Stability of Liposomes *in Vitro* and Their Uptake by Rat Peyer's Patches Following Oral Administration

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To evaluate the usefulness of liposomes as a carrier for the targeted delivery of antigens to gut-associated lymphoid tissue, liposomal stability and uptake by rat Peyer's patches were investigated. Liposomes composed of distearoylphosphatidylcholine, phosphatidylserine, and cholesterol (DSPC-liposome), or dipalmitoylphosphatidylcholine, phosphatidylserine, and cholesterol were stable in acidic solution (pH 2.0), diluted bile, and pancreatin solution. Following the oral administration of liposomes to rats, rhodamine B-PE incorporated in the lipid phase of DSPC-liposomes was preferentially taken up by Peyer's patches in the lower ileum. The uptake of rhodamine B-PE from DSPC-liposomes larger than 374 nm in mean diameter was high. Orally administered DSPC-liposomes of a large diameter thus appear to serve effectively as a vehicle for delivering antigens to Peyer's patches.

KEY WORDS: liposome; oral administration; Peyer's patch; oral vaccine.

INTRODUCTION

The mucosal immune system provides a primary defense against the entry of infectious agents from the luminal mucosa into the body (1). Externally secreted IgA antibodies, following the oral invasion or administration of antigens, perform important functions in this system. Peyer's patches located along the small intestine have the central role of antigen uptake and induction of mucosal immune response. The luminal surface of Peyer's patches is covered with a specialized epithelium called microfold cells (M cells). M cells take up luminal antigens by endocytosis and deliver them to underlying lymphoid cells presented in the dome region of Peyer's patches for antigen sensitization of T cells and precursor IgA B cells (2,3). The mucosal immune system is thus considered essential to immune response against orally administered vaccines (4,5). However, extremely large amounts of antigens are required for adequate immune response owing to their degradation by gastric acidity and proteolytic enzymes in the intestinal lumen.

Various substances can be entrapped into aqueous and/or lipid phase of liposomes, and consequently, liposomes have been the object of considerable attention during the past decades as a carrier of the drug or antigen delivery system (4–6). Liposomal uptake by Peyer's patches is poorly characterized. Recently, we reported the preferential uptake of the negatively charged larger liposomes containing phosphatidylserine (PS) by Peyer's patches in rat *in situ* experiments (7) and suggested that they have potential for developing oral vaccines.

In this study, liposomal stability in various solutions and uptake of liposomes containing PS and various kinds of phosphatidylcholines by Peyer's patches following oral administration were examined to assess their adequacy in as a carrier of antigens to Peyer's patches of rat.

MATERIALS AND METHODS

Materials. Dipalmitoylphosphatidylcholine (DPPC) and distearoylphosphatidylcholine (DSPC) were kindly provided by Nippon Oil and Fat Co., Ltd. (Tokyo). Phosphatidylserine (PS) was provided from Nisshin Seiyu Co., Ltd. (Tokyo). Egg yolk phosphatidylcholine (PC), cholesterol, and pancreatin (porcine) were purchased from Wako Pure Chemicals (Osaka, Japan). 6-Carboxyfluorescein (6-CF) and rhodamine B-phosphatidylethanolamine (rhodamine B-PE) were purchased from Eastman Kodak (USA) and Avanti Polar Lipids (AL, USA), respectively. Male Wistar rats (specific pathogen free) weighing 230–250 g were obtained from Japan SLC (Shizuoka, Japan).

Preparation of Liposomes. A mixture of lipids (total lipid, 66 μmol) in chloroform and methanol was evaporated to make a lipid film. For stability experiments, 2.0 mL of 10 m*M* Tris–HCl-buffered saline (pH 7.4) containing 1% 6-CF were added to the lipid film, and multilamellar vesicles (MLV) were prepared by vortexing at an appropriate temperature for 10 min. Nonencapsulated 6-CF was separated from MLV by a Sephadex G-100 column (3.0 × 40 cm). The encapsulated amount of 6-CF in liposomes was about 9 μg/μmol phospholipid. For the liposomal uptake experiment, rhodamine B-PE (0.33 mg) was added as a membrane marker before preparing the lipid film. The rhodamine B-PE-labeled liposomes were successively extruded through polycarbonate membranes (1.0, 0.4, and 0.1 μm; Nuclepore Co., CA, USA) under N_2 gas pressure of 10–40 kg/cm^2. Mean diameter was determined by dynamic laser light scattering equipment (Nicomp Model 370, Pacific Scientific, MD, USA). Liposomes containing DSPC, DPPC, or egg PC were designated DSPC-, DPPC-, or egg PC-liposomes, respectively.

