, EggPC liposome was stable and not affected by the presence of the intestine, while the release of entrapped marker CF from DSPC liposome was markedly increased by the everted sac. Previous authors have reported that the liposome composed of DSPC-Ch (7:2) is highly stable (3, 12). The destruction of liposomes may be caused by residual phospholipase activity in the tissue, which might be expected to be more active against EggPC than DSPC. However, our result with highly pure DSPC proved it to be unstable. This contradiction is left unsolved.

Fig. 1 shows that perturbation of the liposomal membranes reached an early maximum between 20 and 40 min. Therefore, it can be assumed that the concentration of the non-entrapped free drug throughout the experiment is similar to the free drug concentration at the end of the experiment.

Transport of CF from Liposomal Solutions

Fig. 2 shows the mucosal-to-serosal transport rate of CF from liposomal solutions, in comparison with that from a simple aqueous solution. The clearances of the marker drug from liposomal solutions were markedly reduced regardless of the lipid composition. Furthermore, the clearances calculated from the extraliposomal concentration of CF at the end of each experiment are similar to those of the simple aqueous solution. This result may lead to the interpretation that liposomally-entrapped drugs cannot cross the epithelial layer, while free drugs can be transported. However, CF may represent an inadequate marker for the present study, CF has been widely used as an aqueous space marker of liposomes because of its

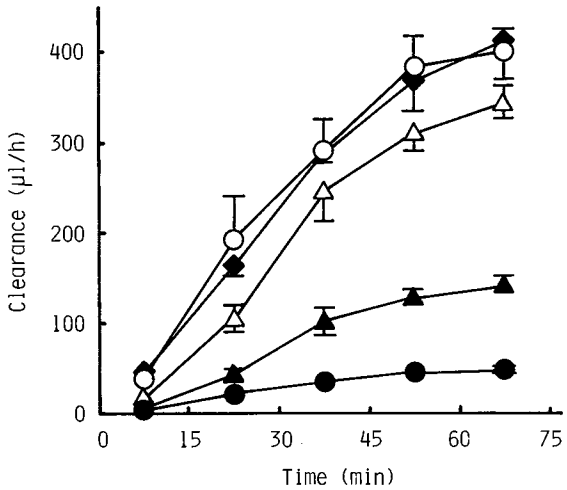

Fig. 2 Mucosal-to-serosal clearance of CF from liposomal solution. Each point represents the mean ± S.E. of 4 experiments. Clearances are calculated from total mucosal drug concentrations (closed symbols) or final mucosal free drug concentrations (open symbols). ◆ Solution; ● EggPC liposome; ▲ DSPC liposome.

Table I. Mucosal-to-Serosal Clearance of Liposomally-Entrapped FITC-D.

Form	FITC-D conc. (μg/ml)	Clearance (μl/h) 0–30 min	30–60 min	60–90 min
Free FITC-D	100	(A) 1.45±0.56(8)	1.68±0.52(8)	12.22±1.62(6)
Free FITC-D	95.7	(A) 1.47±0.33(4)	3.87±0.34(4)	10.45±0.73(4)
+ empty liposome*		(B) 1.71±0.39(4)	4.49±0.40(4)	12.11±0.81(4)
EggPC liposome-	33.5	(A) 1.30±0.62(6)	6.01±0.59(8)	14.07±0.87(8)
entrapped FITC-D	or 44.4	(B) 1.97±0.96(5)	15.61±1.59(8)	36.50±2.33(8)
		(C) 1.54±0.90(6)	6.74±0.95(8)	15.23±1.39(8)
DSPC liposome-	75.3	(A) 2.05±0.25(5)	2.93±0.51(6)	5.58±0.82(7)
entrapped FITC-D	or 24.7	(B) 6.62±0.84(5)	9.22±1.88(6)	16.30±1.80(7)
		(C) 2.37±0.32(5)	2.03±0.72(6)	3.10±0.66(7)

(A): Calculated from total mucosal FITC-D concentrations.
(B): Calculated from final mucosal free FITC-D concentrations.
(C): Calculated clearance for liposomally-entrapped FITC-D.
Results are expressed as the mean ± S.E. with n in parentheses.
* EggPC liposome.

slow leakage (13, 14). Furthermore, CF is poorly absorbed from rat small intestine (unpublished data). Nevertheless, the mucosal-to-serosal clearance of CF was still too high in comparison with those of intact liposomes as discussed later.

