Application of Liposomes for Development of Oral Vaccines: Study of *In Vitro* Stability of Liposomes and Antibody Response to Antigen Associated with Liposomes after Oral Immunization

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ABSTRACT. In order to evaluate the usefulness of liposomes as oral vaccines, the stability of liposomes and serum IgA antibody response to antigen associated with liposomes after oral administration were examined. Liposomes composed of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylserine (DPPS), and cholesterol (Chol) (1:1:2, molar ratio), distearoylphosphatidylcholine (DSPC) and Chol (7:2, molar ratio), and DSPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio) were stable in acidic solution (pH 2.0), bile, and pancreatin solution, whereas liposomes composed of DPPC and Chol (7:2, molar ratio) and DPPC, DPPS, and Chol (7:3:2, molar ratio) were unstable in pH 2.0 and/or bile solutions. After the oral immunization of antigen (ganglioside GM1)-containing liposomes composed of DPPC, DPPS, and Chol (1:1:2, molar ratio) to mice, the serum IgA antibody responses against ganglioside GM1 were found. Furthermore, when monophosphoryl lipid A was incorporated into liposomes containing ganglioside GM1, further augmentation of IgA responses to ganglioside GM1 was observed. On the other hand, the oral administration with liposomes composed of DPPC, Chol, and ganglioside GM1 (unstable liposomes), ganglioside GM1 mixed with liposomes composed of DPPC, DPPS and Chol, and ganglioside GM1 alone was unable to induce any detectable anti-ganglioside GM1 IgA antibody responses. These results suggest that liposomes which showed the stability to acidic solution, bile, and pancreatin solution would serve effectively as an oral delivery vehicle for inducing mucosal immune responses. — KEY WORDS: liposome, mucosal immunity, oral administration, oral vaccine, stability.

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The mucosal surfaces represent the major portal of entry of infectious agents into the body. The gut-associated lymphoid tissue (GALT) (*e.g.*, Peyer's patches) has the central role of antigen uptake and induction of mucosal immune response. The luminal surface of Peyer's patches is covered by a specialized epithelium that consists of cuboidal epithelial cells and unique antigen-sampling cells, termed M cells [17, 19]. 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 [15, 18]. It has been established that mucosal immune system plays an important role to protect animals and humans from mucosal infections, independent of systemic immunity [16]. Externally secreted immunoglobulin A (S-IgA) antibodies, following the oral invasion or administration of antigens, perform important functions in this system. S-IgA can neutralize biologically active antigens such as viruses, toxins, and enzymes, prevent uptake of antigens from the intestinal tract, and inhibit adherence of bacteria to epithelial surfaces [15, 17]. Thus, the selective induction of specific S-IgA antibodies is desirable for the prevention of infectious disease contracted through the large area of mucosal surfaces. The oral route of immunization is a trustworthy way to induce a mucosal S-IgA antibody responses. However, this immunization route has the problem of antigen degradation by gastric acidity and proteolytic enzymes in the intestinal lumen, and extremely large doses are required to achieve adequate immune response.

Bilayer vesicles composed of amphiphilic phospholipids (liposomes) have been used as delivery systems for a wide variety of biologically active substance to specific tissues, and have also been used as immunological adjuvants to enhance the immune response to several bacterial and viral antigens [10]. In particular, since the liposomes-entrapped materials are protected from enzymatic attack until they reach the target sites, the potential usefulness of liposomes as carriers and adjuvants for developing oral vaccines has attracted considerable interests during the last few years [1]. Recently, it was shown that liposomes were taken up by rat Peyer's patches following intraluminal and oral administration [6, 13, 26], suggesting that liposomes have potential for developing oral vaccines. Thus far, the oral administration of liposomes has been undertaken for antigens of parasite [23] and bacteria [5, 7–9, 11, 19, 22, 29]. Their potential as adjuvants has been demonstrated in several studies, in which the use of liposome-associated antigens resulted in protective immunity [9, 23]. However, there have been few reports that when liposomes are used as a carrier of orally administered antigens to enhance the mucosal immune response, they are delivered to Peyer's patches without degradation or release of entrapped antigens during passage through the gastrointestinal tract [3]. Therefore, it is important to elucidate the stability of

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liposomes to acidic solution, bile, and pancreatin solution for their practical application of oral vaccine.

