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ARTICLE *in* BIOCONJUGATE CHEMISTRY · JANUARY 2004

Impact Factor: 4.51 · DOI: 10.1021/bc034134o · Source: PubMed

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Liposomes with Differential Lipid Components Exert Differential Adjuvanticity in Antigen−Liposome Conjugates via Differential Recognition by Macrophages

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We previously reported that liposomes having differential lipid components displayed differential adjuvant effects when antigen was coupled with liposomes via glutaraldehyde. In the present study, antigen−liposome conjugates prepared using liposomes having differential lipid components were added to the macrophage culture, and phagocytosis and the antigen digest of liposome-coupled antigen by macrophages were then investigated. Antigen presentation by macrophages to an antigen-specific T-cell clone was further investigated using the same conjugates. Antigen−liposome conjugates which induced higher levels of antibody production in vivo were recognized more often, and the liposome-coupled antigen was digested to a greater degree by macrophages than antigen−liposome conjugates which induced lower levels of antibody production. These results correlated closely with those regarding antigen presentation by macrophages; when antigen was coupled to liposomes showing higher adjuvant effect, macrophages cocultured with antigen−liposome conjugates activated antigen-specific T-cells at a higher degree. The concentration of OVA in the macrophage culture added as antigen−liposome conjugates was approximately 32 μg/mL. However, the extent of T-cell activation was almost equal to that when 800 μg/mL of soluble OVA was added to the culture. The results of the present study demonstrated that the adjuvant activity of liposomes observed primary in vivo correlated closely with the recognition of antigen−liposome conjugates and antigen presentation of liposome-coupled antigen by macrophages, suggesting that the adjuvant effects of liposomes are exerted at the beginning of the immune response, i.e., recognition of antigen by antigen-presenting cells.

INTRODUCTION

Adjuvants play a pivotal role in vaccination, especially when the vaccine antigen itself possesses a weak immunogenicity (*1*−*3*). At present, aluminum adjuvant is the only adjuvant that is clinically in use, although a number of candidate adjuvants have beeen reported and examined in a preclinical study (*3*). However, aluminum adjuvants are known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity. In addition, aluminum adjuvants are also known to induce the production of IgE antibodies, which is a cause of allergic response against the vaccine (*4*−*6*). Consequently, the development of a novel vaccine adjuvant is indispensable. Among the candidates for adjuvants, liposomes have garnered recent attention for their capacity as carriers of vaccines (*7*−*14*).

We previously reported that antigens chemically coupled to the surface of liposomes induced antigen-specific IgG but not IgE antibody production (*15*). This IgE-selective unresponsiveness was induced by antigen−liposome conjugates regardless of the coupling procedure of the antigen and liposomes (*16*), of the antigens used for the coupling with liposomes (*17*), or of the route of inoculation (i.e., intraperitoneal (*15*) or intranasal (*18*)). The inducibility of antigen-specific IgG antibody production by antigen−liposome conjugates varied among the liposome preparations used for the production of antigen−liposome conjugates; the greater the membrane mobility in liposomes, the more antibody production induced by antigen−liposome conjugates (*19*). In fact, alteration of lipid composition has been reported to modulate immune responses (*20*−*26*). The aim of this study is to examine if the differential adjuvant effects displayed among liposomes with different lipid components were due to differential recognition of liposomal antigens by antigen-presenting cells. Antigen−liposome conjugates prepared using liposomes with differential lipid components were added to the culture of macrophages, and recognition and digest of liposomal antigen were compared. Further, antigen presentation by macrophages was investigated after coculture of macrophages with antigen−liposome conjugates made using liposomes having different lipid components.

MATERIALS AND METHODS

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

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10.1021/bc034134o CCC: $27.50
Published on Web 00/00/0000 **PAGE EST: 5.3**

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

Antigens. Ovalbumin (OVA, Grade VII) was purchased from Sigma (St. Louis, MO). For the analysis of the processing of liposome-coupled OVA by macrophages, DQ-OVA, that exhibits green fluorescence upon proteolytic degradation, was purchased from Molecular Probes, Inc. (Eugene, OR).

