Uptake of Phosphatidylserine Liposomes by Rat Peyer's Patches Following Intraluminal Administration

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Uptake of the nonabsorbable marker 6-carboxyfluorescein was investigated both free and encapsulated in liposomes as a function of their surface charge and hydrodynamic diameter in rat Peyer's patch and nonpatch tissue. Significant uptake of the marker occurred only when encapsulated in liposomes consisting of at least 25 mol% phosphatidylserine and was highest in Peyer's patches. 6-Carboxyfluorescein encapsulated in liposomes equal to or greater than 374 nm was preferentially taken up by Peyer's patches. There was a trend to higher uptake in lower intestinal segments. These findings were supported by fluorescence microscopic observations. Uptake by Peyer's patches was specific for negatively charged liposomes as judged from competition studies.

KEY WORDS: liposome; phosphatidylserine; Peyer's patches; uptake.

INTRODUCTION

Peyer's patches are specific lymphoid follicles located along the small intestine, and distinct from the systemic lymphoid tissue of the body (1). A specialized epithelium covering the luminal surface of Peyer's patch consists of unique antigen sampling cells, the microfold cells (M cells). M cells take up gut luminal antigens by endocytosis and deliver them to underlying lymphoid cells in the dome region containing functional T, B, and antigen presenting cells (2,3). Since the IgA antibody acts as a primary defense mechanism against infection through the mucosal surface (2), many investigators have been able to demonstrate the ability of oral immunization to activate the antibody secretion (2,4). However, this immunization route has the problem of antigen degradation by gastric acidity and proteolytic enzymes, and extremely large doses are required to achieve adequate immune response.

Various substances can be encapsulated into the aqueous and/or lipid phase of liposomes, and consequently, liposomes have been studied much more extensively as a vesicular drug carrier (5). Wolf *et al.* (6) and LeFevre *et al.* (7,8) noted viruses and particulates such as polymer latex to be incorporated into Peyer's patches through M cells. However, the characteristics of liposomal uptake by Peyer's patches are little understood.

Macrophages take up preferentially negatively charged liposomes, especially those containing phosphatidylserine (9,10). M cells transcytose antigenic materials from the lumen into the space between cells where it can be taken up by lymphocytes. For instance, they phagocytize particles and act as antigen sampling cells (11). Thus, here a study was made of PS liposomes uptake by rat Peyer's patches following intraluminal administration.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (PC), cholesterol (Chol), and isoamylalcohol were purchased from Wako Pure Chemicals (Osaka, Japan). Hydrogenated phosphatidylserine (PS), 6-carboxyfluorescein (6-CF), and *N*-(2-*N'*-cholesterylcarboxyaminoethyl)carbamylmethyl mannan (Chol-AECM-mannan) were obtained from Nisshin Seiyu Co., Ltd. (Tokyo), Eastman Kodak (USA), and Dojin Chemicals (Kumamoto, Japan), respectively. Male Wistar rats weighing 230–250 g were purchased from Japan SLC Co. (Shizuoka, Japan).

Preparation of Liposomes. Phospholipids and Chol were dissolved in a mixture of chloroform:methanol (9:1, v/v) at 50 μmol (as total lipid) in a round-bottomed flask, and a dried lipid film was formed with a rotary evaporator. It was rehydrated with 2.0 mL of 10 m*M* Tris–HCl-buffered saline (pH 7.4) containing 1% 6-CF and vortexed for 10 min to form PS liposomes (PC:PS:Chol = 7:3:2) and PC liposomes (PC:Chol = 7:2). Free 6-CF was separated by gel filtration using a Sephadex G-100 column (3.0 × 40 cm) with a flow rate of 12 mL/hr. The liposomes were successively extruded through the polycarbonate membrane (1.0 and 0.4 μm, Nuclepore Co., CA) with a pressure of 5–40 kg/cm^2 at room temperature. Mean diameter of liposomes was analyzed by the dynamic laser light scattering method using a Nicomp Model 370 (Pacific Scientific, MD), and mean diameter of liposomes was estimated as 374 nm. Following the disruption of liposomes by the addition of 1% Triton X-100, encapsulation of 6-CF was determined by fluorescence spectrophotometer (Hitachi MPF-4; excitation, 494 nm; emission, 515 nm) and was 8.66 μg/μmol phospholipid.

