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Enhanced desensitization efficacy by liposomal conjugation of a specific antigen

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Abstract

Since liposomes are known as strong adjuvants, we attempted to use liposomes in immunotherapy as adjuvants, and to achieve desensitization in pre-sensitized mice. At first, we sensitized mice with intraperitoneal injection of model antigen, 100 μg ovalbumin (OVA), with Alum and treated them with liposome composed of distearoylphosphatidylcholine (DSPC) and cholesterol (2:1 as a molar ratio), which was coupled with a small amount of OVA (10 μg OVA in 400 nmol DSPC and 200 nmol cholesterol-liposome was injected into 20 g mouse). It is well known that antigen-specific immunotherapy increases IgG blocking antibodies and decreases in IgE antibodies. The treatment with i.v. injection of OVA-liposome at days 8, 10, and 12 after sensitization strongly suppressed OVA-specific IgE production without affecting IgG level after the boost (100 μg OVA with Alum). Moreover, the treatment with high-density OVA-liposome (10 μg OVA in 80 nmol DSPC and 40 nmol cholesterol-liposome/20 g mouse) not only strongly suppressed IgE levels but also reduced IgG production after the boost of OVA-sensitized mice suggesting the importance of liposomal characteristic in desensitization immunotherapy. Next we reduced the dose of OVA-liposome and the desensitization effect was also observed at the dose of as low as 1 μg OVA on OVA-liposome/mouse. On the contrary, free OVA did not affect the production of both IgG and IgE levels. Biodistribution study indicated that OVA-liposome was highly accumulated in spleen of OVA-sensitized mice compared to control liposome at 3 h after i.v. injection. These results suggest that the liposomal OVA effectively interacts with and desensitizes immune cells, therefore, liposomes coupling with a certain antigen may be effective in allergy immunotherapy.
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Keywords: Liposome; Ovalbumin (OVA); IgE; Desensitization; Allergy

1. Introduction

It is well known that proteins and peptides administered in a body are poorly immunogenic. Therefore, adjuvant is required for a vaccine to specific molecules. Most effective and widely used adjuvants for obtaining adequate immune responses are aluminum based compound Alum and complete Freund's adjuvant (CFA). Alum and CFA induce T helper 2 (Th2)-response and Th1-response, respectively (Beck and Spiegelberg, 1989; Singh and O'Hagan, 1999). Liposomes are also known to have adjuvant effect. Liposomal encapsulation enhanced immune response against encapsulated proteins, peptides and so on (Schijns, 2000). Therefore liposomes have been used as adjuvant for various vaccines for the therapy of tuberculosis, leishmaniasis, meningococcal infection and so on (Holten-Andersen et al., 2004; Sharma et al., 2006; Humphries et al., 2006). By the way the differential immune responses are induced by antigen-encapsulated liposomes and antigen-coupled liposomes. The antigen-coupled liposomes appear to preferentially stimulate CD4 + T cells (Fortin et al., 1996). It is said that antigen-coupled liposome increases IgG blocking antibodies and decreases in IgE antibodies through the shifting of T cell response from Th2 to Th1. Therefore, liposomal adjvant effect is adequate for allergy immunotherapy (Audera et al., 1991). In contrast, Taneichi et al. (2002) reported that suppression of IgE production by using surface-linked liposomal antigen might not be involved T cells. Although mechanism of IgE-selective suppression induced by antigen-coupled liposome is not clear, antigen-coupled liposome may hold great promise for desensitization therapy of

Abbreviations: ADM, adriamycin; DSPC, distearoylphosphatidylcholine; OVA, ovalbumin

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allergy. Naito et al. (2002) showed that oral pre-treatment with ovalbumin (OVA)-liposome suppressed IgE production without increase in IgG levels. However, this reason why OVA-specific IgG levels was not enhanced is unclear.

The most therapy for immune disease is only symptomatic treatment. But the desensitization therapy is prospective as one of the basic therapy for immune disease. Although the present desensitization therapy by only antigen takes a long time to treat the immune disease. Therefore, the therapy by antigen-coupled liposome considered to be effective in less time. In addition, although previously reported studies on desensitization therapy were generally performed as pre-treatment of antigens with oral, nasal (Yoshikawa et al., 2000) or intraperitoneal (Naito et al., 1996) administration, one has to study on the treatment post-sensitization in view of clinical practice. Moreover, to prevent anaphylactic shock of administration, it is necessary to reduce amount of antigen.

In the present study, we examined the suppression of OVA-specific IgE level by the post-treatment with a small amount of OVA-liposome. In fact, liposomal OVA suppressed not only IgE but also IgG levels after the boost, suggesting that liposomes are useful for the treatment of allergy.