Liposome Stability. Liposomes were incubated in a Tris–HCl solution containing 0.9% NaCl (pH 2.0 or 7.4), 10% bile obtained from rat by the bile duct cannulation technique, or a 2.8% pancreatin solution of 10 m*M* Tris–HCl-buffered saline (pH 7.4), at 37°C for 2 hr. Following incubation, each liposomal suspension was ultrafiltrated by Amicon MPS-1 at 3500 rpm for 20 min (4°C). The concentration of 6-CF in the filtrate was measured by a fluorescence spectrophotometer (excitation, 494 nm; emission, 515 nm; Hitachi 640, Tokyo).

Uptake of Liposomes by Peyer's Patches. One milliliter of liposomal suspension (33 μmol total lipid) was orally administered to a rat which had fasted for 18 hr with the aid of

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a feeding needle. At an appropriate time, the small intestine was isolated, washed with a 0.9% NaCl solution, and divided into four segments of 15 cm, each proceeding from the lower end of the duodenum. They were designated the upper jejunum, lower jejunum, upper ileum, and lower ileum. Peyer's patches and neighboring areas (nonpatch tissues) were cut out as an area of 20 mm^2, and then 1% (w/v) homogenate in H_2O was prepared with a Potter–Elvehjem homogenizer. After extracting rhodamine B-PE by the method of Folch *et al.* (8), its concentration was determined with a fluorescence spectrophotometer (excitation, 530 nm; emission, 580 nm).

RESULTS

Stability of Liposomes. To ensure the stability of liposomes during passage through the gastrointestinal tract, the leakage of entrapped 6-CF from liposomes was investigated *in vitro* (Table I). About 60–65% of entrapped 6-CF was released from liposomes composed of egg PC following incubation in a pH 2.0 Tris–HCl solution containing 0.9% NaCl. Consequently, the stability of liposomes containing DSPC or DPPC having saturated fatty acid side chains was relatively high and was improved by raising the cholesterol content.

Liposomal stability in 10% rat bile was essentially the same as that in Tris–HCl solution containing 0.9% NaCl (pH 2.0). The stability was also influenced by the kind of PC constituting liposomes (DSPC- > DPPC- > egg PC-liposomes) and their cholesterol content.

All liposomes examined in the pH 7.4 Tris–HCl solution containing 0.9% NaCl and pancreatin solution were found to cause the release of less than 10% of entrapped 6-CF.

Effects of Lipid Composition on Uptake. Figure 1 shows the uptake of rhodamine B-PE by Peyer's and nonpatch tissues following the oral administration of liposomes. Rhodamine B-PE associated with Peyer's patches when administered as DSPC-liposomes increased and attained a maximum level after 1 hr of administration. The uptake from egg PC-liposomes was less, and the same as that of free rhodamine B-PE (Fig. 1A). In the case of nonpatch tissues, uptake from DSPC- and egg PC-liposomes was low, and the values were basically the same as those of free rhodamine B-PE (Fig. 1B).

Site Specificity of Liposomal Uptake. One hour following the oral administration of liposomes, the rhodamine B-PE concentration in the different segments of the small intestine was determined. As shown in Fig. 2, rhodamine B-PE was preferentially taken up by Peyer's patches located in the lower ileum. That by patches in the upper parts of intestine was low, and no significant difference could be observed between Peyer's patches and neighboring areas.

Effects of Liposomal Size on Uptake. Figure 3 shows the effects of liposomal size on the uptake of rhodamine B-PE from DSPC-liposomes by Peyer's patches in the different segments of the small intestine. The preferential uptake of rhodamine B-PE from DSPC-liposomes 374 and 855 nm in mean diameter was observed for Peyer's patches in the lower ileum. Those with a mean diameter of 162 nm were significantly taken up by Peyer's patches in the lower ileum, but only to about one-third of liposomes with larger diameter.