Mannan-coated liposomes were prepared according to the report by Takada *et al.* (12). To 2.0 mL of a liposome suspension, 1.5 mg of Chol-AECM-mannan was added and incubated at 4°C for 2 hr. Free Chol-AECM-mannan was separated by gel filtration using a Sephadex G-100 column. The mannan concentration was estimated by the method of Dubois *et al.* (13) using yeast mannan as a standard, and the amount of mannan on the liposomal surface was estimated as 53.1 μg/μmol inorganic phosphorus (Pi). The concentration of phospholipids (PL) was determined from Pi following perchloric acid ashing (14). The ζ potential of liposomes was determined using the Laser Zee Model 501 (Pem Kem, USA) in 10 m*M* Tris–HCl-buffered saline (pH 7.4) at room temperature. It was converted to ζ potential (electrokinetic potential) according to the Helmholtz–Smoluchowski equation (15).

Uptake of Liposomes by Peyer's Patches. Male Wistar

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rats (230–250 g) fasted for 18 hr were anesthetized with urethane (100 mg/100 g of body weight). After making an abdominal incision, 1.0 mL of liposome suspension (1.0 μmol phospholipid) was injected into the lower duodenal tract. Two hours later, the intestine was isolated and liposomes remaining within the lumen were washed off by perfusing 100 mL of 0.9% NaCl. Peyer's patches and the neighboring areas (nonpatch tissues) were cut out as a definite area (20 mm^2) and 1% homogenate in water was prepared with a Potter–Elvehjem homogenizer. The amount of 6-CF taken up by the isolated tissues was measured according to the report by Hara *et al.* (16). In some experiments, intestinal loops of four segments were obtained by ligating at every 15 cm from the lower end of the duodenum. The segments were designated the upper jejunum, lower jejunum, upper ileum, and lower ileum. One milliliter of liposomes (approx. 9 μg 6-CF/μmol Pi) was injected into each segment, and the uptake of liposomes was calculated from the uptake of 6-CF (using the encapsulation efficiency of 6-CF) and expressed as nanomoles of Pi per square centimeter of Peyer's or nonpatch tissue.

Statistical analysis was performed by one-way analysis of variance (ANOVA). *P* values of 0.05 or less were considered significant.

Fluorescence Microscopy. For the fluorescence microscopy, rhodamine B-phosphatidylethanolamine (0.66 mg) was embedded in the lipid bilayer of PC:PS:Chol = 7:3:2 (66-μmol) liposomes (rhodamine liposomes). One milliliter of rhodamine liposomes (3.5 μmol phospholipid) was injected into the lower ileum, and after 1 hr Peyer's patches were removed. Isolated Peyer's patches were mounted in OTC compound (Miles Scientific, IL) and cut (7 μm). Cryosections were viewed in a fluorescence microscope (Olympus, Vanox AHB-LB, Tokyo).

RESULTS

We measured and compared the uptake of 6-CF both free and encapsulated in liposomes of various phosphatidylserine concentrations and diameters. In contrast to free, the encapsulated 6-CF in liposomes was taken up in Peyer's patches as well as nonpatch tissue. In Peyer's patches uptake was governed by the amount of PS present in the bilayer, with 25 mol% being best. In nonpatch tissue liposomal uptake was independent of PS (Fig. 1). Uptake of free and encapsulated 6-CF was a function of time, plateauing after 2 hrs (Fig. 2).