2. Materials and methods

2.1. Preparation of liposomes

Distearoylphosphatidylcholine (DSPC, gift from Nippon Fine Chemical Co. Ltd., Hyogo, Japan) and cholesterol (Sigma, St. Louis, MO, USA) were dissolved in chloroform, dried under reduced pressure and stored *in vacuo* for at least 1 h. Liposomes were prepared by hydration of the thin lipid film with PBS, and frozen and thawed for three cycles using liquid nitrogen. Then, the liposomes were sized by extrusion through a polycarbonate membrane filter with 100 nm pores. For a biodistribution study, a trace amount of [^{3}H]cholesterol oleoyl ether (Amersham Pharmacia, Buckinghamshire, UK) was added to the initial chloroform solution.

2.2. Coupling of OVA to liposomes

OVA was purchased from Sigma (St. Louis, MO, USA). OVA solution (33.2 mg of OVA in 0.5 mL of 20 mM borate buffer, pH 8.0) was added with 3-(*N*-succinimidyloxy-glutaryl) aminopropyl, polyethyleneglycol-carbamyl distearoyl-phosphatidylethanolamine (DSPE-PEG-NHS, Nippon Oil and Fat Co. Ltd., Tokyo, Japan) (0.149 mg/0.5 mL OVA solution), and incubated overnight at 4 °C. A mixture of OVA and DSPE-PEG-NHS solution (0.5 mL) was mixed with liposomal solution (20 mM as DSPC), and incubated for 30 min at 65 °C. Uncoupled OVA was removed by Sepharose CL-4B (Amersham Biosciences, Sweden) column chromatography.

2.3. Quantification of OVA by HPLC

The OVA-liposomes were dissolved in 2% *n*-octyl-β-D-glucoside (DOJIN, Japan), and heated for 30 min at 80 °C. The amount of OVA coupled with liposome was analyzed by HPLC (Shimadzu, Japan) with TSK-GEL G3000SW column (TOSOH, Japan). The mobile phase for the HPLC analysis was composed of 0.1 M $NaH_2PO_4 \cdot 2H_2O$, 0.3 M Na_2SO_4 and 0.1% SDS, pH 6.7.

2.4. Schedule of immunization

Female Balb/c mice (Japan SLC Inc.) aged 6 weeks at the onset of sensitization were used in all experiments. Mice were sensitized i.p. with 100 μg OVA and Imject® ALUM (2 mg Al $(OH)_3$, 2 mg Mg $(OH)_2$/mouse, Pierce, USA). At days 8, 10, and 12 after sensitization, mice were injected with liposomes via a tail vein. At day 15, mice were boosted with the same antigen used in sensitization. Serum samples were collected on 7, 14, and 22 days post-sensitization. The animals were cared for according to the animal facility guidelines of the University of Shizuoka.

2.5. IgG antibodies assays by ELISA

Maxisorp plates (Nunk, Roskilde, Denmark) were coated for 2 h at r.t. with OVA (100 μg/mL in PBS). After blocking with 1% gelatin-PBS for overnight at 4 °C, 1/10 000 dilution of serum samples in 1% gelatin-PBS was added and incubated for 2 h at r.t. Mouse monoclonal anti-chicken egg albumin antibody, clone OVA-14 (Sigma) was used for obtaining standard curve of OVA-specific IgG. OVA-specific IgG in the test sample was detected by the addition of HRP-Goat Anti-Mouse IgG (H + L) Conjugate (ZyMaX™ Grade, Zymed Laboratories Inc.), and Sigma Fast™ *o*-phenylendiamine dihydrochloride tablet sets (Sigma, St. Louis, MO, USA). The concentration of antibodies was determined by 490 nm absorbance. PBS containing 0.05% Tween 20 was used as a washing solution.

2.6. IgE antibodies assays by ELISA

Maxisorp plates were coated for 2 h at r.t. with purified rat anti-mouse IgE monoclonal antibody (2 μg/mL) (BD, Pharmingen). After blocking with 1% gelatin-PBS for 2 h at r.t., 1/3 dilution of serum samples were added and incubated for overnight at 4 °C. Monoclonal antibody to ovalbumin, clone 2C6 (Acris Antibodies GmbH, Germany) was used for obtaining standard curve of OVA-specific IgE. The biotinylated OVA (10 μg/mL) prepared with an EZ-LINK sulpho-NHS-LC biotinylation kit (Pierce) was added to the wells, and peroxidase-conjugated goat anti-biotin antibodies were used as a secondary antibody.

