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Research Article

Effect of Oleic Acid Vesicles on Intestinal Absorption of Carboxyfluorescein in Rats

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The effect of oleic acid vesicles (ufasomes) on the intestinal absorption of entrapped carboxyfluorescein (CF) was investigated by an *in situ* closed-loop method in rats. Entrapment of CF in ufasomes enhanced the absorption of CF at the earlier stage following intraduodenal administration, and the threshold concentration of the fatty acid for promoting the absorption of CF was approximately 8 m*M*. The absorption of CF from the large intestine was promoted much more effectively than from the small intestine. These studies suggest that ufasomes have potential as carriers for the oral administration of poorly absorbable drugs.

KEY WORDS: oleic acid; ufasome; promotive absorption; intestinal absorption; drug carrier; 6-carboxyfluorescein.

INTRODUCTION

Liposomes are versatile biological vesicles that can encapsulate a variety of substances and macromolecules (e.g., polypeptides, proteins). Attention has focused on the potential application of liposomes to oral delivery of drugs that are not normally absorbed from the gastrointestinal tract because of their high hydrophilicity, large molecular weight, or instability in the lumen (1–3). However, it is still ambiguous whether liposomes directly increase the absorption rate of these drugs or only protect them from chemical or enzymatic degradation in the lumen. In our preceding report (4), it was suggested that liposomes composed of egg phosphatidylcholine do not substantially promote the absorption of carboxyfluorescein (CF), a water-soluble (i.e., poorly absorbable) model dye, from the rat small intestine. Recent reports (5,6) indicate that liposomes do not permeate the intestinal mucosa in their intact form. It is, therefore, important to develop vesicles with suitable efficiency as oral drug carriers.

We previously reported the marked effect of lipid-surfactant mixed micelles on the intestinal absorption of poorly absorbable drugs, such as gentamicin, streptomycin (7), heparin (8,9), and interferon (10), in rats. As Muranushi *et al.* (11) pointed out, lipids enhanced the intestinal absorption of these drugs are the fusogenic lipids as defined by Ahkong *et al.* (12). On the other hand, unsaturated long-chain fatty acids may form closed vesicles with a bilayer-like structure, so-called "ufasomes" (13). On the basis of these considerations, we have investigated the usefulness of ufasomes as potential oral drug carriers.

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Carboxyfluorescein (CF) was used as a model compound for poorly absorbable drugs. Because most of the absorbed CF is excreted into bile (unpublished data), and in order to avoid decomposition of ufasomes by bile acids in the intestinal lumen, bile fistula rats were used. As a typical unsaturated fatty acid, oleic acid was chosen in view of its low sensitivity for peroxidation, which renders the membrane of ufasomes leaky (14).

MATERIALS AND METHODS

Materials. CF was purchased from Eastman Kodak Co. (Rochester, N.Y.). Oleic acid of high-purity grade (at least over 99%) was kindly supplied by Nippon Oil & Fats Co. Ltd. (Tokyo). All other chemicals and solvents were of reagent grade.

Preparation of Ufasomes. Oleic acid ufasomes containing CF (CF/ufasomes) were prepared according to the method of Gebicki and Hicks (15) with some modification. Oleic acid was dissolved in chloroform to 4% (w/v) and stored at −20°C. For standard preparations, 1 ml of the stock oleic acid solution was placed in a test tube and evaporated to dryness with a stream of nitrogen at 40°C. After the addition of sufficient 1 *N* NaOH to neutralize the fatty acid, residual film was sonicated with an Ohtake Model 5202 sonicator (Ohtake Seisakusho Co., Ltd., Tokyo) at 15 W for 2 min under nitrogen in an ice-water bath to hydrate with 1 ml of 2% (w/v) Tris–HCl-buffered CF solution of pH 8.5. The resulting suspension of ufasomes was then applied to a coarse Sephadex G-25 column (18 × 2 cm) to remove the excess CF unentrapped within the ufasomes. The void-volume fraction was collected using the same buffer as eluent.