In the present study, in order to reveal the potential of liposomes as oral vaccines, we examined the liposomal stability in various solutions. In addition, since it has been reported that, when antigen was given by the oral route, IgA antibody appearing in serum is produced in the intestine and transferred to the serum [12, 20, 25], we also investigated the serum IgA antibody response to antigen associated with liposomes after oral administration.

MATERIALS AND METHODS

Materials: Dipalmitoylphosphatidylserine (DPPS), cholesterol (Chol), monophosphoryl lipid A (MPL) (Sigma Chemical Co., St. Louis, Mo), dipalmitoyl phosphatidylcholine (DPPC) (Nippon Fine Chemical, Osaka), and distearoylphosphatidylcholine (DSPC) (Nippon Oil & Fats, Tokyo) were commercial products. Ganglioside GM1 and pancreatin were purchased from Wako Pure Chemical Industries, Osaka; carboxyfluorescein (CF) from Eastman Kodak (Rochester, NY). Fresh bovine bile was obtained from slaughter-house located in Okayama city.

Mice: Four-week-old BALB/c mice were purchased from Charles River Japan, Tokyo, and maintained in the Animal Center for Medical Research, Okayama University Medical School, Okayama.

Preparation of liposomes: Liposomes entrapping CF were prepared from lipid mixture solution containing DPPC and Chol in a molar ratio of 7:2 (total amount, 14.4 μmol), DPPC, DPPS and Chol in a molar ratio of 7:3:2 (total amount, 14.4 μmol) or 1:1:2 (total amount, 16.0 μmol), DSPC and Chol in a molar ratio of 7:2 (total amount, 14.4 μmol), and DSPC, DPPS, and Chol in a molar ratio of 7:3:2 (total amount, 14.4 μmol) or 1:1:2 (total amount, 16.0 μmol), respectively. For the preparation of liposomes containing CF, the lipids dissolved in chloroform-methanol (1/1,v/v) were dried in a conical flask under reduced pressure and stored *in vacuo* for 30 min. After that, 200 μl of 75 mM CF was added in a lipid film and dispersed by vigorous vortexing after incubation at an appropriate temperature for 3 min. Unencapsulated CF was removed by repeated centrifugation at 95,000 × g for 20 min at 4°C in 10 mM Tris-HCl solution containing 145 mM NaCl (pH 7.4). The final pellet of liposomes was suspended in 200 μl of 10 mM Tris-HCl solution containing 145 mM NaCl (pH 7.4) and used for the analysis of liposomal stability.

Liposomes for oral administration were prepared by the following method. DPPC (0.5 μmol), DPPS (0.5 μmol), Chol (1.0 μmol), and ganglioside GM1 (0.05 μmol), each dissolved in an organic solvent, were mixed in a conical flask. The lipids were dried on a rotary evaporator, followed by standing for 30 min under high vacuum in a desiccator. After addition of 150 μl of saline and incubation at an appropriate temperature for 3 min, the lipid film was dispersed by vigorous vortexing and resulting liposome suspension was orally administered to a mouse. Liposomes prepared from a lipid mixture comprising DPPC (1.58 μmol), Chol (0.45 μmol), and ganglioside GM1 (0.05 μmol) and liposomes prepared from a lipid mixture containing DPPC (0.5 μmol), DPPS (0.5 μmol), Chol (1.0 μmol), MPL (2 or 4 μg), and ganglioside GM1 (0.05 μmol) as described above were also used for oral immunization.