2.7. Biodistribution of liposomes

Six-week-old BALB/c female mice (SLC, Shizuoka, Japan) were intrapertoneally sensitized with a mixture of OVA and Imject® Alum (2 mg $Al(OH)_3$, 2 mg Mg $(OH)_2$/mouse). Ten days after sensitization, the mice were injected with radiolabeled-OVA-liposome (OVA-lipo) or radiolabeled-antigen uncoupled-liposome (Cont.lipo) via a tail vein. After

3 h, the mice were sacrificed under diethylether anesthesia. The radioactivities in injected solution and in each organ were determined with a liquid scintillation counter (Aloka LSC-3100).

2.8. Statistical analysis

Student's test was used for statistical analysis, and $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Suppression of OVA-specific IgE by the treatment of OVA-liposome

At first, we examined the desensitization by the treatment with OVA-liposome by use of OVA-sensitized mice. OVA-liposome (10 μg OVA, 400 nmol DSPC, and 200 nmol cholesterol/20 g mouse) was injected intravenously into OVA-sensitized mice, and serum IgG and IgE levels in these mice were determined. Table 1 shows the amounts of OVA-specific IgG and IgE after the treatment with OVA-liposome. Two days after the thrice administrations of OVA-liposome (14 days after sensitization), OVA-liposome-treated group showed high IgG level compared to that of the control group indicating that OVA-liposome acted as booster. One week after the boosting by the i.p. injection of 100 μg of free OVA with Alum (22 days after sensitization), OVA-liposome-treated group and control group showed similarly high IgG levels. On the contrary, OVA-specific IgE level was low in OVA-liposome-treated group compared with that in control.

Next, we prepared high-density OVA-liposome for the treatment of OVA-sensitized mice: Namely, 10 μg OVA was conjugated to liposome composed of 80 nmol DSPC and 40 nmol cholesterol, instead of that of 400 nmol DSPC, and 200 nmol cholesterol. High-density OVA-liposome was prepared with a small amount of lipids. Therefore, the density of OVA on each liposome was increased. By use of this liposome, the effect of OVA-liposome treatment on IgG and IgE production was investigated. As shown in Fig. 1, IgG level of OVA-liposome-treated group was about 6 times higher than that of control group 2 days after thrice administration of OVA-liposome (14 days after sensitization). This result indicates that liposomal OVA had a little booster effect. Interestingly, 1 week after the first boost (22 days after sensitization), OVA-liposome-treated group induced suppression of IgG1 level: The OVA-specific IgG level was about a half of that of control. On the other hand, IgE level of OVA-liposome-treated group was greatly suppressed compared to that of control. These data suggest that the liposomal adjuvant effect is dependent on the concentration of antigen in one liposome rather than the total amount of antigen injected.

Table 1
The amount of OVA-specific antibodies in OVA-sensitized mice after the treatment with low-density OVA-coupling liposome

Treatment	IgG (μg/mL)		IgE (ng/mL)
	Day 14	Day 22	Day 22
Control	8.2 ± 2.5	648.7 ± 419.7	448.3 ± 343.7
OVA-lipo	58.9 ± 30.3*	574.4 ± 148.6	109.3 ± 76.9

Six-week-old female Balb/c mice ($n = 6$) were sensitized with an i.p. injection of 100 μg OVA per 20 g body weight. OVA-sensitized mice were i.v. injected with control (0.3 M glucose) or OVA-liposome (10 μg OVA, 400 nmol DSPC, 200 nmol cholesterol/20 g mouse) 8, 10, 12 days after sensitization. At day 15, mice were boosted with OVA (100 μg/20 g mouse) in Alum. Before (day 14) and after (day 22) boosting, blood samples were collected. The levels of OVA-specific IgG and IgE in serum were determined by ELISA. Results are represented the mean ± S.D. Significant difference, $^{*}p < 0.001$ vs. control group.

3.2. Suppression of the IgE level by the treatment of a small amount of OVA (1 μg)-liposome

Since desensitization effect was more obvious for the treatment of mice with 10 μg lipids-reduced liposomal OVA than for that with 10 μg lipids-rich liposomal OVA, we next examined further reduced the amount of high-density OVA-liposome for desensitization. OVA-liposome (1 μg OVA, 8 nmol DSPC and 4 nmol cholesterol/20 g mouse) was injected intravenously into OVA-sensitized mice, and effect of OVA-liposome on IgG1 and IgE production was investigated. After the boost,