In Vitro *Stability of Ufasomes.* CF/ufasomes in suspension with pH 8.5 Tris–HCl buffer were incubated in a water bath at 37°C. One-milliliter aliquots of the suspension were

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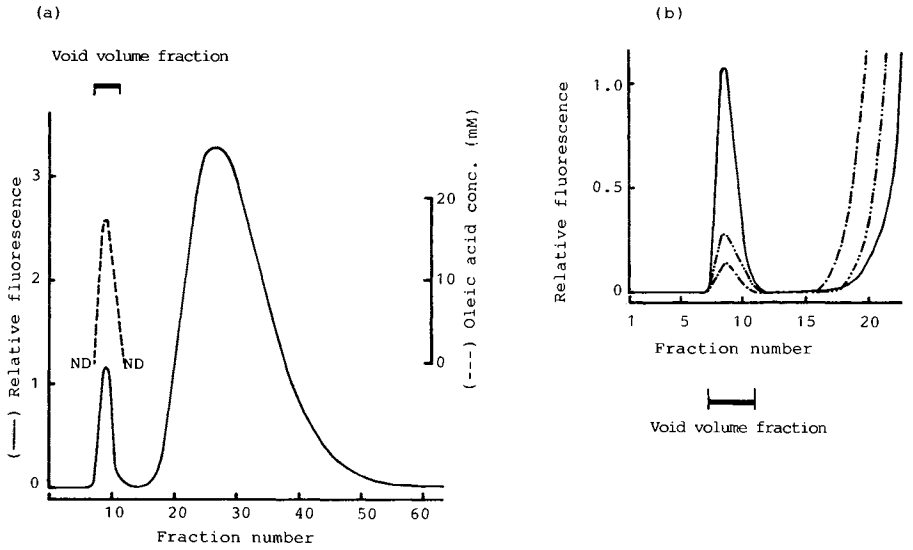

Fig. 1. Gel filtration pattern of ufasomes. (a) Elution chromatogram of CF/ufasomes for the standard preparation. ND < 0.01 m*M*. (b) CF/ufasomes prepared with oleic acid at 40 mg (——), 10 mg (— · · —), and 5 mg (— · —). Chromatography was performed on a coarse Sephadex G-25 column at a flow rate of ca. 20 ml/hr (2 ml/tube).

periodically taken and immediately ultrafiltered using an Amicon MPS-1 micropartition system with a YMT membrane (Amicon Ltd.) at 700*g* for 8 min. The filtrates and another aliquot of the suspension were used for CF assay as described below.

Absorption Experiments in Rats. Absorption experiments were performed in male Wistar albino rats (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan) weighing 230–280 g by the *in situ* closed-loop method as described previously (4). Animals were fasted for 16 hr prior to experiments (but given water ad libitum) and anesthetized intraperitoneally with sodium pentobarbital (32 mg/kg of body weight) during the experiments. The intestine was exposed through the midline incision, and an intestinal loop was prepared by cannulation of 3-cm silicon tubing (i.d., 3 mm; o.d., 5 mm) at the proximal and distal ends of the small intestine or the entire large intestine (colon and rectum). The bile duct was cannulated with polyethylene tubing (i.d., 0.5 mm; o.d., 0.8 mm), and bile was removed from the body during the experiments. The test solution warmed to 37°C, was introduced into the intestinal loop, which was closed by clipping with a forceps at the cannulated position of each tubing. The administered volume was 5 ml for the small intestine and 2.5 ml for the large bowel. The dose of CF was 1 mg/kg of rat body weight. Blood samples were collected periodically via a polyethylene catheter (i.d., 0.5 mm; o.d., 0.8 mm) placed into the carotid artery.

In Situ *Stability of Ufasomes.* At the predetermined time after the administration of the test solution, the remaining solution in the lumen was thoroughly collected by forcing it out with syringe air. The collected solution was ultrafiltered under the conditions described above, and its pH was measured.

Analytical Methods. The fluorescence of CF in the diluted samples was measured with a Hitachi Model 650-10S fluorescence spectrophotometer (Hitachi Ltd., Tokyo) at 520 nm at an excitation wavelength of 490 nm. For measurement of the total fluorescence, to 0.1 ml of the ufasome suspensions 0.1 ml of 12.5% (w/v) Triton X-100 solution was added to release all of the CF and then diluted with the appropriate buffer volume.

