

An Increased Adjuvant Activity of Liposomes by the Inclusion of Phosphatidylserine in Immunization with Surface-Coupled Liposomal Antigen

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Key Words

Phosphatidylserine · Liposome · Antigen presentation · Adjuvant · Lipid component

Abstract

Background: Exposure of phosphatidylserine (PS) on apoptotic cells is known to result in the enhanced recognition of apoptotic cells by phagocytes. By the inclusion of PS in the lipid component of liposomes, increased liposome immune adjuvant activity was expected. **Methods:** In the present study, two different liposome preparations containing either PS, i.e. PS-liposome, or phosphatidylcholine (PC), i.e. PC-liposome, were made, and macrophage recognition, processing, and antigen presentation of surface-coupled liposomal antigen were compared. **Results:** When ovalbumin-liposome conjugates were added to a culture of macrophages, enhanced recognition and processing of ovalbumin by the macrophages were observed by the inclusion of PS in the liposomes. The results correlated well with those regarding macrophage antigen presentation of liposome-coupled ovalbumin. Furthermore, in vivo immunization in mice with ovalbumin-liposome conjugates made with PS-liposomes induced a significantly higher

level of anti-ovalbumin IgG antibody production than was induced by ovalbumin-liposome conjugates made with PC-liposomes. IgE-selective unresponsiveness was induced by ovalbumin-liposome conjugates regardless of the lipid components of liposomes. **Conclusions:** These results suggest that the inclusion of PS in liposomes enhances recognition and processing of surface-coupled liposomal antigen by macrophages, and increases liposome immune adjuvant activity.

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Introduction

Apoptotic cells are recognized and removed by professional phagocytes, such as macrophages, dendritic cells, or neighboring semiprofessional cells including fibroblasts and epithelial cells. The clearance of apoptotic cells by phagocytes is essential to prevent tissue damage and inflammation due to the release of toxic intracellular contents, and to prevent an unwanted immune reaction, as apoptotic cells are a potential source of autoantigens [1–4]. Among the elements displayed on the apoptotic cells, phosphatidylserine (PS) is exposed on the surface of apoptotic lymphocytes and triggers the specific recognition

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and removal of apoptotic cells by phagocytes [5]. Therefore, the enhanced liposome uptake by phagocytes, which leads to an enhanced induction of the immune response, would be expected by the inclusion of PS in liposomes.

Liposomes have attracted increasing attention as carriers (vehicles) of antigens because they are known to act as powerful adjuvants when physically associated with a protein antigen [6–9]. Most of liposomal vaccines proposed to date have been prepared by antigen entrapment within the aqueous lumen of the liposome [10]. However, it is known that encapsulated and surface-linked liposomal antigens induce differential immune responses in both humoral- [11] and cell-mediated [12] immunity. We previously reported that surface-linked liposomal antigens induce antigen-specific IgG antibody production, but not antigen-specific IgE antibody production [13], by a mechanism not involving T cells [14]; such findings have suggested the existence of an alternative mechanism not involving T cells in the regulation of IgE synthesis. Furthermore, in the secondary immune response, ovalbumin (OVA)-liposome conjugates were shown to enhance anti-OVA IgG antibody production, but they did not enhance ongoing IgE production; those findings suggested that the IgE-selective unresponsiveness induced in vivo by the liposomal antigen involved direct effects on IgE, but not IgG switching [14].

Thus, surface-linked liposomal antigens are expected to serve as a novel protocol for the development of vaccines that induce only a minimal allergic response and also for the development of allergen vaccines for allergen immunotherapy. This study is aimed at investigating the effect of the inclusion of PS in liposomes on liposome immune adjuvant activity.

Materials and Methods

Mice

BALB/c mice (8 weeks of age, female) were purchased from Charles River (Yokohama, Kanagawa, Japan).