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

Liposomes. Liposomes consisting of two different lipid components were used in this study: "Stearoyl" liposomes composed of distearoyl phosphatidylcholine, distearoyl phosphatidyl ethanolamine, cholesterol, and distearoyl phosphatidyl glycerol acid in 4:3:7:2 molar ratios, and "Oleoyl" liposomes composed of dioleoyl phosphatidylcholine, dioleoyl phosphatidyl ethanolamine, cholesterol, and dioleoyl phosphatidyl glycerol acid in 4:3:7:2 molar ratios. The crude liposome solution was passed through a membrane filter (Nucleopore polycarbonate filter, Coster, Cambridge, MA) with a pore size of 0.2 μm.

Coupling of OVA to Liposomes. Liposomal conjugates with plain OVA, Alexa-labeled OVA, or DQ-OVA were prepared essentially in the same way as described previously (*15*). Briefly, to a mixture of 90 mg of liposomes and 6 mg of OVA in 2.5 mL phosphate buffer (pH 7.2) was added in drops 0.5 mL of 2.5% glutaraldehyde solution. The mixture was stirred gently for 1 h at 37 °C, and then 0.5 mL of 3 M glycine−NaOH (pH 7.2) was added to block excess aldehyde groups. This 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., Upsala, Sweden). The amount of lipid in the liposomal fraction was measured using a Phospholipid-Test-Wako phospholipid content assay kit (Wako Pure Chemical Industries). The OVA−liposome solution was adjusted to 10 mg lipid/mL in RPMI-1640, sterile-filtered using Millex-HA syringe filter unit (0.45 μm, Millipore Corp., Bedford, MA), and kept at 4 °C until use.

Quantification of OVA Coupled to Liposome. For the measurement of OVA coupled to liposome, radiolabeled OVA ([methyl-^{14}C]: purchased from New England Nuclear, Boston, MA) was mixed with cold OVA and used for coupling with liposome and for determining the calibration curve. The radioactivity of the resulting OVA−liposome solution was counted using a calibration curve. The amounts of OVA coupled to "Stealoyl" and "Oleoyl" liposomes were 47.0 and 46.8 μg/mg lipid, respectively.

Immunization. Mice were immunized intraperitoneally (ip) with OVA−liposome at a dose of 2 mg lipid/200 μL/mouse or with 80 μg/200 μL/mouse of plain OVA solution in PBS. Four weeks after primary immunization, mice were boosted in the same manner as that used for primary immunization. For the measurement of serum antibodies, mice were bled from the tail vein.

Measurement of Serum Antibodies. Six weeks after primary immunization, serum anti-OVA IgG and IgG isotypes were determined by ELISA using HRP-labeled goat anti-mouse IgG (Zymed Lab., San Francisco, CA).

Cloned Macrophage Hybridoma. Macrophage hybridoma clone #39, obtained from the fusion of splenic adherent cells from CKB mice and P388D$_1$ (*27*), was used.

Antigen-Specific T-Cell Clone. OVA-specific T-cell clone, 42−6A (*28*), was kindly provided by Dr. T. Kakiuchi, Toho University School of Medicine, Tokyo.

Macrophage Culture. Macrophage hybridoma clone #39 was maintained in RPMI-1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco Laboratories) in a 75 cm^2 flask (No. 3111, Becton Dickinson Labware, Franklin Lakes, NJ).

Flow Cytometry. To investigate the capture of OVA−liposome by macrophages, #39 macrophage clone were incubated for 60 min at 37 °C in the presence of Alexa-labeled OVA−liposome conjugates that contained a final concentration of 4 μg/mL OVA. After the incubation, cells were washed with ice cold PBS. Then they were analyzed on a FACS Calibur flow cytometer (BD Biosciences, Mountain View, CA). The histograms of fluorescence distribution were plotted as the number of cells versus fluorescence intensity on a logarithmic scale.

Confocal Laser Scanning Microscopy To investigate degradation of liposome-coupled OVA by macrophages, #39 macrophage clone were cultured for 18h at 37 °C on eight-hole heavy Teflon-coated slides (Bokusui Brown, New York, NY) and were then incubated with DQOVA−liposome conjugates, prepared using "Stearoyl" or "Oleoyl" liposomes, for 60 min at 37 °C. They were washed with MEM and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After fixation, they were incubated for 10 min in 0.1 M glycine-HCl, pH 7.0, to block the remaining aldehyde residue. They were then washed two times in PBS. After being washed, these slides were sealed with PBS/glycerin (PBS:glycerin = 1:9). These slides were analyzed under a confocal laser scanning microscope system, LSM410 (Carl Zeiss Co., Germany).