Transport of FITC-D from Liposomal Solutions

The mucosal-to-serosal transport of liposomally-entrapped drugs was similarly investigated with the use of FITC-D as the marker. The mucosal-to-serosal clearance of this macromolecule was much smaller (approximately 1/30) than that of CF and was not concentration-dependent within the range examined (0.05–1.0 mg/ml) (data not shown). The fluorescence in the serosal solution derived from mucosal FITC-D could not be filtered by Micropartition System, suggesting that metabolic breakdown was negligible and that FITC-D was transported as the macromolecular form. The results of transport experiments are summarized in Table I. The clearance of FITC-D calculated from the total mucosal concentration was significantly reduced in DSPC liposome, similarly to the result obtained from CF as a marker. However, the overall clearance did not change in the case of EggPC liposomes. Furthermore, the clearances calculated from the concentration of non-entrapped FITC-D (which was not increased in the presence of the everted sac in contrast to the case of CF, as measured by ultracentrifugation) did not differ from those of the simple solution for the DSPC liposome preparation, while they were markedly higher for the EggPC liposome preparations. These results suggest the transport not only of the free marker but also of liposomally-entrapped marker, in the case of the EggPC liposomes. As shown in Table I, the clearance of free FITC-D was not affected by the presence of empty liposomes, suggesting that liposomes do not change permeability characteristics of the mucosal membrane. Thus, the mucosal-to-serosal clearance of liposomally-entrapped FITC-D was calculated as follows:

$$\text{Clearance} = \frac{(\text{Transport rate}) - (\text{Clearance of free FITC-D}) \times (\text{Concentration of free FITC-D})}{(\text{Concentration of liposomally-entrapped FITC-D}).}$$

The calculated values are listed in column (C) of Table I. These values suggest that FITC-D entrapped in EggPC liposome can pass through the epithelial layer at a rate similar to or even higher than the free marker, which is not observed in the case of the DSPC liposomes. Since the transport rate of drugs entrapped in EggPC liposome is slow, it may be overlooked when using marker drugs with higher transport rates, as in the case of CF.

Effect of Inhibitors on Transmucosal Passage of Liposomally-Entrapped Drugs

In order to clarify the mechanism of the transmucosal passage of liposomally-entrapped drugs, the effect of potential inhibitors on the clearance of liposomal FITC-D was examined. Inhibitors used were colchicine, an inhibitor of microtubular assembly, cytochalasin B, an inhibitor of microfilament polymerization, and iodoacetate, an inhibitor of glycolysis. None of these affected the leakage of FITC-D from the liposomes. They tended to reduce the transport of free FITC-D, while the differences were not statistically significant (data not shown). As shown in Table II, the calculated clearance of liposomal FITC-D was significantly inhibited in EggPC liposome by those inhibitors, but not in DSPC liposome. These results strongly suggest that the passage of EggPC liposomes,

Table II. Effect of Inhibitors on Mucosal-to-Serosal Clearance of Liposomally-Entrapped FITC-D

Inhibitor	Clearance (μl/h) 0–30 min	30–60 min	60–90 min
EggPC liposome			
Control	1.54±0.90(6)	6.74±0.95(8)	15.23±1.39(8)
0.1 mM Colchicine	0 ±0 (2)	3.69±2.18(4)	5.15±1.26(3)[b]
41.7 μM Cytochalasin B	1.36±0.74(4)	3.08±0.38(4)[c]	5.34±0.84(4)[a]
10 mM Iodoacetate	0.40±0.11(2)	1.69±0.32(4)[b]	4.31±0.50(4)[a]
DPPC liposome			
Control	2.37±0.32(5)	2.03±0.72(6)	3.10±0.66(7)
0.1 mM Colchicine	0 ±0 (3)[b]	1.15±0.63(4)	2.10±0.70(4)
41.7 μM Cytochalasin B	0.34±0.17(4)[b]	1.85±0.28(4)	4.17±1.00(4)

Results are expressed as the mean ± S.E. with n in parentheses.
[a] $p < 0.001$; [b] $p < 0.01$; [c] $p < 0.05$, compared with each control.

but not DSPC liposomes, may involve endocytosis rather than fusion. Significant reductions of FITC-D transport were observed over the initial period of the experiments with DSPC liposomes; however, these results cannot be explained at present.

Effect of Concentration of Liposomal Lipids

Table III shows the effect of the concentration of liposomal lipids on the transport of liposomal FITC-D. An apparent saturation phenomenon was observed which further supports the endocytosis mechanism.