Chemicals

All phospholipids were obtained from NOF Co., Tokyo, Japan. Reagent grades of cholesterol were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Antigens

OVA (grade VII) was purchased from Sigma (St. Louis, Mo., USA). For the analysis of the processing of liposome-coupled OVA by macrophages, DQTM-OVA, which exhibits green fluorescence upon proteolytic degradation, was purchased from Molecular Probes, Inc. (Eugene, Oreg., USA).

Fluorescence Labeling of OVA

OVA was labeled with fluorescence using an Alexa FlourTM 488 protein labeling kit (Molecular Probes, Inc.) following the manufacturer's protocol.

Liposomes

Liposomes that consisted of two different lipid components were used in this study. The first type was phosphatidylcholine (PC)-liposomes composed of dipalmitoyl PC, dipalmitoyl phosphatidyl ethanolamine, cholesterol, and dimyristoyl phosphatidyl glycerol in 4:3:7:2 molar ratios. The other type was PS-liposomes, which were composed of dipalmitoyl PS, dipalmitoyl phosphatidyl ethanolamine, cholesterol, and dimyristoyl phosphatidyl glycerol in 4:3:7:2 molar ratios. The crude liposome solution was passed through a membrane filter (Nucleopore polycarbonate filter; Coster, Cambridge, Mass., USA) with a pore size of 0.2 μ m.

Coupling of OVA to Liposomes

Liposomal conjugates with plain OVA, fluorescence-labeled OVA, or DQ-OVA were prepared essentially in the same manner as described previously [13]. Briefly, a mixture of 90 mg of liposomes and 6 mg of OVA in 2.5 ml phosphate buffer (pH 7.2) was used, to which 0.5-ml drops of 2.5% glutaraldehyde solution were added. The mixture was stirred gently for 1 h at 37 °C, and then 0.5 ml of 3 M glycine-NaOH (pH 7.2) were added in order to block excess aldehyde groups. This step was followed by incubation overnight at 4 °C. The liposome-coupled OVA and uncoupled OVA in the resulting solution were separated using CL-4B column chromatography (Pharmacia Fine Chemical Co., Uppsala, Sweden). The amount of lipid in the liposomal fraction was measured using a Phospholipid-Test-WakoTM phospholipid content assay kit (Wako Pure Chemical Industries). The OVA-liposome solution was adjusted to 10 mg lipid/ml in RPMI-1640 and then sterile-filtered using MillexTM-HA syringe filter unit (0.45 μ m; Millipore Corp., Bedford, Mass., USA), and kept at 4 °C until use.

Quantification of OVA Coupled to Liposome

Radiolabeled OVA ([methyl-¹⁴C], purchased from New England Nuclear, Boston, Mass., USA) was mixed with cold OVA and used for coupling to liposome and for determining the calibration curve. The radioactivity of the resulting OVA-liposome solution was counted using a calibration curve. The amount of OVA coupled to the PC- and PS-liposomes was 47.1 and 47.0 μ g/mg lipid, respectively.

Cloned Macrophage Hybridoma

Macrophage hybridoma clone No. 39, obtained from the fusion of splenic adherent cells from CKB mice and P388D₁ [15], was used.

Macrophage Culture

Macrophage hybridoma clone No. 39 was maintained in RPMI-1640 (Gibco Laboratories, Grand Island, N.Y., USA) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, Utah, USA), 100 U/ml of penicillin and streptomycin (Gibco Laboratories) in a 75-cm² flask (No. 3111, Becton Dickinson Labware, Franklin Lakes, N.J., USA).

Flow Cytometry

In order to investigate the capture of OVA-liposome by macrophages, the No. 39 macrophage clone was incubated for 60 min at 37°C in the presence of Alexa-labeled OVA-liposome conjugates containing 4 µg/ml OVA. After incubation, the cells were washed with ice-cold PBS, and were then analyzed on a FACS Calibur™ flow cytometer (BD Biosciences, Mountain View, Calif., USA). The fluorescence distribution histograms were plotted as the number of cells versus fluorescence intensity on a logarithmic scale.

