

Evaluation of Stearylamine-Modified Liposomes for the Oral Vaccine Adjuvant

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Abstract

The usefulness of stearylamine (SA)-modified liposomes for the oral vaccine adjuvant in the induction of immune responses was evaluated. Mice were orally immunized with unmodified liposomes containing ovalbumin (OVA) (group I), OVA-containing monophosphoryl lipid A (MPL)-modified liposomes (group II), OVA-containing SA-modified liposomes (group III) or OVA alone (group IV). After immunization, significant OVA-specific antibodies were detected in the serum and intestine from mice of groups I to III, but not in group IV. Especially, intestinal IgG and IgA antibody responses against OVA were significantly higher in mice of group III than in groups I and II. When sera were analyzed for isotype distribution, OVA-specific IgG1 antibody responses were noted in mice of groups I and II, whereas the induction of OVA-specific IgG1 and IgG3 antibody responses was observed in mice of group III. Moreover, substantial production of IFN- γ (Th1-type) and IL-4 (Th2 type) was demonstrated in spleen cells from mice of group III in vitro. These results suggest that the SA-modified liposomes would serve effectively as mucosal vaccine adjuvant for inducing humoral and cell-mediated immune responses.

Keywords: Liposomes; Vaccines; Protective immunity; Th1; Th2

Introduction

Many pathogens cause initially disease by colonizing or penetrating through the mucosal surface of the enteric epithelium [1-4]. The mucosal immune system plays a central role in the primary defense against pathogens by preventing binding of the microbes or their toxins to the epithelium [5-7]. Induction of mucosal immune responses is achieved by the deposition of antigen via the mucosa (e.g., oral route) but not the systemic route [8]. Further, mucosal immunization has been shown to induce antigen-specific immune responses in both mucosal and systemic compartments [8-12]. Although systemic vaccination (e.g., intramuscular injection) can induce effective immune responses in the systemic compartment, it does not result in the generation of antigen-specific mucosal immune responses. Considering infection of pathogens, mucosal vaccination can offer two layers of immunity (mucosal and systemic immune responses) and provide an effective barrier against invasion of pathogens. Externally secreted IgA and local IgG antibodies produced in response to the mucosal invasion or administration of antigens perform important functions in this system [13,14]. It has been reported that these local antibodies are effective in inhibiting the binding of pathogen to the mucosal cells [13]. However, it has been shown that delivery of antigen alone is insufficient for the induction of maximum levels of antigen-specific immune response by mucosal vaccine [8,9]. Thus, it is necessary to coadminister with new adjuvants and carriers for the induction of mucosal immune responses.

Liposomes have been used as immunological adjuvants to enhance the immune response to several bacterial and viral antigens [15-18]. In particular, the potential usefulness of liposomes as adjuvants for developing vaccines has led to considerable interests during the last few years because the materials encapsulated within the liposomes are protected from degradation until they reach the target sites [19]. Several studies have demonstrated the potential of liposomes as

adjuvants [20-29]. In these studies, it is revealed that, depending on the liposomal composition, charge and size, liposomes may have different pharmacokinetics and be formulated to obtain optimal retention and presentation of the vaccine antigens and are avidly taken up by the dendritic cells (DCs) owing to their particulate nature. It is well known that cationic liposomes are able to both enhance and modulate the immune responses. The adjuvant mechanisms of cationic liposomes have been reviewed elsewhere [30]. Different amphiphilic cationic compounds have been tested for inclusion into liposomes and hold promise for vaccine delivery [31-35]. However, relatively little data on their potential mucosal vaccine is inconclusive. Moreover, since many of them are very expensive, cationic liposome vaccines are in limited clinical application. Thus, for the clinical application of the cationic liposome vaccine, inexpensive cationic compounds are required.