Liposome stability: One hundred μl of liposome suspension (1 μmol total lipid) was incubated with 1.9 ml of Tris-HCl solution containing 145 mM NaCl (pH 2.0 or 7.4), phosphate-buffered saline (PBS; pH 7.2) containing 10% bovine bile, or 2.8% pancreatin solution at 37°C for 1 hr. After incubation, 0.5 ml of liposome suspension incubated with 10 mM Tris-HCl solution containing 145 mM NaCl (pH 2.0) was mixed with an equal volume of 10 mM Tris-HCl solution containing 145 mM NaCl (pH 9.5) and centrifuged at 95,000 × g for 10 min (4°C). On the other hand, liposome suspensions incubated with PBS containing 10% bovine bile or 2.8% pancreatin solution were ultrafiltrated by CENTRICUT mini (Kurabo Ltd., Osaka) at 3,300 × g for 30 min (4°C). The concentration of CF in supernatant (or filtrate) and precipitate was measured with Hitachi F-3010 fluorometric spectrophotometer (Hitachi Ltd., Tokyo) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Immunization of mice: Five BALB/c mice were orally immunized in groups of 4 as follows: group A, liposomes composed of DPPC (0.5 μmol), DPPS (0.5 μmol), Chol (1.0 μmol), and ganglioside GM1 (0.05 μmol); group B, liposomes composed of DPPC (1.58 μmol), Chol (0.45 μmol), and ganglioside GM1 (0.05 μmol); group C, ganglioside GM1 (0.05 μmol) mixed with liposomes composed of DPPC (0.5 μmol), DPPS (0.5 μmol) and Chol (1.0 μmol); group D, ganglioside GM1 (0.05 μmol). Other groups of 5 BALB/c mice were also orally immunized with liposomes composed of DPPC (0.5 μmol), DPPS (0.5 mmol), Chol (1.0 μmol), MPL (2 or 4 μg), and ganglioside GM1 (0.05 μmol) in a volume of 0.15 ml per mouse. Sera were collected at 14 days after immunization and used for antibody assay.

Antibody assay: For determining specific antibody against antigen associated with liposomes present in serum, the enzyme-linked immunosorbent assay (ELISA) was performed as follows: Ganglioside GM1 was dissolved in ethanol to a concentration of 100 μg/ml. This solution was dispensed in 10 μl/well into a 96-well microtiter plate (Sumitomo MS-7896F; Sumitomo Bakelite, Tokyo), followed by standing for 60 min under high vacuum in a desiccator to evaporate the organic solvents. The wells were treated with 100 μl of PBS containing 1% bovine serum albumin (solution A), and incubated for 2 hr at 37°C to block nonspecific binding, and then washed 5 times with PBS containing 0.1% Tween 20 (washing solution). After washing with the washing solution, 50 μl of the serum diluted with solution A was added to each well. The plates were incubated overnight at 4°C, and washed 5 times with the washing solution, and then 50 μl of horseradish peroxidase-conjugated anti-mouse IgA (at 1:2,000 dilution in PBS; Cappel), anti-mouse IgM (at 1:2,000 dilution in PBS; Cappel), or anti-mouse IgG (at 1:1,000 dilution in

PBS; Cappel) solution was added as the second antibody. After incubation for 2 hr at 37°C, the wells were washed 5 times with the washing solution, 50 μl of *o*-phenylenediamine dihydrochloride substrate solution (Sumitomo ELISA Color Reagent Kit; Sumitomo Bakelite) was reacted for 15 min at room temperature. The enzyme reaction was stopped by addition of the stopping solution (Sumitomo ELISA Color Reagent Kit; Sumitomo Bakelite), and absorbance at 492 nm was measured with a microplate spectrophotometer (MTP-32; Corona Electric, Hitachi-naka).

Statistical analysis: Mann-Whitney U-test was performed for statistical evaluation of the results. Less than 0.05 of p value was taken as the level of significance. Results are expressed as the arithmetic mean with the standard deviation of the mean (mean ± S.D.).