Antigen-Specific T-Cell Clone

An OVA-specific T-cell clone, 42-6A [16], was kindly provided by Dr. T. Kakiuchi of the Toho University School of Medicine, Tokyo.

Antigen Presentation by Macrophages Cocultured with OVA-Liposome

OVA-liposome conjugates made with PC- or PS-liposomes were added to cultures of macrophage clone No. 39 at a final concentration of 0.5 mg lipid/ml and the cultures were incubated overnight. The macrophages were then washed 3 times in ice-cold media and 5×10^4 cells were cocultured with 2×10^4 cells of the OVA-specific T-cell clone, 42-6A, on 96-well plates (No. 3072, Becton Dickinson Labware). After 18 h of incubation, the amount of IL-2 in the culture supernatants was assayed using a Biotrak™ mouse ELISA system (Amersham International, Buckinghamshire, UK). The final concentration of OVA-liposome added to the macrophage culture was 800 µg lipid/ml, which included 32 µg OVA/ml. As a control, soluble OVA was continuously added to the macrophage culture, achieving a final concentration of 32 µg/ml.

Immunization

Mice were immunized intraperitoneally with OVA-liposome at a dose of 2 mg lipid/200 µl, with OVA solution, or with OVA-alum. OVA solution and OVA-alum contained the same doses of OVA as in OVA-liposome (94 µg OVA/200 µl in PBS and in 0.5 mg alum, respectively). Four weeks after primary immunization, the mice were administered a booster in the same manner as that used for the primary immunization. For the measurement of serum antibodies, the mice were bled weekly from the tail vein.

Measurement of Serum Antibodies

Anti-OVA IgG antibodies in the sample sera were determined by an ELISA assay using peroxidase-labeled rabbit antibodies against mouse IgG or mouse Ig subclasses (Zymed Lab., San Francisco, Calif., USA). For the quantitative analysis of antigen-specific antibody, murine monoclonal antibodies against OVA were produced and affinity-purified in our laboratory and used as the assay standard. The levels of antigen-specific IgE were determined by monoclonal antibody-captured ELISA using monoclonal antibody against murine IgE produced by clone B1E3.

Statistic Analysis

Student's t test was employed for the statistical analysis.

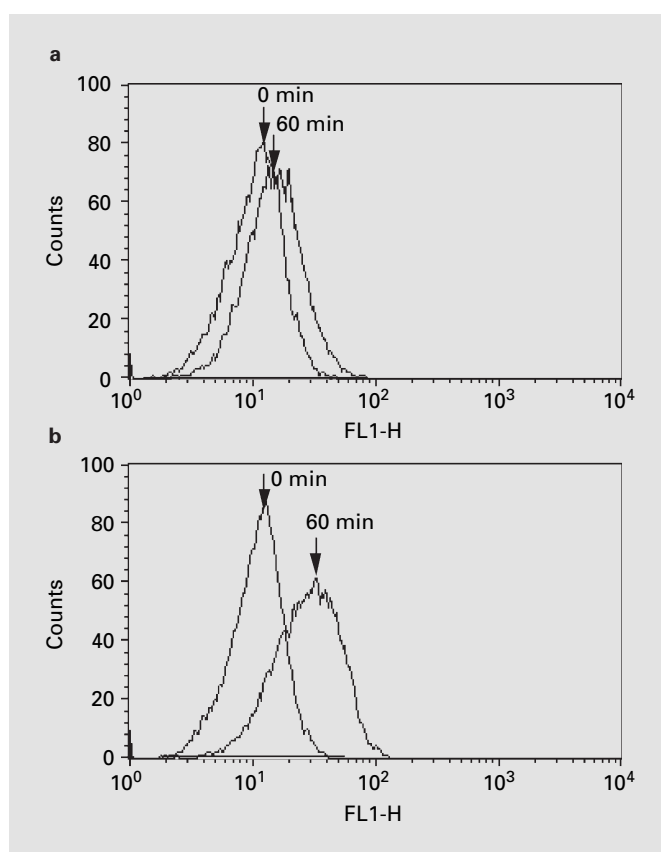


Fig. 1. Phagocytosis of OVA-liposome conjugates by macrophages. Fluorescence-labeled OVA was coupled to either PC-liposomes (**a**) or PS-liposomes (**b**) and added to a culture of macrophages as described in Materials and Methods. Macrophages recovered from the culture were analyzed using flow cytometry.