Stearylamine (SA) is one of low-priced cationic compounds and SA modification of the liposomes represents an important factor for enhancing their immunoadjuvancy in the induction of antigen-specific immune responses by conventional (injection) route [36]. Therefore, SA-modified cationic liposomes are expected as mucosal (oral) vaccine adjuvant. However, data on the SA-modified cationic liposomes as oral vaccine adjuvant is relatively little. To know the usefulness of SA-modified cationic liposomes as oral vaccine adjuvant, the present study, mice were orally immunized with OVA-containing SA-modified cationic liposomes, and immune responses were evaluated. Our data suggests that SA-modified cationic liposomes can induce strong antigen specific humoral (Th2) and cell-mediated (Th1) immunity as oral vaccine adjuvant.

Materials and Methods

Materials

Dipalmitoyl phosphatidyl choline (DPPC), cholesterol (Chol), monophosphoryl lipid A (MPL), SA, trypsin inhibitor (soybean type I-S), ovalbumin (OVA), and bovine serum albumin (BSA) (SIGMA) were commercial products.

Animals

Female BALB/c mice (6 weeks old) were purchased from Charles River Japan, Tokyo, Japan. They were maintained according to the Standards Relating to the Care and Management of Experimental Animals of Japan. The experiments were carried out in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

Preparation of liposomes

SA-modified liposomes that entrap OVA were prepared according to the method of Watarai et al. [37]. DPPC (4 μ mol), Chol (4 μ mol), and SA (0.4 μ mol), 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 1 ml of PBS containing OVA (5mg/ml) and incubation at an appropriate temperature for 3 min, the lipid film was dispersed by vigorous vortexing. Any unencapsulated OVA were removed by repeated centrifuging at 14,000 x g for 20 min at 4°C in PBS, and the resulting liposome suspension was used for immunization. MPL-modified liposomes were also prepared according to the above procedure using lipid mixture of DPPC (4 μ mol), Chol (4 μ mol), and MPL (16 μ g). Furthermore, unmodified liposomes were prepared according to the above procedure using lipid mixture solution without SA and MPL. The amount of OVA entrapped in liposomes was determined by the following method. Ninety μ l of isopropyl alcohol was added to a 10 μ l suspension of liposome-entrapped OVA (at 3-fold dilution in PBS), followed by vortex mixing. The protein concentration of the resulting solutions was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories), with bovine plasma gamma globulin used as a standard.

Immunization of mice

Mice were divided into 3 groups (5 mice per a group). Each group was orally immunized as follows: group I, unmodified liposomes that entrap OVA (200 μ g protein/150 μ l/mouse); group II, MPL-modified liposomes that entrap OVA (200 μ g protein/150 μ l/mouse); group III, SA-modified liposomes that entrap OVA (200 μ g protein/150 μ l/mouse); group IV, OVA alone (200 μ g protein/150 μ l/mouse). Immunization was repeated two times at 2-week intervals. Two weeks after final immunization, the mice were killed and sera, small intestine (30cm from pylorus), and spleen were harvested. Intestinal antibodies from small intestine regions were collected as described previously [37]. Sera and intestinal antibodies obtained were used for antibody assay. Spleen cells isolated as described previously [38] were used for cytokine measurements.

Antibody assay

Antibody assay was performed according to the method described previously [39].

Cytokine measurements

Measurement of cytokine levels was performed according to the method described previously [39] using spleen cells from non-treated control and group III mice.

Statistical analysis

Student's t-test was employed in the statistical evaluation of the results.

Results

Immune responses in mice immunized orally with OVA-containing SA-modified liposomes:

Mice were administered orally with OVA antigen, such as unmodified liposomes containing OVA (group I), MPL-modified liposomes containing OVA (group II), SA-modified liposomes containing OVA (group III) or OVA alone (group IV), and antibodies against OVA were evaluated at 14 days after final immunization. As shown in Figure 1, in serum from mice receiving OVA alone (group IV), production of anti-OVA IgG and IgA antibody was not demonstrated. On the other hand, serum IgG and IgA activity against OVA could be seen in the groups I, II and III. IgG and IgA antibody responses against OVA in the groups I, II and III were significantly higher than those in group IV (IgG: $p < 0.05$, $p < 0.0001$, $p < 0.005$ compared to groups I, II and III, respectively; IgA: $p < 0.0001$ compared to groups I, II and III). Furthermore, mice immunized with OVA-containing MPL-modified liposomes (group II) showed significantly higher OVA-specific serum IgG antibodies than did mice immunized unmodified liposomes entrapping OVA (group I) ($p < 0.02$) and mice immunized with OVA-containing SA-modified liposomes (group III) ($p < 0.0095$).