DISCUSSION

Peyer's patches have the central role of antigen uptake and induction of mucosal immune response (1). Previously, we have reported that negatively charged liposomes containing PS were preferentially taken up by rat Peyer's patches in *in situ* experiments (7). When liposomes are used as a carrier of orally administered antigens to enhance the mucosal immune response, they should be delivered to M cells without degradation or release of entrapped antigens during passage through the gastrointestinal tract. Thus, the stability of liposomes containing PS and various kinds of PC has been examined in acidic solution, bile, and pancreatin solution. Liposomes containing DSPC or DPPC which have saturated, relatively long fatty acid chains and higher phase transition temperatures (T_c) were stable in each solution, the extent depending on the cholesterol content (Table I). Okuhata and Seki (9) reported the pH-sensitive permeation of a fluorescent probe through synthetic membrane bilayers, and this permeation depended on temperature and occurred only above T_c. The T_c of egg PC, DPPC, and DSPC is −15 to −7, 41.5, and 58.0°C, respectively. Since the membrane fluidity of liposomes composed of PC having a high T_c is usually low, DPPC- and DSPC-liposomes should have a greater solidity than egg PC-liposomes. Consequently, the membrane permeability of DPPC- and DSPC-liposomes may be low and show a high stability in pH 2.0 and 7.4 solutions. Liposomes composed of PC having a high T_c such as DPPC and DSPC

Table I. Stability of Liposomes *in Vitro*

Lipid composition (molar ratio)	Leakage of 6-CF (%)			
	pH 7.4	pH 2.0	10% bile	Pancreatin
Egg PC:Chol, 7:2	6.2 ± 0.1	64.8 ± 2.8	58.7 ± 1.7	4.4 ± 1.7
Egg PC:PS:Chol				
7:3:2	4.9 ± 0.1	62.2 ± 5.4	34.1 ± 0.8	0.4 ± 1.3
1:1:2	3.6 ± 0.4	59.6 ± 3.8	14.1 ± 0.8	8.0 ± 0.6
DPPC:PS:Chol				
7:3:2	7.1 ± 0.8	42.8 ± 4.2	27.2 ± 0.6	6.0 ± 2.3
1:1:2	4.3 ± 0.3	30.0 ± 2.3	6.4 ± 0.6	1.4 ± 0.1
DSPC:PS:Chol				
7:3:2	5.4 ± 0.6	29.6 ± 1.3	10.0 ± 0.6	10.2 ± 1.4
1:1:2	5.5 ± 0.3	20.0 ± 0.1	4.1 ± 0.1	3.7 ± 0.1

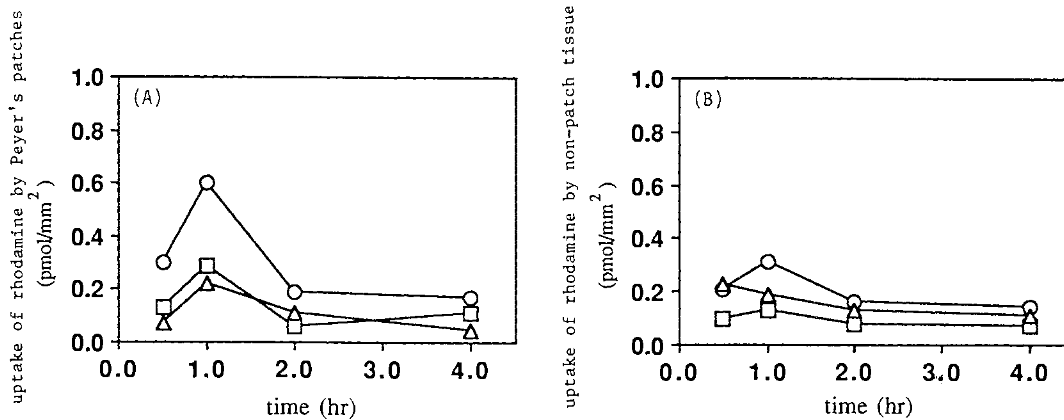

Fig. 1. Uptake of rhodamine B-PE by Peyer's patches (A) and nonpatch tissues (B) following oral administration of liposomes. Each liposome passed through a polycarbonate membrane of 0.4 μm was used. (○) DSPC:PS:Chol, 1:1:2 (molar ratio); (△) egg PC:PS:Chol, 1:1:2 (molar ratio); (□) free rhodamine-PE.

were relatively stable in bile solution, this being consistent with the report of Rowland and Woodley (10). Liposomes containing a high molar ratio of cholesterol showed a high stability in pH 2.0 and in bile solutions (Table I). The membrane fluidity of liposome was previously shown to decrease with increasing cholesterol content by the fluorescence polarization technique (11). Increases in the solidity of liposomes by cholesterol presumably enhance the stability. The relatively low leakage of all kinds of liposomes in pancreatin solution is consistent with the findings that liposome containing cholesterol showed resistance to pancreatic lipase (10). It thus follows that DPPC- and DSPC-liposomes may facilitate the delivery of incorporated materials after oral administration without leakage or degradation.