Results

Phagocytosis of Fluorescence-Labeled OVA by Macrophages

Phagocytosis of OVA-liposome by macrophages was investigated by adding fluorescence-labeled OVA coupled with PC- or PS-liposomes to the macrophage culture. FACS analysis was performed 60 min after the addition of OVA-liposome conjugates to the macrophage culture. Figure 1 shows the fluorescence intensity of macrophages cultured for 60 min with the OVA-liposome conjugates. More OVA was incorporated into the macrophages when OVA was coupled to PS-liposomes (OVA-PS-liposome) than when OVA was coupled to PC-liposomes (OVA-PC-liposome). The mean channels of fluorescence intensity were 11.6 and 16.7 at 0 and 60 min, respectively, in macrophages cultured with OVA-PC-liposome, and 11.8 and

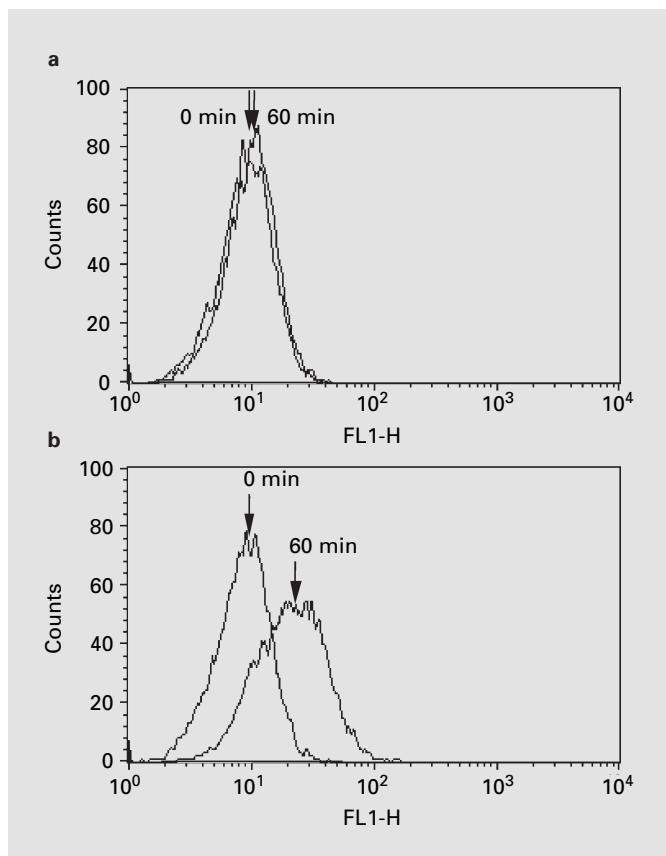


Fig. 2. Processing of liposome-coupled OVA by macrophages. PC-liposomes (**a**) or PS-liposomes (**b**) coupled with DQ-OVA were added to macrophage cultures. Sixty minutes after the onset of the culture period, the macrophages were recovered and analyzed using flow cytometry.

31.7 at 0 and 60 min, respectively, in macrophages cultured with OVA-PS-liposome.