RESULTS

Stability of liposomes: In order to confirm the stability of liposomes during passage through the oral-gastrointestinal route, the effect of pH, bile, and pancreatin on the release of entrapped CF from liposomes was studied *in vitro*.

As shown in Table 1, incubation at pH 2.0 Tris-HCl solution containing 145 mM NaCl caused the release of 49.7 ± 10.6% of entrapped CF from liposomes composed of DPPC and Chol in a molar ratio of 7:2. However, liposomes composed of DPPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio), DSPC and Chol (7:2, molar ratio), and DSPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio) were stable at pH 2.0 Tris-HCl solution containing 145 mM NaCl.

Treatment with 10% bovine bile resulted in 74.9 ± 49.4 and 99.9 ± 23.3% leakage of CF from liposomes composed of DPPC and Chol (7:2, molar ratio) and DPPC, DPPS, and Chol (7:3:2, molar ratio), respectively. On the other hand, 10% bovine bile had very little effect on liposomes composed of DPPC, DPPS, and Chol (1:1:2, molar ratio), DSPC and Chol (7:2, molar ratio), and DSPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio).

In contact with 2.8% pancreatin, all liposomes tested were stable. Liposomes composed of DPPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio), DSPC and Chol (7:2, molar ratio), and DSPC, DPPS, and Chol (1:1:2, molar ratio) were particularly stable.

These results suggest that the stability of liposomes containing DSPC, *i.e.*, liposomes composed of DSPC and Chol in a 7:2 molar ratio and DSPC, DPPS, and Chol in a 7:3:2 or 1:1:2 molar ratio, is relatively high, and also indicate that the stability of liposomes composed of DPPC, DPPS, and Chol is improved by the increase of the Chol content.

Antibody responses of sera from mice inoculated orally with antigen-containing liposomes: One of stable liposomes, *i.e.*, liposomes composed of DPPC, DPPS, and Chol in a molar ratio of 1:1:2, was selected and used as a carrier for oral immunization. As a model antigen, ganglioside GM1 was inserted in the liposomes. The stability of liposomes made of DPPC, DPPS, Chol, and ganglioside GM1 in a molar ratio of 10:10:20:1 was also examined in acidic solution (pH 2.0), bile, and pancreatin solution. Liposomes containing ganglioside GM1 tested were stable in each solution (Table 1). Consequently, liposomes made of DPPC, DPPS, Chol, and ganglioside GM1 (10:10:20:1, molar ratio) were administered orally to mice, and antibody response to liposomal antigen was evaluated at 14 days after primary immunization.

As shown in Table 2, production of anti-ganglioside GM1 IgA antibody was demonstrated in serum from mice receiving liposomes composed of DPPC (0.5 μmol), DPPS (0.5 μmol), Chol (1.0 μmol), and ganglioside GM1 (0.05 μmol) (Group A). Whereas, no serum IgG and IgM activity against ganglioside GM1 could be seen in the group A. Furthermore, no anti-ganglioside GM1 antibody responses could be detected in serum of mice immunized with liposomes composed of DPPC (1.58 μmol), Chol (0.45 μmol), and ganglioside GM1 (0.05 μmol) (Group B), ganglioside GM1 (0.05 μmol) mixed with liposomes composed of DPPC (0.5 μmol), DPPS (0.5 μmol) and Chol (1.0 μmol) (Group C), and ganglioside GM1 alone (0.05 μmol) (Group D). IgA antibody responses against ganglioside GM1 in the group A was significantly higher than those in other groups (p<0.01).