Determination of the concentration of CF in rat plasma was carried out as previously reported. Briefly, 50 μl of plasma was mixed wth 0.1 ml of 12.5% (w/v) Triton X-100 and 3 ml of 1 *N* HCl. To the resulting mixture 6 ml isoamyl alcohol was added, and CF was extracted. After centrifugation, 5 ml of the isoamyl alcohol was pipeted, 4 ml of pH 10 Na_2CO_3–$NaHCO_3$ buffer solution was added, and CF was reextracted into the buffer phase. The aqueous phase was fluorometrically analyzed. Determination of oleic acid was carried out with a commercial kit for the measurement of nonesterified fatty acids by the ACS-ACOD metho (NEFA Kit-U, Nippon Shoji Kaisha Ltd., Osaka , Japan).

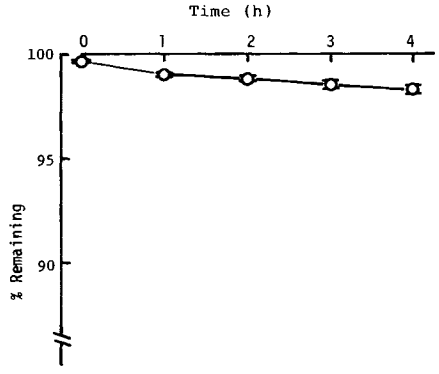

Fig. 2. Release of CF from ufasomes *in vitro*. The percentage of CF remaining in the ufasomes is plotted. Vertical bars represent the standard deviations of the means for three determinations.

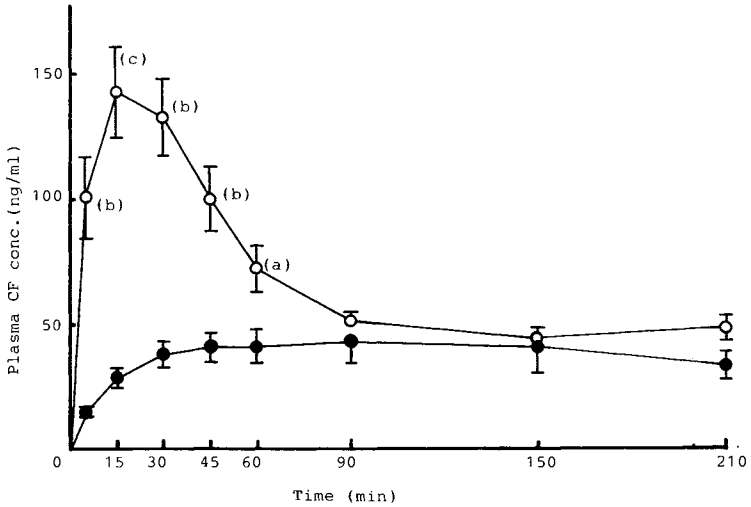

Fig. 3. Plasma concentration of CF following administration into the small intestine. Results are expressed as the mean ± SE of three or four experiments. Statistical comparison of plasma level of CF at each time was done by Student's *t* test: (a) $P < 0.05$; (b) $P < 0.01$; (c) $P < 0.001$. (●) Free CF; (○) CF/ufasomes.

RESULTS

Preparation and *in Vitro* Stability of Ufasomes

Figure 1 shows typical column chromatograms of ufasome suspensions obtained by fluorescence detection of CF. More than 90% of the fatty acid on a column was recovered as ufasomes in the void fraction. The CF entrapped in ufasomes coeluted in the void volume. In the case of the standard preparation, the absolute recovery was 4.07 ± 0.08% (mean ± SD; $N = 4$), which was proportionally increased with the concentration of fatty acid used for preparation. The CF in the filtrate through Amicon membranes was less than 1% of the total CF in the eluate applied on the ultrafiltration system, while fatty acid was not detected in the filtrate. The CF eluted in the void volume was substantially entrapped within the vesicles, and the CF/ufasomes did not pass through the ultrafiltration membrane. Consequently, the ratio of the CF concentration in the ultrafiltrate to that in the void-volume fraction may be regarded as the proportion of CF leaked out of the vesicles.