Processing of Liposome-Coupled OVA by Macrophages

In order to compare the macrophage processing of OVA coupled either to PC- or PS-liposomes, the fluorescence intensity of the macrophages was investigated by adding DQ-OVA-coupled liposome to the macrophage culture. FACS analysis was performed 60 min after the addition of DQ-OVA-liposome to the macrophage culture. Figure 2 shows the FACS results at 60 min after the addition of DQ-OVA-liposome to the culture. More OVA appeared to be processed by macrophages when DQ-OVA was coupled to PS-liposome and added to the culture than when DQ-OVA were coupled to PC-liposome and added to the culture. The mean channels of fluorescence intensi-

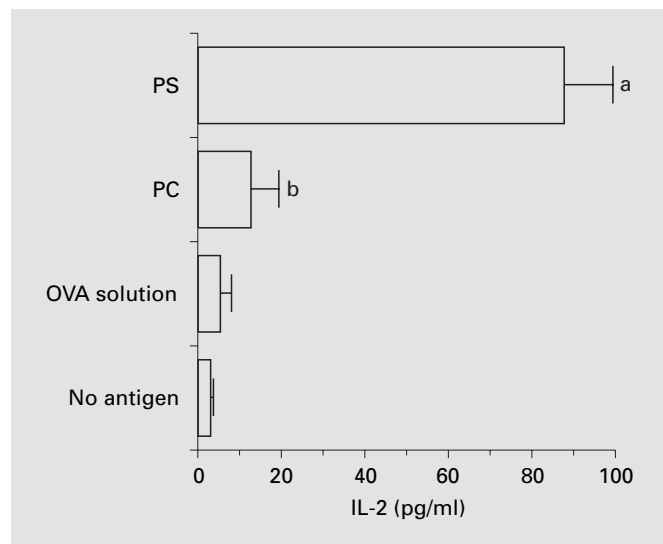


Fig. 3. Antigen presentation by macrophages pulsed with OVA-liposomes. Macrophages preincubated with OVA-liposomes were cocultured with OVA-specific T-cell clones as described in Materials and Methods. Data represent the mean IL-2 concentration and the SE of the culture supernatant in triplicate cultures. ^a $p < 0.01$: significant difference as compared with the other groups; ^b $p < 0.01$: significant difference as compared with the OVA solution.

ty were 9.6 and 11.9 at 0 and 60 min, respectively, in macrophages cultured with PC-liposome, and 9.4 and 28.3 at 0 and 60 min, respectively, in macrophages cultured with PS-liposome.

Antigen Presentation by Macrophages Cocultured with OVA-Liposome Conjugates

Macrophages were cultured overnight in the presence of OVA-liposome prior to coculture with the OVA-specific T-cell clone, 42-6A. IL-2 production by the T-cell clone was then monitored. Because no cytokine production was observed in the absence of OVA under the culture condition described in Materials and Methods, the data shown in figure 3 are considered to represent antigen-specific cytokine production. Figure 3 shows the amount of IL-2 in the culture supernatant. A significantly high level of IL-2 production was observed when the macrophages were precultured with OVA-PS-liposome. The amount of OVA in the culture to which the OVA-liposome conjugates were added was 32 $\mu\text{g/ml}$. However, the addition of 32 $\mu\text{g/ml}$ of plain OVA to the culture resulted in no or very low production of IL-2. Although a significant level of IL-2 was produced when the macrophages were precultured with OVA-PC-liposome, the amount of

IL-2 was more than threefold lower than that produced when PS-liposome was added to the culture. No IL-2 production was observed when the macrophages were precultured with PS- or PC-liposome to which OVA was not coupled (data not shown).

Comparison of Anti-OVA Antibody Production in Mice Induced by OVA-Liposome Conjugates Made with PC- or PS-Liposomes

Mice were immunized with OVA-PC-liposome, OVA-PS-liposome, plain OVA solution, or with alum-adsorbed OVA. Figure 4 shows serum anti-OVA IgG and IgE titers. Levels of anti-OVA IgG antibody production induced by the two different types of OVA-liposome conjugate were significantly different; OVA-PS-liposome induced significantly higher levels of anti-OVA IgG antibody production than that observed in association with OVA-PC-liposome at 2, 4, 5, and 6 weeks after primary immunization (fig. 4a). Plain OVA solution induced significantly lower levels of anti-OVA IgG antibody production than those in groups of mice immunized with OVA-liposome. An enhanced induction of anti-OVA IgG production was observed by the immunization of mice with OVA-alum as compared with that in the group of mice immunized with OVA solution. The levels of anti-OVA IgG at 4–6 weeks were comparable in groups of mice immunized either with OVA-PS-liposome or with OVA-alum. On the other hand, anti-OVA IgE antibody production was observed in mice immunized either with OVA solution or with OVA-alum but not in mice immunized with OVA-liposome (fig. 4b).