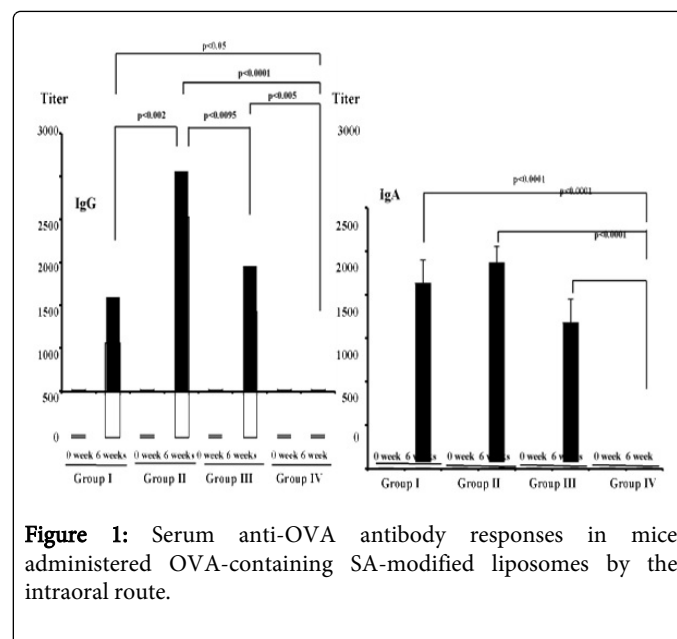


Figure 1: Serum anti-OVA antibody responses in mice administered OVA-containing SA-modified liposomes by the intraoral route.

Mice were immunized orally with unmodified liposomes entrapping OVA (group I) or MPL-modified liposomes entrapping OVA (group II) or SA-modified liposomes entrapping OVA (group III) or OVA alone (group IV), and antibody titers were determined by

ELISA on day 14 following final immunization. Results are expressed as the mean \pm SEM in 5 different mice.

Furthermore, serum antibody responses were characterized by analyzing the pattern of IgG subclasses present in sera from mice in groups I to III. As shown in Figure 2, OVA-specific serum IgG1 antibody responses were demonstrated in the serum from the three groups. In particular, production of IgG1 antibody against OVA was significantly enhanced by the oral administration of MPL-modified liposomes containing OVA (group II) than by unmodified liposomes entrapping OVA (group I) ($p < 0.018$) or by SA-modified liposomes containing OVA (group III) ($p < 0.026$). The induction of OVA-specific serum IgG3 antibody responses was demonstrated in sera from mice in group III ($p < 0.024$ vs group I; $p < 0.023$ vs group II), although the serum IgG2a antibody activities against OVA were not detected in any mice in groups I to III.