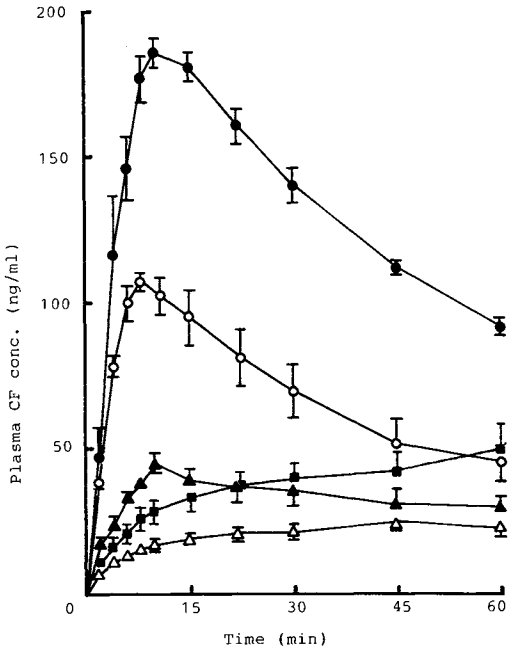

Fig. 4. Plasma level of CF following intraduodenal administration of ufasomal solutions with different fatty acid concentrations. Results are expressed as the mean ± SE of four experiments. (●) 32 m*M*; (○) 16 m*M*; (▲) 8 m*M*; (△) 4 m*M*; (■) free CF.

The releasing property of CF from the isolated ufasomes was studied to estimate their stability under *in vitro* conditions. As shown in Fig. 2, not more than 3% of the dye was released when CF/ufasomes were incubated with the Tris–HCl buffer at 37°C for 4 hr. In this report, we did not correct for any adsorption of CF onto the YMT membranes since it was minimal. For instance, when the YMT membrane was incubated with 1 ml of 50 ng/ml CF solution, which represents a rather low concentration, at 37°C for half an hour, the percentage CF adsorbed was only 3.98 ± 0.05% ($N = 4$). Moreover, the adsorption of CF onto the YMT membrane was negligible when a higher CF concentration (for example, more than 30 μg/ml) was applied to the micropartition system.

Administration of CF/Ufasomes into the Small Intestine

The plasma concentrations of CF were compared after the intraduodenal administration of free CF and CF/ufasomes. As Fig. 3 shows, a steeper rise of CF plasma levels was observed after the administration of CF/ufasomes than after that of free CF. The presence of ufasomes clearly enhanced the absorption of the dye from the small intestinal tract in the early phase. However, no significant difference was detected between plasma CF concentrations at 1.5 hr after dosing with CF/ufasomes and free CF (Student's *t* test,

Table I. Relationships Between Oleic Acid Concentration and Absorption Efficiency (AUC 0–60 min and C_{max}) of CF[a]

Oleic acid conc. (mM)	C_{max} (ng/ml)	AUC 0–60 min (ng · min · ml^{-1})	
32	185.55 ± 5.00	7882.72 ± 154.08	(4)
16	107.40 ± 2.90	3924.76 ± 230.38	(4)
8	46.80 ± 5.58	1984.38 ± 166.16	(4)
4	21.58 ± 3.26	1096.08 ± 121.17	(4)
Free	50.20 ± 8.30	2134.54 ± 132.55	(4)

[a] Results are expressed as the mean ± SE with the number of animals in parentheses.

$P < 0.05$). On the basis of these results, all of the subsequent experiments were performed within 1 hr after administration.

Effect of the Concentrations of Oleic Acid on CF Absorption

The effects of the oleic acid concentration as a constituent of ufasomes on the absorption of CF from the small bowel were investigated. As seen in Fig. 4, the promoting effect of ufasomes on CF absorption was observed above a concentration of 8 mM of the fatty acid. The plasma concentration–time curves showed a similar pattern in both experiments. Plasma CF levels peaked within 10 min after dosing and declined thereafter. The maximal concentration (C_{max}) and area under the curves (AUC; 0–60 min) correlated well with the fatty acid concentration (Table I). On the contrary, 4 mM oleic acid in ufasomes significantly suppressed the absorption of CF from the intestine, and the concentration–time curve followed a pattern similar to that of free CF dosing.