Table III. Effect of Lipid Concentration on Mucosal-to-Serosal Clearance of FITC-D Entrapped in EggPC Liposome

EggPC conc. (mg/ml)	Clearance (μl/h) 0–30 min	30–60 min	60–90 min
0.46	2.51 ± 0.88(4)	8.77 ± 1.41(4)	19.53 ± 1.70(4)
0.91	0.75 ± 0.25(4)	3.20 ± 0.57(4)	8.59 ± 0.81(4)
1.83	0.66 ± 0.16(4)	1.56 ± 0.19(4)	3.64 ± 0.50(4)

Results are expressed as the mean ± S.E. with n in parentheses.

Can Intact Liposomes Carry the Content through the Intestinal Wall?

In order to investigate whether liposomally-entrapped drugs can be carried through the intestinal wall by an intact liposomal form, the mucosal-to-serosal clearance of ^{14}C-DPPC, a marker substance of the lipid phase was examined. The result is shown in Table IV. At the condition where liposomal FITC-D can be efficiently transported, the lipid phase marker failed to be transported into the serosal fluid. Therefore, the serosal appearance of liposomal drugs is not associated with the liposomal structure. This result coincides with papers of Seiden and Lichtenberg (4) and Whitmore and Wheeler (5).

Table IV. Mucosal-to-Serosal Clearance of Liposomal Components

Component	Clearance (μl/h) 0–30 min	30–60 min	60–90 min
FITC-D	1.54 ± 0.90(6)	6.74 ± 0.95(8)	15.23 ± 1.39(8)
^{14}C-DPPC	0.66 ± 0.43(4)	0.10 ± 0.06(4)	0.20 ± 0.08(4)

Lipid composition of the liposome was EggPC-Ch (2:1). Results are expressed as the mean ± S.E. with n in parentheses.

On the contrary, Rowland and Woodley (6, 7) claimed that the majority of macromolecules reaching the serosal fluid was still entrapped within liposomes. Their interpretation was based on the finding that the water-soluble macromolecules in the serosal fluid were sedimented by centrifugation; however, one cannot be sure that the precipitation is due to the association with liposomes.

From the results described above, we conclude that intestinal epithelial cells take up liposomes by endocytosis at a slow rate. Drugs are released from liposomes in the cell and then transported into the serosal side. In addition, the uptake by endocytosis is dependent on the lipid composition; it is operative in EggPC liposome, but not in DSPC liposome.

Acknowledgement

This work was supported by a Grant-in-Aid for Scientific Research provided by the Ministry of Education, Science and Culture of Japan.

References

(1) Gregoriadis, G. (1978) Ann. N. Y. Acad. Sci. 308, 343–370.
(2) Ryman, B. E., Jewkes, R. F., Jeyasingh, K., Osborne, M. P., Patel, H. M., Richardson, V. J., Tattersall, M. H. N., Tyrrell, D. A. (1978) Ann. N.Y. Acad. Sci. 308, 281–307.
(3) Richards, M. H., Gardner, C. R. (1978) Biochim. Biophys. Acta 543, 508–522.
(4) Seiden, A., Lichtenberg, D. (1979) J. Pharm. Pharmacol. 31, 414–415.
(5) Whitmore, D. A., Wheeler, K. P. (1979) Biochem. Soc. Trans. 7, 929–931.
(6) Rowland, R. N., Woodley, J. F. (1981) Biochim. Biophys. Acta 673, 217–223.
(7) Rowland, R. N., Woodley, J. F. (1981) FEBS Lett. 123, 41–44.
(8) Rhodes, D. N., Lea, C. H. (1957) Biochem. J. 65, 526–533.
(9) Bangham, A. D., Standish, M. M., Watkins, J. C. (1965) J. Mol. Biol. 13, 238–252.
(10) Jorgensen, C. R., Landau, B. R., Wilson, T. H. (1961) Am. J. Physiol. 200, 111–116.
(11) Koizumi, T., Arita, T., Kakemi, K. (1964) Chem. Pharm. Bull. 12, 421–427.
(12) Rowland, R. N., Woodley, J. F. (1980) Biochim. Biophys. Acta 620, 400–409.
(13) Weinstein, J. N., Yoshikami, S., Henkart, P., Blumenthal, R., Hagins, W. A. (1977) Science 195, 489–492.
(14) Szoka, F. C., Jr., Jacobson, K., Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 551, 295–303.