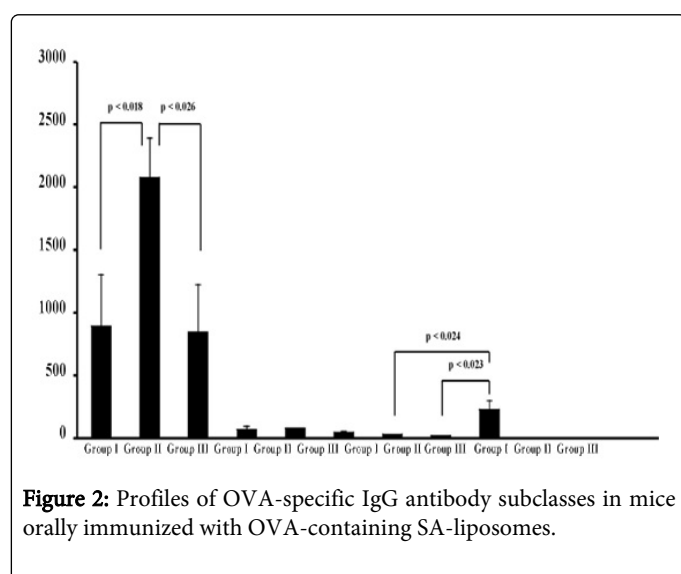


Figure 2: Profiles of OVA-specific IgG antibody subclasses in mice orally immunized with OVA-containing SA-liposomes.

Mice were immunized orally with unmodified liposomes entrapping OVA (group I) or MPL-modified liposomes entrapping OVA (group II) or SA-modified liposomes entrapping OVA (group III) or OVA alone (group IV), and antibody titers were determined by ELISA on day 14 following final immunization. Results are expressed as the mean \pm SEM in 5 different mice.

None of mice in groups I, II and III induced detectable levels of OVA-specific IgE (Figure 2). Next, we investigated whether OVA-specific antibody responses were effectively induced in mucosal compartment by oral immunization. The intestinal anti-OVA antibody responses were evaluated after immunization. Figure 3 shows the OVA-specific intestinal IgG and IgA responses in mice of groups I, II, III and IV. Production of anti-OVA IgG and IgA antibody was demonstrated in intestinal fluid from mice in the groups I, II and III, but not in the group IV. The levels of OVA-specific IgG and IgA in mice of groups I, II and III were much higher than those detected in the group IV (IgG: $p < 0.0001$, $p < 0.0001$, $p < 0.003$ compared to groups I, II and III, respectively; IgA: $p < 0.04$, $p < 0.012$, $p < 0.012$ compared to groups I, II and III, respectively). Among groups I to III, furthermore, IgG and IgA antibody responses against OVA were significantly higher in group III than in group I and group II ($p < 0.05$).

Th1 and Th2 cytokine production by spleen cells from mice immunized intranasally with OVA-containing SA-modified liposomes:

OVA-containing SA-modified liposome-induced antigen-specific serum IgG1 and IgG3 responses suggest efficient major histocompatibility complex presentation of the antigen leading to both humoral (IgG1) (Th2) and cell-mediated (IgG3) (Th1) response (Figure 2). To confirm antigen-specific Th1 and Th2 responses, therefore, cytokine measurements were done by cytokine ELISA. As shown in Figure 4, higher levels of both Th1 (IFN- γ) and Th2 (IL-4) cytokines were detected in the culture supernatant harvest from in vitro OVA-stimulated spleen cells from mice in group III than did spleen cells from non-treated control mice (IFN- γ , $p < 0.035$; IL-4, $p < 0.0007$).

Discussion

The mucosal immune system plays a central role in the primary defense against pathogens by preventing binding of the microbes or their toxins to the epithelium [40-42]. Thus, the development of mucosal vaccines is of great importance in veterinary medicine and new adjuvants are essential to this aim. In addition, efficient vaccine delivery systems have also been required for achievement of protective immunity. Previously, we have reported that liposomes are an effective antigen-delivery vehicle for the induction of systemic and mucosal immune responses [18,24,37,43]. In addition, it has been shown that cationic liposomes are the most effective liposomal delivery systems for vaccine antigens compared with other liposome system (anionic and neutral liposomes) [30]. The use of cationic liposomes as antigen-delivery vehicles in vaccine is well-documented method to increase the immune recognition against otherwise inert or poorly immunogenic subunit proteins [44]. SA is one of cationic compounds. Incorporated in liposomal membrane, it leads to a positive (cationic) surface charge and can enhance liposomal immunoadjuvancy in the induction of antigen-specific immune responses [36]. However, data on the SA-modified cationic liposomes as mucosal vaccine adjuvant is relatively little. In this study, thus, we used the SA-modified cationic liposomes as adjuvant for mucosal vaccine, especially oral vaccine, and induction of systemic and local (mucosal) immune responses was evaluated.