Discussion

Inclusion of PS in the lipid component of liposomes resulted in enhanced recognition (fig. 1), processing (fig. 2), and antigen presentation (fig. 3) of liposomal antigens by macrophages. The results correlated well with the results observed in a study of the induction of in vivo antibody production in mice (fig. 4). Enhanced recognition of liposomal antigen may lead to an enhanced T helper cell activation which in turn would lead to increased activation of B cells and antibody production. It is unlikely that substitution of neutral PC with negatively charged PS in liposomes could result in a change in surface charge and better uptake by some mechanism not involving the PS receptor, since addition of antigen-free PS-liposomes but not PC-liposomes in the macrophage culture inhibited macrophage recognition and processing of liposomal antigens (data not shown).

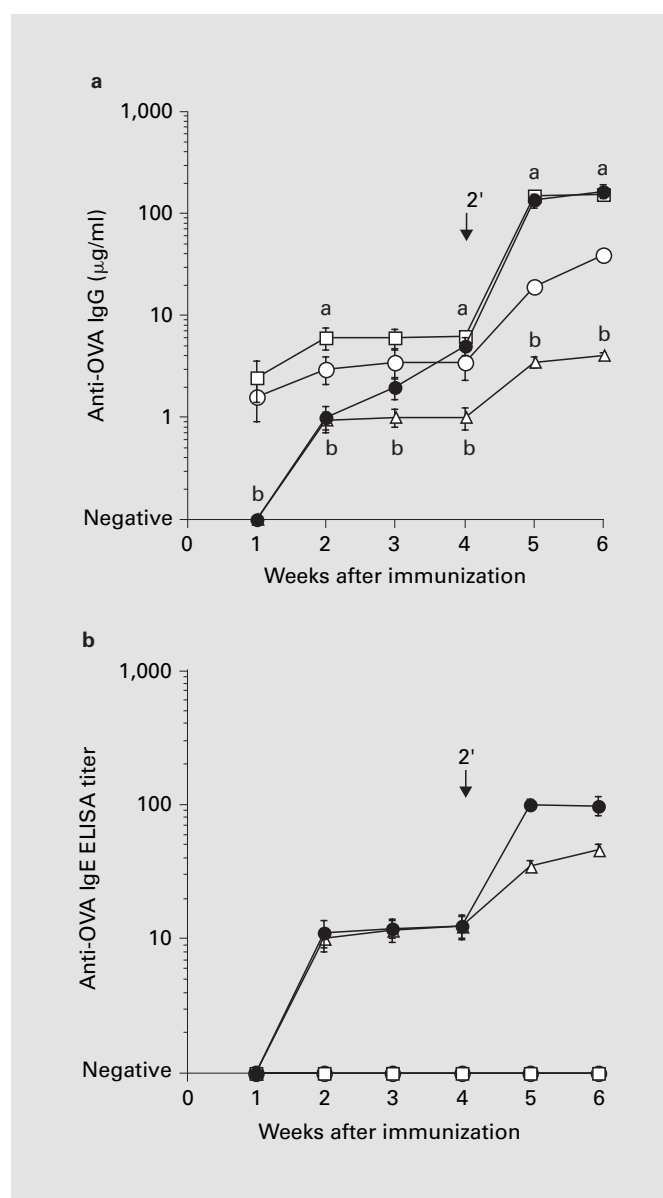


Fig. 4. Anti-OVA IgG (a) and IgE (b) antibody production in mice immunized with OVA-liposome, plain OVA solution, or with OVA-alum. BALB/c mice were immunized with OVA-liposome made using PC-liposomes (○) or PS-liposomes (□), plain OVA solution (△), or with OVA-alum (●) at 0 and 4 weeks as described in Materials and Methods. Mice were bled weekly from the tail vein, and serum anti-OVA IgG and IgE were measured. Data represent mean and SE of 5 mice per group. ^a $p < 0.01$: significant difference between the PC- and PS-liposome groups; ^b $p < 0.01$: significant difference between the PC-liposome and OVA solution groups. 2' = Secondary immunization.

A variety of cell surface molecules (e.g. lectin-like molecules [17], CD14 [18], scavenger receptor antagonists [19], and PS [5]) have been implicated in the recognition of apoptotic cells. Among them, PS is known to trigger the specific recognition and removal of apoptotic lymphocytes by macrophages [5], suggesting that the inclusion of PS in the lipid components of liposomes would lead to enhanced recognition by macrophages, and thereby result in an enhanced induction of the immune responses. On the other hand, a number of reports have shown that PS possesses immunosuppressive properties, such as an inhibition of the T-cell mitogen response [20], a reduction of macrophage NO synthesis [21], the inhibition of tumor cytotoxicity by macrophages [22], and the suppression of antigen-specific antibody production when PS was orally administered [23]. However, in this study, the inclusion of PS in the liposome composition significantly enhanced the induction of antigen-specific antibody production in mice immunized with antigen-liposome conjugates. These results therefore suggest that the inclusion of PS in liposomes increases the adjuvant activity of liposomes.