Antigen Presentation by Macrophages Cocultured with OVA−Liposome. OVA−liposome conjugates made using "Stearoyl" or "Oleoyl" liposomes were added to the culture of macrophage clone #39 and incubated overnight. The macrophages were then washed three times in ice-cold media, and 5×10^4 cells were cocultured with 2×10^4 cells of OVA specific T-cell clone, 42−6A, in a 96-well plate (No. 3072, Becton Dickinson Labware). After 18 hours of incubation, IL-2 in the culture supernatants were 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. For controls, OVA was added to the macrophage culture continuously at final concentrations of 32 or 800 μg/mL.

RESULTS

Comparison of Anti-OVA Antibody Production in Mice Induced by OVA−Liposome Conjugates Made Using Liposomes of Two Different Lipid Components. Mice were immunized with OVA−liposome conjugates which were made using "Stearoyl" or "Oleoyl" liposomes. Figure 1 shows serum anti-OVA IgG titers six weeks after the primary immunization with OVA−liposome or with plain OVA solution. Levels of anti-OVA IgG antibody production induced by two OVA−liposome conjugates were significantly different; OVA−liposome made using "Oleoyl" liposome induced more than a 10-fold higher level of anti-OVA IgG production than that induced by OVA−liposome made using "Stearoyl" liposome. The same dose of plain OVA solution induced a

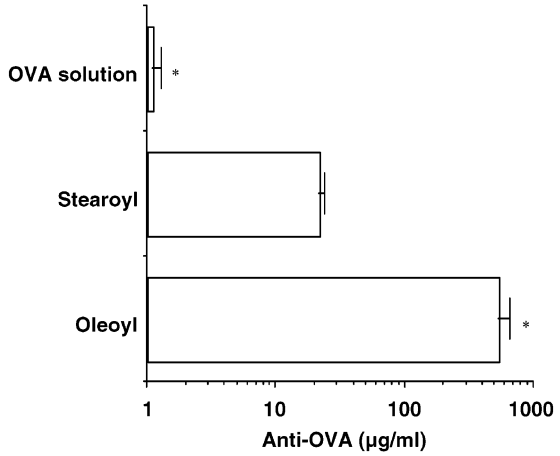

Figure 1. Anti-OVA IgG antibody production in mice immunized with OVA−liposome conjugates. BALB/c mice were immunized with OVA−liposome made using "Stearoyl" or "Oleoyl" liposomes or with plain OVA solution at 0 and 4 weeks. Six weeks after primary immunization, mice were bled from the tail vein, and serum anti-OVA IgG was measured. Data represent mean and SE of five mice per group. Asterisk, significant ($p < 0.01$) difference as compared with "Stearoyl" group.

far lower level of anti-OVA IgG antibody production as compared with that induced by OVA−liposome conjugates.

Phagocytosis of Fluorescence-Labeled OVA by Macrophages. To examine if the differential adjuvant effects between two liposome preparations observed in the above experiment were due to differential recognition of liposomal antigens by antigen-presenting cells, phagocytosis of OVA−liposome by macrophages was investigated by adding fluorescence-labeled OVA coupled with "Stearoyl" or "Oleoyl" liposomes to the macrophage culture. FACS analysis was performed 15, 30, 60, and 120 min after the addition of OVA−liposome conjugates to the macrophage culture, and a maximal incorporation was observed at 60 min. Figure 2a shows the fluorescence intensity of macrophages cultured for 60 min with OVA−liposome conjugates. More OVA was incorporated when OVA was coupled to "Oleoyl" liposomes than when OVA was coupled to "Stearoyl" liposomes. A similar result was observed in the confocal microscopic analysis; more OVA was incorporated by macrophages when OVA coupled to "Oleoyl" liposome was added to the culture (Figure 2b).