It has been established that liposomes have an application possibility as an adjuvant for use in vaccines [15,16, 43]. In this study, none of mice receiving OVA alone (group IV) showed the production of anti-OVA IgG and IgA antibody in serum and intestine (Figures 1 and 3). However, the oral administration of unmodified liposomes containing OVA (group I), MPL-modified liposomes containing OVA (group II), and SA-modified liposomes containing OVA (group III) induced not only good serum IgG and IgA responses against OVA (Figure 1), but also good intestinal IgG and IgA responses against OVA (Figure 3). In particular, MPL-modified liposomes containing OVA (group II) induced serum IgG responses in mice greater than those induced by OVA-containing unmodified liposomes (group I) and by OVA-containing SA-modified liposomes (group III) (Figure 1). Intestinal IgG and IgA antibody responses against OVA, on the other hand, were significantly higher in group III than in group I and group II ($p < 0.05$) (Figure 3). These results indicate that liposomes function as effective mucosal adjuvant for increasing IgG and IgA responses in the serum and intestinal mucosa when immunized by oral route and that the adjuvant effect of liposomes can be further elevated by inclusion of MPL in liposomes for potentiating IgG antibody responses in the serum, whereas the adjuvant effect of

liposomes resulted in further potentiating IgA antibody responses in the intestinal mucosa by inclusion of SA in liposomes.

Furthermore, we estimated whether OVA entrapped within liposomes induce IgE production, because IgE shows harmful effects, such as allergy. In the present study, an induction of IgE antibody against OVA was not observed in mice orally immunized with OVA in association with liposomes, such as unmodified liposomes containing OVA (group I), MPL-modified liposomes containing OVA (group II), SA-modified liposomes containing OVA (group III) (Figure 2). This suggests that liposomes might serve as a mucosal adjuvant without detrimental effects, such as allergic responses.

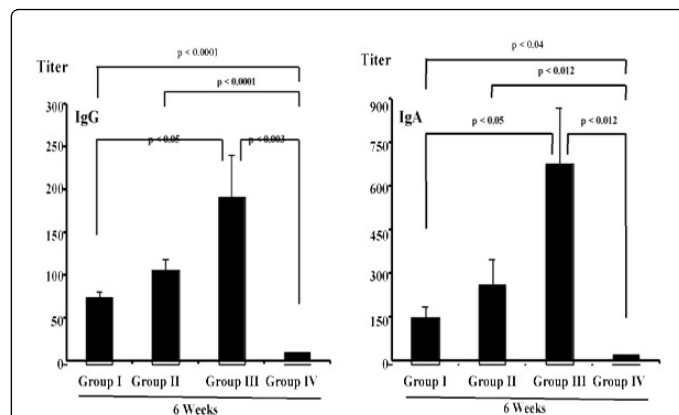


Figure 3: Intestinal anti-OVA antibody responses in mice administered OVA-containing SA-modified liposomes by the intraoral route.

Mice were immunized orally with unmodified liposomes entrapping OVA (group I) or MPL-modified liposomes entrapping OVA (group II) or SA-modified liposomes entrapping OVA (group III) or OVA alone (group IV), and antibody titers were determined by ELISA on day 14 following final immunization. Results are expressed as the mean \pm SEM in 5 different mice.

Th2-type cytokines are pivotal for regulation of IgG1 antibody responses, while Th1-type cytokines support IgG2a and IgG3 antibody responses [45]. In this study, it was shown that oral immunization with OVA-containing unmodified liposomes (group I), with MPL-modified liposomes having OVA (group II) and with SA-modified liposomes in association with OVA (group III) induced antigen-specific IgG1 antibody responses (Figure 2). Especially, a significant increase in the titer of IgG1 antibody was noted in mice immunized MPL-modified liposomes containing OVA (group II). This result suggests that oral immunization with antigen-containing MPL-modified liposomes induces antigen-specific Th2 responses predominantly. In this study, on the other hand, oral immunization with SA-modified liposomes in association with OVA (group III) induced not only antigen-specific IgG1 antibody production, but also IgG3 antibody production (Figure 2). This finding suggests that SA-modified liposomes were potent to induce both a humoral (Th2-type) (IgG1) and a cell-mediated (Th1-type) (IgG3) response. Actually, this was corroborated by the production of cytokines IFN- γ (Th1) and IL-4 (Th2) (Figure 4). After antigens are taken up by DCs, in general, most of the antigens are probably delivered into processing pathways for MHC class II presentation of peptides (Th2-type response), but some antigens might escape from phagosomes into the cytoplasmic