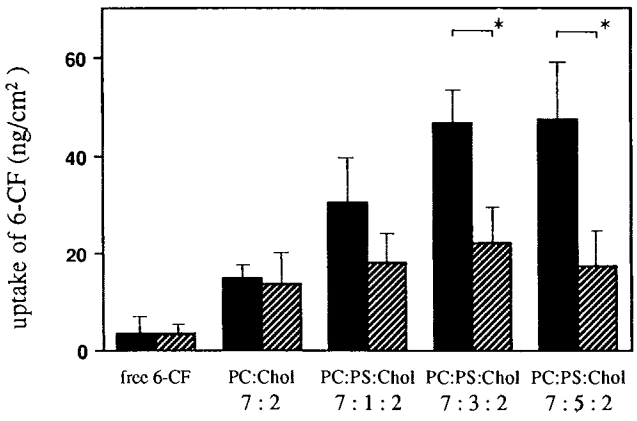

Fig. 1. Effect of lipid composition on the uptake of liposomes by Peyer's patches. PC liposomes (PC:Chol = 7:2) and PS liposomes with different concentration of PS (PC:PS:Chol = 7:1:2, 7:3:2, 7:5:2) were used. The ζ potentials of PC and PS liposomes were −2.5 ± 1.5, −22.4 ± 4.5, −27.1 ± 5.8, and −40.7 ± 5.6 mV, respectively. Liposomes 374 nm in mean diameter were used. Each bar represents the mean ± SD (n = 3–5). (*) $P < 0.05$. (■) Peyer's patches; (□) nonpatch tissue.

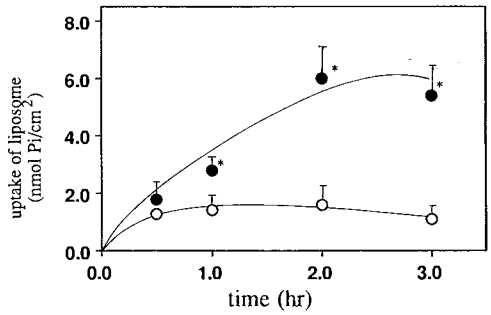

Fig. 2. Time course of liposome uptake. Lipid composition of liposomes was PC:PS:Chol = 7:3:2. Liposomes 374 nm in mean diameter were used. The uptake of liposomes was calculated from the uptake of 6-CF (using encapsulation efficiency of 6-CF) and expressed as nanomoles of Pi per square centimeter of Peyer's or nonpatch tissues. Each point represents the mean ± SD (n = 3). (*) $P < 0.05$. (●) Peyer's patches; (○) nonpatch tissue.

In situ perfusion with liposomes of different sizes, however, at the same amount of 6-CF (approx 9 μg), yields maximum uptake at a hydrodynamic diameter equal to or greater than 374 nm in both Peyer's patches and nonpatch tissue, but significant differences in uptakes between Peyer's patches and nonpatch tissues were observed for larger liposomes (Fig. 3). There was a trend for higher uptake in Peyer's patches from lower intestinal segments observed. Uptake in nonpatch tissue was independent from the location (Fig. 4).

The uptake of liposomes by Peyer's patches at lower ileum was also examined using rhodamine liposomes. As shown in Fig. 5, fluorescence was observed under follicular-associated epithelium, but no fluorescence could be observed under epithelium of nonpatch tissue. Rhodamine B alone, used as a control, did not show any fluorescence in Peyer's patches and nonpatch tissue.

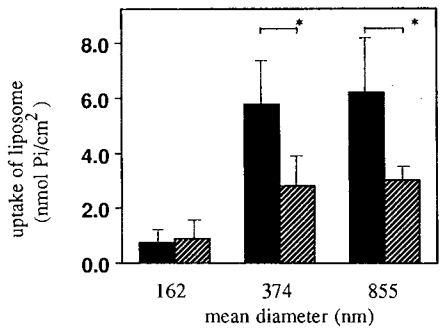

Fig. 3. Effect of diameter on the uptake of liposomes. Lipid composition of liposomes was PC:PS:Chol = 7:3:2. Liposomes having various diameters were prepared by the successive extrusion method. Each bar represents the mean ± SD (n = 4). (*) $P < 0.05$. (■) Peyer's patches; (□) nonpatch tissue.