This possibility was supported by the results that orally administered rhodamine B-PE incorporated in the membrane of DSPC-liposomes was preferentially taken up by Peyer's patches compared to egg PC-liposomes (Fig. 1).

From the duodenum to the cecum of rat intestine, about 20 Peyer's patches could be visually observed. The function of Peyer's patches differs according to site in the intestine, birth, and bleeding environment (12), and age of the animal (13). The uptake of liposomes by Peyer's patches at different segments of intestine was thus investigated, and preferential uptake was observed for Peyer's patches located in the lower ileum (Fig. 2). LeFevre *et al.* noted latex particles to be preferentially taken up by Peyer's patches in the lower and upper small intestine of conventional and germfree mice, respectively (12). Results obtained from specific pathogen free rats in our experiment were in agreement with those for conventional mice. This fact may result from the differences in number and function of M cells overlying Peyer's patches and/or in exposure time of Peyer's patches to liposomes located in different regions of the intestine (12).

The uptake of particulates such as bacteria (14), virus (15), and polymer latex (16) was preferentially taken up by M cells overlying Peyer's patches through endocytosis, and M cells were called antigen sampling cells. The effects of particle size on uptake by Peyer's patches was extensively examined by Eldridge *et al.* (17) and Ebel (18). Microspheres with a mean diameter exceeding 5 μm were taken up by Peyer's patches and retained, but those smaller than 5 μm could be seen in mesentric lymph nodes and spleen as well as Peyer's patches (17), suggesting that particles of adequate size are taken up by Peyer's patches and transported to mac-

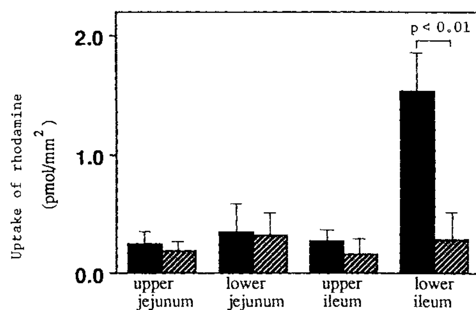

Fig. 2. Uptake of rhodamine B-PE by Peyer's patches located in different sites in the intestine. Lipid composition of liposomes was DSPC:PS:Chol = 1:1:2 (molar ratio). Each liposome passed through a polycarbonate membrane of 0.4 μm was used. Filled and hatched bars indicate the uptake of liposomes by Peyer's patches and nonpatch tissues, respectively. Each bar represents the mean ± SD (n = 3).

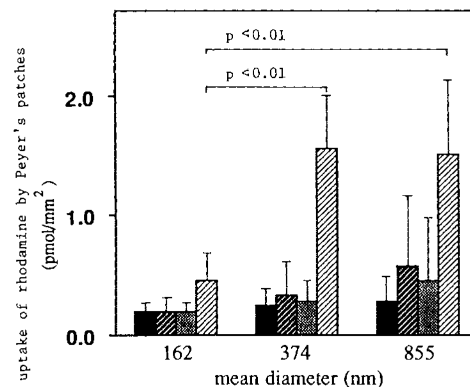

Fig. 3. Effects of liposomal diameter on the uptake of rhodamine B-PE by Peyer's patches located in different sites in the intestine. Lipid composition of liposomes was DSPC:PS:Chol = 1:1:2 (molar ratio). Each bar represents the mean ± SD (n = 3). (■) Upper jejunum; (▨) lower jejunum; (▩) upper ileum; (▧) lower ileum.

rophages through efferent lymphatics. Thus, liposomes taken up by Peyer's patches may be transported into lymph nodes or spleen and show a maximum value at 1 hr following oral administration (Fig. 1). In this study, liposomes of larger size (374 and 855 nm) were preferentially taken up by Peyer's patches in the lower ileum. Allen *et al.* (19) found liposomes of larger size to be preferentially taken up by the reticuloendothelial system such as Kupffer cells of the liver and macrophages of the spleen. M cells have endocytic activity as do macrophages. Thus DSPC-liposomes larger in size may be preferentially taken up by M cells overlying Peyer's patches through endocytosis.

Taking the above findings into consideration, orally administered DSPC-liposomes containing PS of a larger mean diameter may be an effective carrier of unstable antigens attainable adequate mucosal immune response. In addition, as liposomes act as an adjuvant (20), they have potential in developing oral vaccines for weak antigens.

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