compartments and be delivered into MHC class I presentation pathways (Th1-type response) [46-50]. Although little is known about this "leakage" from phagosome to the cytoplasm, it is conceivable that a large number of antigens should be incorporated into DCs in order for them to be presented MHC class I. Interactions between liposomes with immune cells such as DCs have been studied. Liposomal properties such as charge, size, and lipid composition have been shown to affect liposomal uptake by macrophages [51]. Cellular uptake of liposomes is generally believed to be mediated by the adsorption of liposomes onto the cell surface and subsequent internalization and endocytosis. It would be accepted that positively charged SA-modified liposomes could be taken up in large quantities. Internalized SA-modified cationic liposomes may induce both a humoral (Th2-type) and a cell-mediated (Th1-type) response.

A new immunizing method using cationic compound (SA)-modified liposomes would clearly be worth. To our knowledge, this study is the first report about usefulness of cationic compound (SA)-modified liposomes as mucosal vaccine adjuvant. We have provided here evidence for induction of antigen-specific humoral and cell-mediated immune responses. The present results will provide useful information for the design of oral vaccine. Furthermore, this cationic compound (SA)-modified liposome vaccine adjuvant would be effective in inducing protective immunity, thereby facilitating extirpation of the disease.

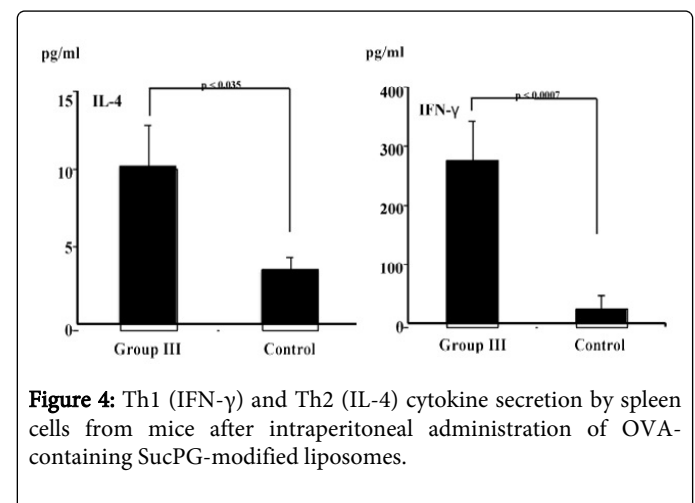


Figure 4: Th1 (IFN- γ) and Th2 (IL-4) cytokine secretion by spleen cells from mice after intraperitoneal administration of OVA-containing SucPG-modified liposomes.

Spleen cells were harvested on day 14 after final immunization and cultured with OVA for 5 days. Subsequently, culture supernatants were collected for the analysis of cytokine production by ELISA. Values represent the mean \pm SEM of cytokine production by spleen cells of mice in each group (OVA-containing SA-modified liposome-immunized mice [Group III] and non-treated control mice [Control]).

Conclusions

In conclusion, this study was carried out to evaluate the usefulness of cationic compound (SA)-modified liposomes as mucosal (oral) vaccine adjuvant. It was confirmed that SA-modified liposomes could serve effectively as mucosal (oral) vaccine adjuvant for inducing humoral and cell-mediated immune responses.

In summary, it is expected to use SA-modified liposomes as a mucosal (oral) vaccine adjuvant for the induction of protective humoral and cell-mediated immunity.

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