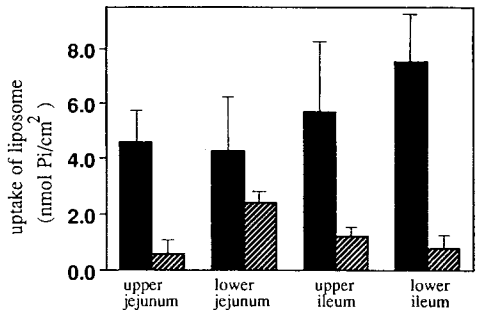

Fig. 4. Uptake of liposome by Peyer's patches at different locations. Lipid composition of liposomes was PC:PS:Chol = 7:3:2. Liposomes 374 nm in mean diameter were used. The designation of each segment of small intestine is elucidated under Materials and Methods. Each bar represents the mean ± SD (n = 4). (■) Peyer's patches; (□) nonpatch tissue.

The effect of mannan coating on the uptake of liposomes by Peyer's patches was investigated. The uptake of mannan-coated PS liposomes (Man-PS liposomes) appeared to be somewhat higher than that of PS liposomes (Fig. 6). Competition experiments of mannan-coated liposomes with either free mannan (5.3 mg) or PS liposomes demonstrated the interaction of PS and mannose residues with the surface of Peyer's patches, whereas no interaction was noticed for nonpatch tissue (Fig. 6).

DISCUSSION

Peyer's patches are indispensable to antigen and induction of mucosal immune responses (1). M cells overlying Peyer's patches have been shown to take up particulate materials such as bacteria (17), virus (6), and polymer latex (7,8) by endocytosis and act as antigen sampling cells. Fidler and Schroit (18) reported that receptors for negatively charged phospholipids are present on the surface of macrophages. The importance of PS, an acidic phospholipid, in the recognition of aged erythrocytes by macrophages was reported by Schroit *et al.* (19,20). They found that during the aging of erythrocytes, PS located mainly in inner leaflets of lipid bilayers is transferred to outer leaflets to possibly trigger this recognition.

The authors thus studied the uptake of PS liposomes by rat Peyer's patches and compared the results for those of PC liposomes deficient in PS as a lipid component. Negatively charged PS liposomes were preferentially taken up depending on the PS concentration (Fig. 1). Patel *et al.* (21) observed liposomal transport across rabbit ileum and found negatively charged liposomes containing dicetylphosphate to be absorbed much more than neutral or positively charged liposomes. The involvement of Peyer's patches in liposomal transport is still unclear.

Following the intravenous injection of liposomes, larger liposomes are rapidly removed from the circulation and taken up by reticuloendothelial system such as Kupffer cells of the liver and macrophages of the spleen (16). The results in Fig. 3 are consistent with these observations. M cells might take up PS liposomes of a larger size effectively and deliver them to Peyer's patches. Thus, liposomal size may be a factor for the preferential uptake by Peyer's patches.

From the duodenum to the cecum of adult rat intestine, about 20 Peyer's patches could be visually observed. The functions of these patches differ according to their particular site in the intestine (22), age (8), and environment (22) for the animals. There is a trend toward higher uptake in lower intestinal parts, at best (Fig. 4).

Pappo and Ermake examined the uptake and translocation of fluorescent latex particles by rabbit Peyer's patches, and transepithelial transport of particles was suggested (23). In this study, fluorescence was observed only by rhodamine liposomes at Peyer's patches, suggesting that the liposomes were translocated through follicle epithelium at Peyer's patch.