Effect of MPL on IgA antibody response to orally administered liposomal antigen: We evaluated the adjuvant MPL for its ability to potentiate the IgA immune response

Table 1. Stability of liposomes *in vitro*

Lipid composition (molar ratio)	Leakage of CF (%)			
	pH 7.4	pH 2.0	10% bile	2.8% pancreatin
DPPC: Chol (7:2)	20.8 ± 6.5	49.7 ± 10.6	74.9 ± 49.4	7.2 ± 2.2
DPPC: DPPS: Chol				
(7:3:2)	11.7 ± 5.1	17.5 ± 11.4	99.9 ± 23.3	2.7 ± 0.1
(1:1:2)	1.8 ± 0.8	16.3 ± 4.8	7.6 ± 0.9	1.9 ± 0.6
DSPC: Chol (7:2)	2.9 ± 2.5	7.5 ± 4.9	10.8 ± 1.3	4.4 ± 1.4
DSPC: DPPS: Chol				
(7:3:2)	0.7 ± 0.2	15.0 ± 11.6	3.8 ± 0.8	11.4 ± 2.6
(1:1:2)	1.0 ± 0.1	22.0 ± 5.5	5.0 ± 0.5	4.1 ± 1.2
DPPC: DPPS: Chol: GM1				
(10: 10: 20: 1)	——	15.6 ± 5.7	1.8 ± 0.3	1.5 ± 0.1

Table 2. Serum antibody responses in mice to ganglioside GM1, alone or in different types of liposomes, administered orally

Immunized mice[b)]	Anti-ganglioside GM1 antibody[a)]					
	IgA		IgG		IgM	
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
Group A	0.27 ± 0.03	0.76 ± 0.43[c)d)e)]	0.05 ± 0.03	0.08 ± 0.03	0.23 ± 0.08	0.32 ± 0.15
Group B	0.15 ± 0.07	0.17 ± 0.03	< 0.02	< 0.01	0.12 ± 0.04	0.20 ± 0.05
Group C	0.14 ± 0.01	0.22 ± 0.03	< 0.02	< 0.02	0.22 ± 0.01	0.17 ± 0.03
Group D	0.08 ± 0.02	0.09 ± 0.01	0.05 ± 0.01	< 0.03	< 0.01	0.09 ± 0.04

a) Antibody activity was detected against ganglioside GM1 by ELISA with absorbance at 492 nm using a 1/10 dilution of serum. b) Four groups of 5 BALB/c mice were orally immunized as follows: group A, liposomes composed of DPPC (0.5 μmol), DPPS (0.5 μmol), Chol (1.0 μmol), and ganglioside GM1 (0.05 μmol); group B, liposomes composed of DPPC (1.58 μmol), Chol (0.45 μmol), and ganglioside GM1 (0.05 μmol); group C, ganglioside GM1 (0.05 μmol) mixed with liposomes composed of DPPC (0.5 μmol), DPPS (0.5 μmol) and Chol (1.0 μmol); group D, ganglioside GM1 alone (0.05 μmol). c) $p<0.01$ compared with value for group B. d) $p<0.01$ compared with value for group C. e) $p<0.01$ compared with value for group D.

Table 3. Effect of monophosphoryl lipid A (MPL) dose on serum anti-GM1 antibody responses in mice after oral administration of ganglioside GM1-containing liposomes[a)]

MPL (μg/mouse)	Anti-ganglioside GM1 antibody[b)]					
	IgA		IgG		IgM	
	Day 0	Day 14	Day 0	Day 14	Day 0	Day 14
0	0.27 ± 0.03	0.76 ± 0.43	0.05 ± 0.03	0.08 ± 0.03	0.23 ± 0.08	0.32 ± 0.15
2	0.27 ± 0.04	1.32 ± 0.57	0.12 ± 0.04	0.07 ± 0.03	0.25 ± 0.06	0.16 ± 0.07
4	0.23 ± 0.13	1.14 ± 0.78	0.07 ± 0.07	0.20 ± 0.14	0.26 ± 0.13	0.34 ± 0.17

a) Three groups of 5 BALB/c mice were orally immunized with liposomes composed of DPPC (0.5 μmol), DPPS (0.5 μmol), Chol (1.0 μmol), MPL (0, 2, or 4 μg), and ganglioside GM1 (0.05 μmol) in a volume of 0.15 m*l* per mouse. Sera were collected at 14 days after immunization and assayed for anti-ganglioside GM1 IgA, IgG, and IgM antibody by ELISA. b) Antibody activity was detected against ganglioside GM1 by ELISA with absorbance at 492 nm using a 1/10 dilution of serum.

to liposomal antigen given by the oral route. Antibody response to liposomal antigen was evaluated at 14 days after the immunization.