Stability of Ufasomes in the Small Intestinal Loop

To estimate the stability of CF/ufasomes *in situ*, the magnitude of CF leakage from the vesicles in the lumen was examined. Figure 5 shows the time course of the free CF fraction and the pH change in the intraluminal fluid. The fraction of free CF after the administration of CF/ufasomes was approximately 30% at 0.5 hr and 53% at 1 hr. The pH changed by only 0.6 unit at 1 hr after dosing.

Administration of CF/Ufasomes into the Large Intestine

The promoting effects of the fatty acids in a mixed micellar state on the absorption of poorly absorbable drugs were stronger in the large intestine than in the small bowel, as previously reported from our laboratory (7,9). Hence, the effect of ufasomes on the absorption of CF was similarly investigated in the large intestine of rat. Much more absorption of CF was caused in the large intestine by ufasomal entrapment as shown in Fig. 6, although free CF was less absorbed from the large intestine than from the small intestine.

DISCUSSION

Among the therapeutic drugs with low molecular weights are many highly hydrophilic compounds, which are, in general, poorly absorbable via the gastrointestinal tract. Carboxyfluorescein, widely used as a vesicle-entrapped marker of the aqueous space is a weekly acidic trivalent anion at neutral pH (16). Gebicki and Hicks have reported that ufasomal preparations are capable of encapsulating glucose, a nonelectrolyte (15). In this work, CF was shown to be also enclosed within these vesicles (Fig. 1). As demonstrated in *in vitro* stability experiments, ufasomes are able to retain the entrapped CF after dilution with Tris–HCl buffer and incubation at 37°C (Fig. 2). Therefore, the influence of free CF in the preparations on the total absorption rate of CF can be neglected. Initial experiments with ufasomal encapsulation of CF given into the small and large intestines of rats indicated that ufasomal entrapment may improve the absorption of CF at the early phase after dosing (Figs. 3 and 6). In addition, the absorption of CF was enhanced much more effectively in the large intestine than in the small intestine (Fig. 6); at present, no convincing explanation can be proposed for this finding. However, these results are consistent with our previous observation on the lipid-surfactant mixed micelles (7–9), namely that the essential element for

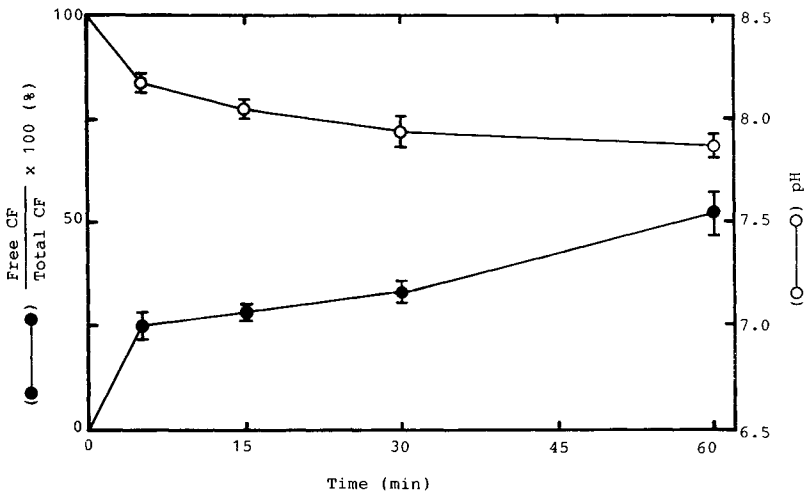

Fig. 5. Fraction of free CF and pH in the intestinal lumen after the administration of CF/ufasomes. Each point is represented as the mean ± SE of separate triplicate experiments.