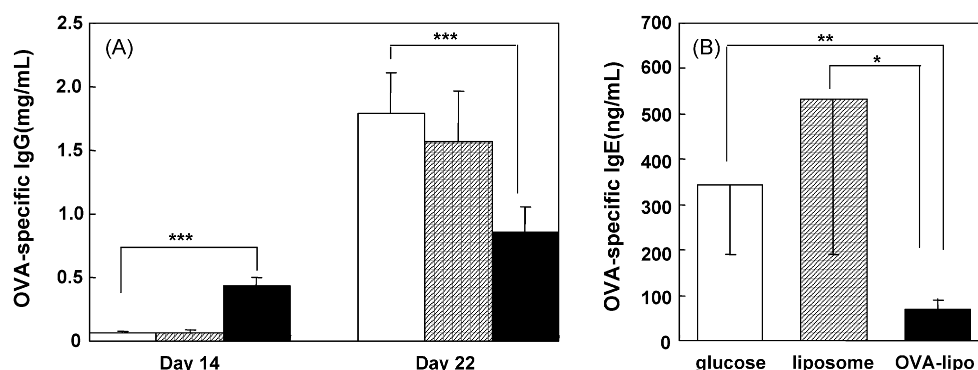

Fig. 1. The levels of OVA-specific antibodies after the treatment of OVA-sensitized mice with high-density OVA-coupling liposome. OVA-sensitized mice were intravenously treated with 0.3 M glucose (open bar) or liposome (hatched bar) or OVA-liposome (10 μg OVA, 80 nmol DSPC, and 40 nmol cholesterol/20 g mouse, closed bar) 8, 10, 12 days after sensitization. At day 15, mice were boosted with OVA (100 μg/mouse) in Alum. 14, 22 days after sensitization, blood samples were collected. The levels of OVA-specific IgG and IgE in serum were determined by ELISA. (A) OVA-specific IgG levels 14 and 22 days after sensitization. (B) OVA-specific IgE levels 22 days after sensitization. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

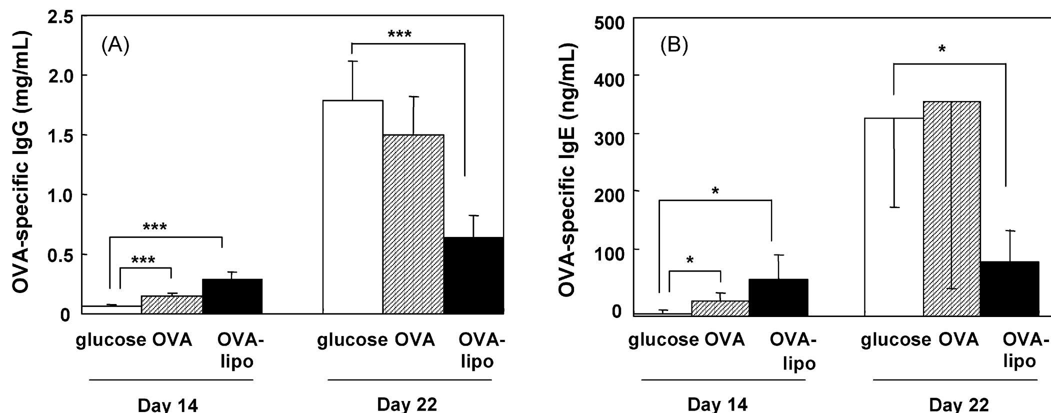

Fig. 2. Effect of low dose of free OVA or OVA-liposome on the levels of OVA-specific antibodies. OVA-sensitized mice were intravenously treated with 0.3 M glucose (control), OVA solution (1 μg OVA/20 g mouse), or OVA-liposome (1 μg OVA, 8 nmol DSPC, and 4 nmol cholesterol/20 g mouse) 8, 10, 12 days after sensitization. At day 15, mice were boosted with OVA (100 μg/20 g mouse) in Alum. At 14, 22 days after sensitization, blood samples were collected. The levels of OVA-specific IgG and IgE in serum were determined by ELISA. (A) OVA-specific IgG levels 14 and 22 days after sensitization. (B) OVA-specific IgE levels 22 days after sensitization. $^{*}p < 0.05$, $^{***}p < 0.001$.

OVA-liposome-treated group induced suppression of IgE levels even at the dose of as low as 1 μg OVA per mouse (Fig. 2).