As shown in Table 3, mice given liposomes containing ganglioside GM1 and MPL exhibited higher serum IgA antibody responses than animals given liposomes containing ganglioside GM1 alone (without MPL) (data is not statistically significant). The maximum augmentation of IgA antibody responses was obtained when a dose of 2 μg of MPL was given orally with the antigen. Higher dose of MPL (4 μg per mouse) did not potentiate the IgA response to the antigen to a greater degree than a dose of 2 μg of the adjuvant. On the other hand, both major immunoglobulin isotypes in serum, *i.e.*, IgG and IgM, were not enhanced by the oral administration of liposomes containing ganglioside GM1 and MPL.

DISCUSSION

It has been established that mucosal immune system plays an important role to protect animals and humans from mucosal infections, independent of systemic immunity [16]. S-IgA antibodies perform important functions in this system. The oral route of immunization is a trustworthy way to induce a mucosal S-IgA antibody responses. However, this immunization route has the problem of antigen degradation by gastric acidity and proteolytic enzymes in the intestinal lumen. Recently, it was shown that liposomes containing phosphatidylserine were taken up by rat Peyer's patches following intraluminal and oral administration [3, 26]. Thus far, however, there have been few reports that when liposomes are used as a carrier of orally administered antigens to enhance the mucosal immune response, they are delivered to Peyer's patches without degradation or release of entrapped antigens during passage through the gastrointestinal tract [3]. In the present study, therefore, we examined the stability of liposomes to acidic solution, bile, and pancreatin solution. Since it has been shown that the serum IgA titers correlate with S-IgA antibody synthesis in intestine [12, 20, 25], furthermore, we also investigated the serum IgA antibody response to the antigen after oral administration of the antigen associated with liposomes.

In this study, liposomes containing DSPC which have saturated, relatively long fatty acid chains and higher phase

transition temperatures (T_c), *i.e.*, liposomes composed of DSPC and Chol (7:2, molar ratio) and DSPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio), were stable in acidic solution, bile and pancreatin solution. On the other hand, DPPC containing liposomes, such as liposomes composed of DPPC and Chol in a molar ratio of 7:2, were unstable in acidic and bile solutions (Table 1). This may be due to the difference between the T_c of DSPC (58.0°C) and that of DPPC (41.5°C). The membrane fluidity of liposomes composed of PC having a high T_c is usually low. Thus, liposomes containing DSPC should have a greater solidity than liposomes containing DPPC. Consequently, the membrane permeability of liposomes containing DSPC may be low and show a high stability in acidic solution, bile, and pancreatin solution. Indeed, it has been reported that liposomes composed of DSPC having a high T_c are relatively stable in acidic and bile solutions [4]. Furthermore, the stability of liposomes composed of DPPC, DPPS, and Chol in bile solution was improved by the increase of the Chol content (Table 1). This may be mainly attributed to the modification of the T_c by the increase of Chol content. Previously, it has been shown that the membrane fluidity of liposomes decrease with increasing Chol content [27]. An increase in the solidity of liposomes by Chol presumably enhances the stability. The low leakage of all kinds of liposomes tested in pancreatin solution is consistent with the finding that liposomes containing Chol showed resistance to pancreatic lipase [24]. Thus, we concluded that liposomes which showed the stability to acidic solution, bile, and pancreatin solution, such as liposomes composed of DPPC, DPPS, and Chol (1:1:2, molar ratio), DSPC and Chol (7:2, molar ratio), and DSPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio), may serve as an oral delivery vehicles for inducing mucosal immune responses.