Processing of Liposome-Coupled OVA by Macrophages. To compare the processing of OVA coupled either with "Stearoyl" or "Oleoyl" liposomes by macrophages, the fluorescence intensity of the macrophages was investigated by adding DQ-OVA−coupled liposome to the macrophage culture. FACS analysis was performed 15, 30, 60, and 120 min after the addition of OVA−liposome in the macrophage culture, and a maximal processing of OVA was observed at 60 min. Figure 3 shows the results of FACS and confocal microscopic analysis at 60 min after the addition of OVA−liposome in the culture. More OVA appeared to be processed in 60 min by macrophages when OVA was coupled to "Oleoyl" liposome and added to the culture than that when OVA was coupled to "Stearoyl" liposome and added

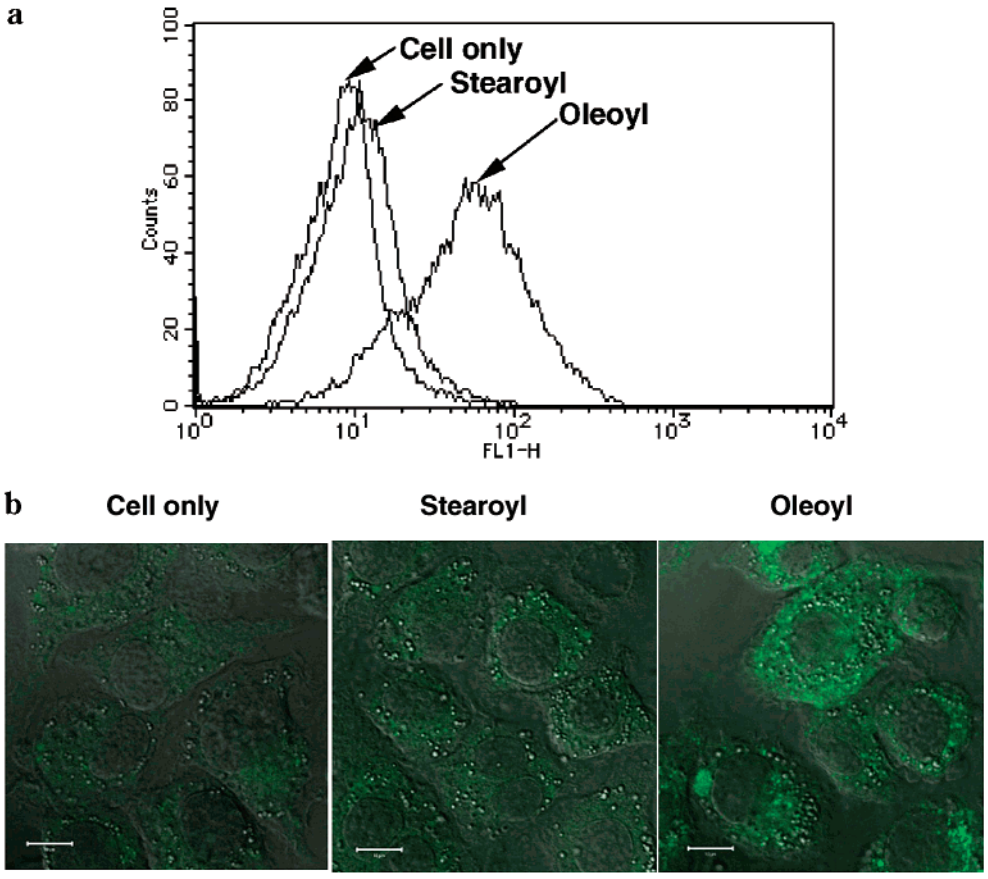

Figure 2. Phagocytosis of OVA−liposome conjugates by macrophages. Fluorescence-labeled OVA was coupled to either "Stearoyl" or "Oleoyl" liposomes and added to the culture of macrophages as described in Materials and Methods. Macrophages recovered from the culture were analyzed using flow cytometry (a) and confocal laser scanning microscopy (b).