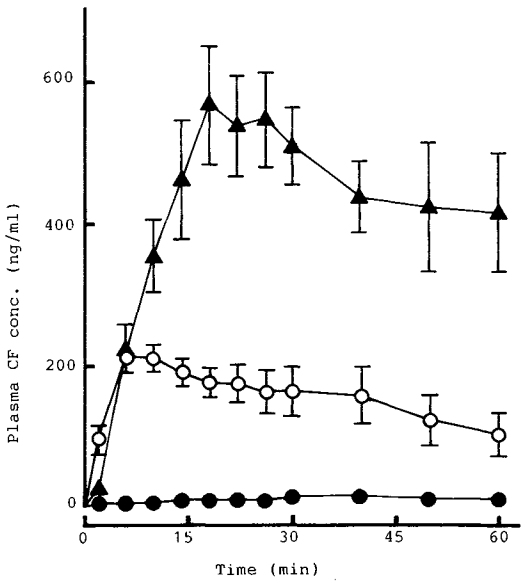

Fig. 6. Plasma concentration of CF after small intestinal or large intestinal administration. Results are expressed as the mean ± SE of four or five experiments. (▲) CF/ufasome into the large intestine; (○) CF/ufasomes into the small intestine; (●) free CF into the large intestine. All of the test solutions were administered at 2.5 ml/rat.

their adjuvant effect is derived from the fusogenic lipids such as oleic acid, linoleic acid, and glyceryl monooleate. On the other hand, either endocytosis or fusion has been proposed to be the dominant mechanism as to drug delivery with liposomes (17–19). The results shown in Fig. 4 suggest that endocytosis does not participate in the mechanism by which ufasomes enhance the intestinal absorption of CF, as a function of the ufasome concentration, because a saturation phenomenon should be observed with an endocytosis mechanism (6). Alternatively, the fusogenic fatty acids incorporated into the mucosal membrane as a component of ufasomes may temporarily induce a membrane perturbation (20,21), resulting in increased permeation of the poorly absorbable dye. However, the exact mechanism for the impact of fusogenic fatty acids on CF absorption remains to be elucidated and is under investigation.

A significantly larger release of CF from ufasomes was observed in the intestinal lumen, compared with that detected *in vitro* (Fig. 5). Incubation of ufasomes with Tris–HCl buffer, adjusted to different pH levels with hydrochloric acid, did not significantly induce the release of the marker at pH 7 to 9. Hence, CF release from ufasomes in the lumen may not be a function of its neutralization. It is probably due to their destruction resulting from alterations of the physicochemical environments and/or the interaction with the mucosal membrane following administration.

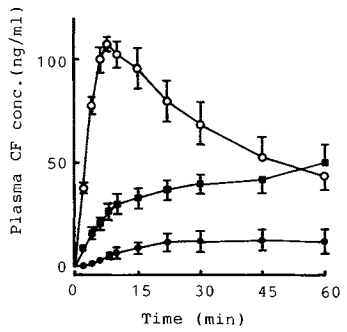

Fig. 7. Effect of CHL incorporation into ufasome membrane on the small intestinal absorption of entrapped CF. (●) CF CHL ufasomes; (○) CF/ufasomes; (■) free CF.

The absorption-promoting effect of ufasomes in the small intestine was detected immediately after administration when the fatty acid concentration was above 8 m*M*, i.e., near the threshold. In contrast, at the fatty acid concentration of 4 m*M*, ufasomal entrapment suppressed the absorption of CF in the small intestine. Under these conditions, the appearance of CF in plasma can be ascribed to the nonenhanced absorption of CF leaked out of the vesicles. Relatively large amounts of cholesterol (CHL) can be incorporated into the ufasome membrane, similar to the phospholipid liposome. However, the incorporation of CHL failed to improve the ufasome preparations because of the leakage of the marker under *in vitro* conditions. Further *in situ* investigation revealed that the incorporation CHL inhibited the promoting effect of ufasomes on CF absorption in the small intestine, but in contrast, drastically suppressed it (Fig. 7), as reported previously with liposomes (4). Possibly the incorporation of CHL renders the ufasome membrane more leaky but provides a rigid structure that resists destruction in the lumen. The stability of CHL-containing ufasomes in the intestinal lumen was not investigated in this study. Also, the possibility that the absorption-promoting effect of ufasomes might be induced following their destruction on the mucosal membrane was not pursued further. However, the optimization of the composition of ufasomes, accompanied by the improvement of its encapsulating properties and delivery of the entrapped materials, is considered to be an important subject in the future for the pharmaceutical application of ufasomes.

In conclusion, ufasomes may represent potentially useful and safe carriers for oral delivery of poorly absorbable drugs, in view of their suitable bioavailability and biodegradability.

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