We previously reported that surface-linked liposomal antigen induces IgE-selective unresponsiveness [13]. This phenomenon was consistently observed regardless of the particular antigen coupled to the liposomes [24, 25], the lipid components of the liposomes [26], or the procedure for coupling the antigen and the liposome [27]. Antigen-specific, IgE-selective unresponsiveness was induced by antigen-liposome conjugates only when antigen and liposomes were chemically coupled [28]. As regards liposome immune adjuvant activity, the lipid composition of the liposomes was demonstrated to affect liposomal adjuvant activity, resulting in the induction of antigen-specific IgG antibody production [26]. However, no correlation was found between liposomal adjuvant activity and the induction of IgE-selective unresponsiveness. During the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-coupled liposomal antigens, we discovered an alternative approach to regulate the production of IgE. We found a mechanism that is independent of the activity of T cells [14]; OVA-liposome conjugates were found to induce IgE-selective unresponsiveness without induction of apparent Th1-type immune response. CD4⁺ T cells of mice immunized with OVA-liposome produced a significant level of IL-4 upon *in vitro* stimulation with OVA and were capable of inducing antigen-specific IgE antibody production in athymic nude mice which were immunized with OVA-alum, suggesting that IgE production was regulated by a mechanism that did not involve polarization of Th1 in

mice immunized with OVA-liposome. On the other hand, the enhancement of liposome adjuvant activity by the inclusion of PS in liposomes was found to correlate with the enhanced induction of antigen-specific T helper cells.

Adjuvants play a key role in the development of vaccines, particularly in the case of vaccine antigens with weak immunogenicity. Adjuvants may increase the immunogenicity of weak antigens, enhance the speed and duration of the immune response, modulate antibody avidity, or decrease the dose of antigen in the vaccine [29]. However, the precise mechanism underlying so-called adjuvant activity is unknown. In the present study, inclusion of PS in liposomes was shown to increase liposome immune adjuvant activity via enhanced recognition and also by the processing of surface-coupled liposomal antigen by macrophages, in the context of immunization with a surface-coupled liposomal antigen. Thus, in the present study, the inclusion of PS in liposomes was shown to enhance recognition and processing of surface-coupled liposomal antigen by macrophages, and increase liposome immune adjuvant activity.

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