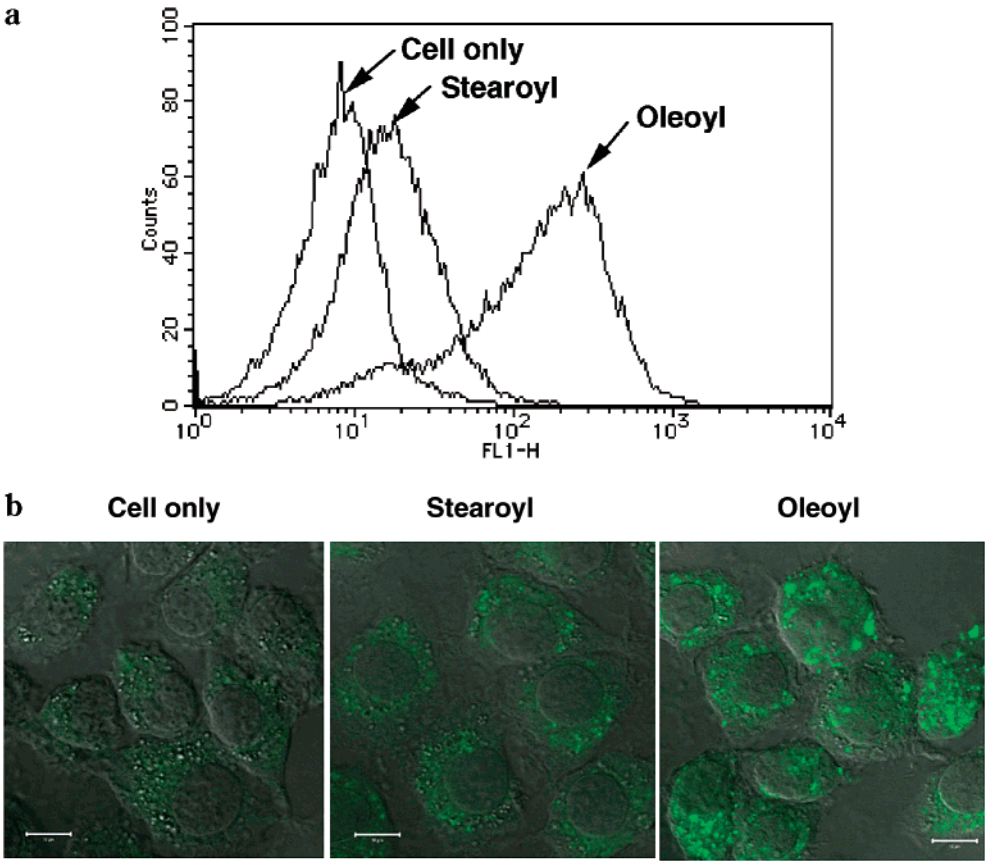

Figure 3. Digestion of liposome-coupled OVA by macrophages. "Stearoyl" or "Oleoyl" liposomes coupled with DQ-OVA were added to the macrophage culture. Sixty minutes after the onset of the culture, macrophages were recovered and analyzed using flow cytometry (a) and confocal laser scanning microscopy (b).

to the culture (Figure 3a). A consistent result was observed by confocal microscopic analysis (Figure 3b).

Antigen-Presentation by Macrophages Cocultured with OVA−Liposome Conjugates. Macrophages were cultured in the presence of OVA−liposome prior to the coculture with OVA-specific T-cell clone, 42−6A, and IL-2 production by the T-cell clone was monitored. Figure 4 shows the amount of IL-2 in the culture supernatant. A significantly high level of IL-2 production was observed when the macrophages were pre-cultured with OVA−liposome made using "Oleoyl" liposomes. The amount of IL-2 was comparable with that when 800 μg/mL of plain OVA was added to the culture. The amount of OVA in the culture to which OVA−liposome conjugates were added was 32 μg/mL. However, the addition of 32 μg of plain OVA to the culture resulted in production of a far lesser amount of IL-2. Although a significant level of IL-2 was produced when macrophages were pre-cultured with OVA−liposome made using "Stearoyl" liposomes, it was more than 10-fold less than that in the "Oleoyl" liposome group.