3.3. Biodistribution of OVA-liposome

Since one liposome bore about 20 molecules of OVA for lipids-rich OVA-liposome and about 200 molecules for high-density OVA-liposome on their surface by a calculation, both types of liposomes might interact with immune cells stronger than a free OVA molecule due to multi valency: This might cause stronger immune response. If this is true, it is possible that OVA-liposome accumulate in organs which bear a number of immune cells, namely spleen. Therefore, we finally examined the biodistribution of OVA-liposome by using a high-density OVA-liposome composed of 1 μg OVA, 8 nmol DSPC, 4 nmol cholesterol in OVA-sensitized mice. At 10 days after sensitization, mice were injected intravenously with radiolabeled OVA-liposome and the biodistribution was determined 3 h after the injection. Both OVA-liposome and control liposome were accumulated in liver nearly 90% of injected dose, and specific accumulation was not observed in other organs except spleen (data not shown). In spleen, OVA-liposome was highly accumulated compared to control liposome as expected (Fig. 3).

4. Discussion

Immunotherapy by antigen desensitization has been achieved in atopic asthma (Adkinson et al., 1997), rhinitis (Tulic et al., 2004) and dermatitis (Juji et al., 2003). Since liposome has been known as safe and efficient carrier of antigen, liposome-mediated desensitization is expected to be effective in immunotherapy. In the desensitization study by using antigen-coupled liposome, antigen-specific IgG level is reported to be increased, while IgE level is decreased (Van Neerven et al., 2006). Because liposome suppressed IgE level, liposome may be good adjuvant in clinical practice. However, actual therapy of allergy needs to treat post-sensitized patients with antigen. In the present study, we performed desensitization of pre-sensitized mice with OVA, a model antigen. OVA-liposome suppressed OVA-specific IgE production after boost without or with only partially affecting IgG level. In fact, liposome is reported to reduce the efficient amount of antigen in allergy immunotherapy (Mueller et al., 2005). In the present experiment, 1 μg of free OVA did not affect the production of both IgG and IgE after the boost. Therefore, free antigen is not effective for desensitization. These results indicate that the liposomal formulation of OVA is important for the suppression of IgE production.

We speculate that the IgG produced by the first sensitization of OVA bound OVA-liposomes injected next, and OVA-liposome might suppress OVA-specific IgE production

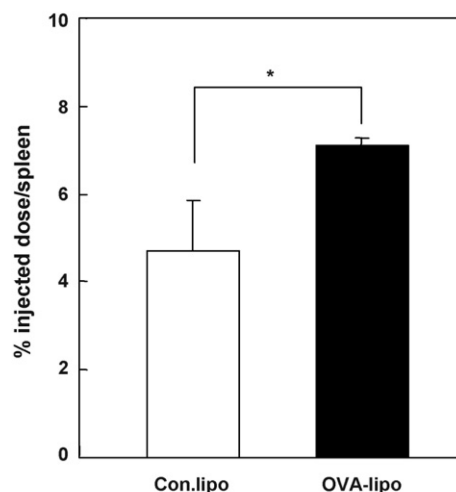

Fig. 3. Biodistribution of liposome in spleen of OVA-sensitized mice. Radiolabeled liposomes were prepared as described in the text. Ten days after sensitization, OVA-sensitized mice were injected with control liposome or OVA-liposome (1 μg OVA, 8 nmol DSPC, 4 nmol cholesterol/20 g mouse) into the tail vein. The mice were sacrificed under diethyl ether anesthesia at 3 h after the injection, and the liver, the spleen, the lung, the kidney, and the heart were removed. Then the radioactivity in each organ was determined with a liquid scintillation counter. Since the specific accumulation of OVA-liposome was only observed in spleen, only the data obtained from spleen was represented. Significant differences are indicated as follows: $^{*}p < 0.05$.

after boosting. Alternatively, liposomal antigen affects immune systems as follows. In general, Th1 is involved in the suppression of IgE production induced by antigen. Sefra and coworkers reported that repeated injection of liposomal antigen preferentially induced Th1-type response dominated by INF-γ and IL-2 production, and the induction of a high IFN-γ:IL-4 ratio resulted in decreased synthesis of IgE and reduced histamine release on boost of free antigen (Sefra et al., 1998). However, Taneichi reported a polarized Th1 response was not observed in mice immunized with OVA-liposome (Taneichi et al., 2002). They demonstrated IgE-selective unresponsiveness induced by OVA-liposome did not involve CD4 + T cells and splenic non-B cells. They suggested that B cells might play a key role in the induction of IgE-selective unresponsiveness by OVA-liposome.

The biodistribution study of OVA-liposome resulted in higher accumulation of OVA-liposome than control liposome in spleen (Fig. 3). It is reasonable, since spleen bears a number of immune cells. Although the majority of both OVA-liposome and control liposome were accumulated in liver, another organ functioning reticuloendothelial system, in which both liposomes might be degraded.

In conclusion, our present study suggests that antigen-coupled liposome is applicable not only as a vaccine adjuvant but also desensitization therapy after sensitization.

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