In general, most of liposomes intravenously injected into animals are preferentially taken up by the phagocytic cells of the reticuloendothelial system such as Kupffer cells of the liver and macrophages of the spleen [21]. M cells overlying Peyer's patches have endocytic activity as do macrophages. Thus, liposomes which are stable in the gastrointestinal tract would be preferentially taken up by M cells through endocytosis when orally administered. In this study, the oral administration of ganglioside GM1-containing liposomes composed of DPPC, DPPS, and Chol (stable liposomes) (Group A) induced good serum IgA antibody response directed against ganglioside GM1. On the other hand, the oral administration of ganglioside GM1-containing liposomes composed of DPPC and Chol (unstable liposomes) (Group B), ganglioside GM1 mixed with stable liposomes (Group C), and ganglioside GM1 alone (Group D) was ineffective for the induction of the serum IgA antibody responses against ganglioside GM1 (Table 2). These results suggest that stable liposomes composed of DPPC, DPPS, and Chol (1:1:2, molar ratio) are able to deliver to Peyer's patches without leakage or degradation of incorporated antigens during passage through the gastrointestinal tract and then to induce the intestinal IgA response against antigen incorporated into liposomes.

The poor immunogenicity of ganglioside antigen such as ganglioside GM1 is well documented [14]. In the present study, oral immunization of stable liposomes containing ganglioside GM1 in mice produced a serum IgA antibody response to ganglioside GM1, whereas that of ganglioside GM1 alone was unable to induce any detectable serum IgA response (Table 2, Groups A and D), suggesting that stable liposomes possess innate adjuvant properties. Actually, the effectiveness of liposomes as adjuvants for mucosal immune responses when administered orally has been demonstrated in several studies [7]. Incorporation of immunostimulants into liposomes is also useful for the enhancement of the potency of liposomal vaccines. It has been established that MPL has the applicability as an adjuvant for use in human vaccines [28]. In addition, it has also been found that liposomes can serve as a vehicle that allows expression of the adjuvant activity of MPL [1, 2]. We, therefore, proposed MPL as an ideal immunopotentiating agent for liposomal vaccine. In this study, liposomes containing ganglioside GM1 and MPL induced IgA antibody responses in mice greater than those induced by liposmes containing ganglioside GM1 alone (Table 3). This suggests that MPL act as effective adjuvant for potentiating IgA antibody responses on the intestinal mucosa when administered by oral route and that the adjuvant properties of liposomes can be further enhanced by inclusion of adjuvant such as MPL in liposomes.

After oral administration of ganglioside GM1-containing stable liposomes, furthermore, a serum IgA response directed against ganglioside GM1 was induced, but neither the serum IgG nor the IgM antibody response against ganglioside GM1 was detected (Table 2, Group A and Table 3). Previously, it was shown that S-IgA response is typical of antigen administration by oral route [17, 19], which would explain the above observation.

Taking the findings obtained here into consideration, not only liposomes composed of DPPC, DPPS, and Chol (1:1:2, molar ratio) (stable liposomes) but also other liposomes which are stable in acidic solution, bile and pancreatin solution, such as liposomes composed of DSPC and Chol (7:2, molar ratio), and DSPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio), when administered by the oral route, would be useful for the effective carriers of antigens that are susceptible to degradation in the gastrointestinal environment to Peyer's patches. Thus, to elucidate whether other stable liposomes, such as liposomes composed of DSPC and Chol (7:2, molar ratio), and DSPC, DPPS, and Chol (7:3:2 or 1:1:2, molar ratio), are useful for the carriers of unstable antigens to Peyer's patches and are able to induce an immune response in the intestine, studies are in progress. In this study, furthermore, ganglioside GM1 was virtually nonimmunogenic alone but was highly immunogenic in stable liposomes and was even more immunogenic in stable liposomes containing MPL (Tables 2 and 3). Thus, they would have potential in developing oral vaccines for weak antigens.

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