Takada *et al.* (12) found that, by coating the liposome surface with mannan, liposomal stability increased and uptake through mannose-binding protein of macrophage was

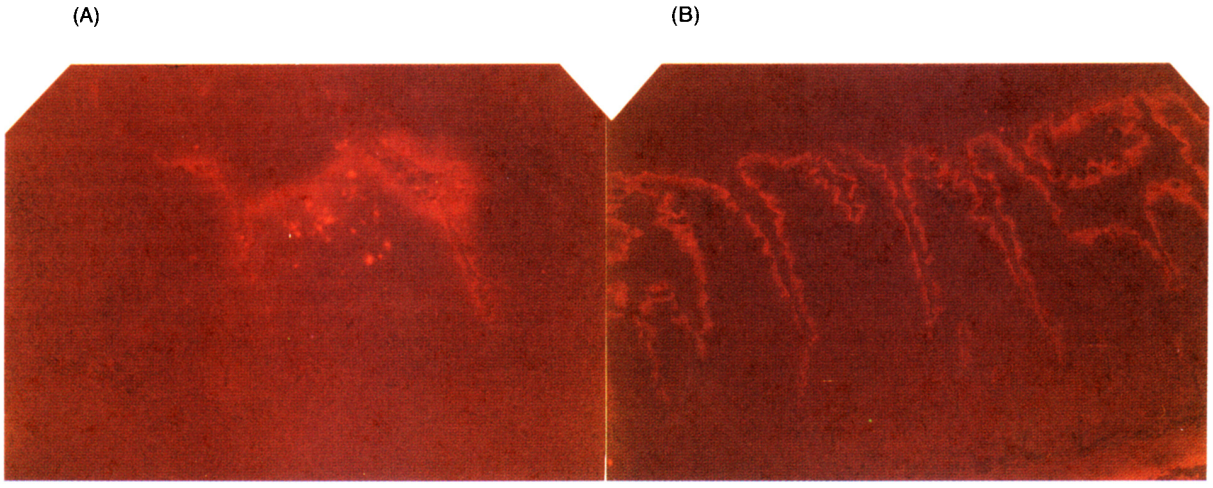

Fig. 5. Fluorescence microscopy of localization of rhodamine liposomes. (A) Peyer's patches; (B) nonpatch tissue. Rhodamine liposomes were injected into the loop of lower ileum. After 1 hr, the Peyer's patches and nonpatch tissue at the lower ileum were removed. ×100; reduced to 70% for reproduction.

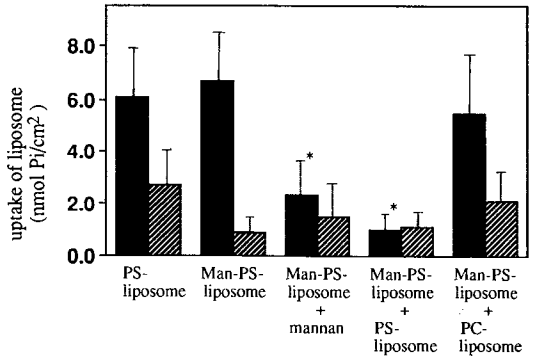

Fig. 6. Effect of mannan coating on the uptake of liposomes. Lipid composition of liposomes was PC:PS:Chol = 7:3:2. PS liposomes 374 nm in mean diameter were used. Following mannan coating, the mean diameter of Man-PS liposomes was estimated as 416 nm. Each bar represents the mean ± SD ($n = 5$). (*) $P < 0.05$, mean uptake of PS liposome by Peyer's patch vs mean uptake of the liposomes in the presence of competitors. (■) Peyer's patches; (□) nonpatch tissue.

enhanced. The effect of the mannan coating on liposome uptake was thus investigated. The uptake of Man-PS liposomes increased slightly over that of PS liposomes (Fig. 6). It was not known that mannose-binding protein is present on the M cell surface, but by adding 100 times as much free mannan, the uptake was reduced to about 35% that of Man-PS liposomes. Mannan thus appears to be involved in the recognition process by M cells. The liposomal surface could not be coated by mannan completely, the ζ potential of Man-PS liposomes being -19.2 mV, which is about 70% of the negative charge of PS liposomes (legend to Fig. 1).

The uptake of Man-PS liposomes was reduced by about 85% by the addition of empty PS liposomes but not PC liposomes (Fig. 6). Some specific mechanism(s) recognizing PS thus appears to be involved in liposomal uptake by Peyer's patches through overlying M cells.

Based on the present results, negatively charged liposomes with larger mean diameters may be effective carriers to Peyer's patches.

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