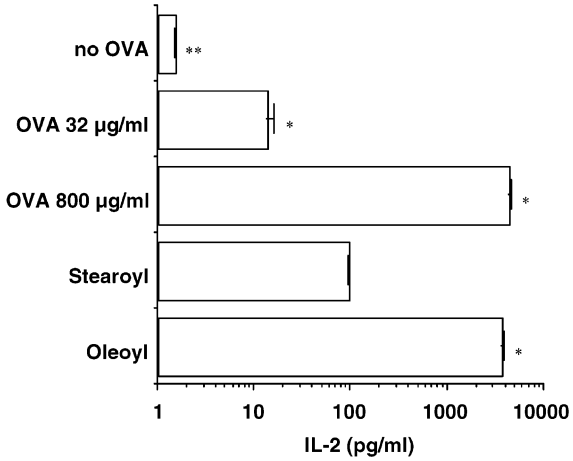

Figure 4. Antigen presentation by macrophages pulsed with OVA−liposomes. Macrophages preincubated with OVA−liposomes were cocultured with OVA−specific T-cell clone as described in Materials and Methods. Data represent mean IL-2 concentration and the SE of the culture supernatant in triplicate cultures. Asterisk, significant ($p < 0.01$) difference as compared with "Stearoyl" group. Two asterisks, significant ($p < 0.01$) difference as compared with the other groups.

DISCUSSION

We previously demonstrated that the adjuvanticity of liposomes in the surface-linked liposomal antigen differs among liposomes of differing lipid compositions (*19*). The results obtained in the present study suggested that the adjuvanticity in liposomes with differential lipid components would correlate with the recognition of liposomal antigens by macrophages, resulting in the differential extent of digestion and antigen presentation. When OVA was coupled to "Oleoyl" liposomes, which expressed high adjuvant activity, OVA was incorporated more efficiently by macrophages than when OVA was coupled to "Stearoyl" liposomes, which expressed lower adjuvanticity. These results further correlated closely with those obtained in the succeeding investigations regarding antigen processing and presentation by macrophages; more OVA was processed in macrophages when OVA was coupled to "Oleoyl" liposomes than when coupled to "Stearoyl" liposomes. Moreover, OVA coupled to "Oleoyl" liposomes was effectively presented to OVA-specific T-cells. When

OVA coupled to "Oleoyl" liposomes was presented to T-cells, the amount of IL-2 released from OVA-specific T-cells was comparable to that when 800 μg/mL of plain OVA was added to the culture, although the amount of OVA added to the culture as OVA−liposome was 32 μg/mL. A significant difference was observed among "Oleoyl"- and "Stearoyl" liposomes in the results, although OVA coupled to "Stearoyl" liposomes was presented even more effectively as compared with that when 32 μg/mL of plain OVA was added to the culture. These results suggest that the adjuvant effect of liposomes correlates well with antigen recognition and presentation by macrophages, at the initiation of immune response.

In the vaccines, adjuvants are indispensable especially for antigens with weak immunogenicity. However, the currently used aluminum adjuvants are known to stimulate only humoral responses (*1*) and are known to induce IgE antibody production, which elicits an allergic response in the vaccinee (*4*). Therefore, the availability of improved adjuvants suitable for clinical use is highly desirable. Among the candidates for adjuvants for novel vaccines, liposomes are garnering attention as antigen carriers (vehicles) because they are known to act as powerful adjuvants when physically associated with a protein antigen (*7*, *10*, *12*, *13*). Most of the liposomal vaccines proposed have been prepared by antigen entrapment within the aqueous lumen of liposomes (*8*). However, it is known that encapsulated and surface-linked liposomal antigens induce differential immune responses in both humoral- (*26*) and cell-mediated (*25*) immunity.

We previously reported that surface-linked liposomal antigen induced IgE-selective unresponsiveness (*15*). The results were consistent even when different procedures for coupling antigen with liposomes (*16*), or for liposomes with different lipid components (*19*), were employed. 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, one that is independent of the activity of T-cells (*29*). In the secondary immune response, OVA−liposome enhanced anti-OVA IgG antibody production but it did not enhance ongoing IgE production, suggesting that the IgE-selective unresponsiveness induced by the liposomal antigen involved direct effects on IgE but not IgG switching in vivo. Another advantage of liposomes as an adjuvant is that they are capable of inducing both humoral and cellular immunity. A liposome delivery system could be tailored to induce either a preferential cellular or humoral immune response (*22*, *30*).

The mechanism of action of adjuvants remains unclear. Adjuvants may increase cellular infiltration into the injection site, may directly contribute to the delivery of antigen to the local lymph node, or may directly promote the uptake of antigen into antigen-presenting cells. The results in this study clearly demonstrated that liposomes promote the uptake of liposome-coupled antigen by antigen-presenting cells and enhance antigen presentation to T-cells, which may result in the enhanced induction of systemic immune responses.

ACKNOWLEDGMENT

This work was supported in part by a grant from The Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation).

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