Clinical Application of Surface-Linked Liposomal Antigens

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Abstract: The potential usefulness of surface-linked liposomal antigens for application to vaccine development was investigated. During the course of this investigation, a significant difference was observed in the recognition of liposomal antigens by antigen-presenting cells (APCs) between liposomes with different lipid components, and this difference was closely correlated with the adjuvant activity of liposomes. In addition to this "quantitative" difference between liposomes with differential lipid components, a "qualitative" difference (i.e., a differential ability to induce cross-presentation) was also observed between liposomes with different lipid components. Although the precise mechanism underlying this difference is currently unclear, the significant difference in membrane mobility observed between these liposomes might affect their ability to induce cross-presentation. Thus, surface-linked liposomal antigens may be applicable for the development of vaccines with minimal allergic side effects and for a novel protocol of allergen immunotherapy. In addition, by utilizing their ability to induce cross-presentation, surface-linked liposomal antigens could be used to develop virus vaccines that induce a cytotoxic T-cell (CTL) response, as well as tumor vaccine preparations that present tumor antigens to APCs and induce effective antitumor responses. These data suggest that differential lipid components in liposomes lead to differential processing and presentation of liposomal antigens in APCs.

Key Words: Liposome, IgE, allergy, vaccine, cross-presentation, antitumor immunity.

INTRODUCTION

Adjuvants are indispensable in vaccines, especially for antigens with weak immunogenicity. However, the currently used aluminum adjuvants are known to stimulate only humoral responses [1] and are also known to induce IgE antibody production, which elicits an allergic response in some individuals following vaccination [2]. Therefore, there is need of improved adjuvants suitable for clinical use. 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 [3-6]. Most of the liposomal vaccines proposed have been prepared by antigen entrapment within the aqueous lumen of liposomes [7]. However, it is known that encapsulated and surface-linked liposomal antigens induce differential immune responses in both humoral- [8] and cell-mediated [9] immunity. We previously reported that surface-linked liposomal antigens induced IgE-selective unresponsiveness [10]. The results were consistent even when different procedures for coupling antigens with liposomes [11], or for producing liposomes with different lipid components [12], 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 regulating the production of IgE, one that is independent of the activity of T cells [13]. The IgE-selective unresponsiveness induced by the liposomal antigen involved direct effects on IgE, but not IgG switching *in vivo*. Thus, surface-linked liposomal antigen is expected to be applicable for the development of a novel vaccine that induces minimal IgE synthesis. Moreover, given the relatively low allergic response to and increased antigenicity of the allergen, this form of antigen preparation would be applicable for allergen immunotherapy [14, 15]. In addition, we recently found that, by choosing lipid components for liposomes, surface-coupled liposomal antigens are cross-presented to $CD8^+$ T cells *via* MHC class I [16]. Therefore, surface-linked liposomal antigens might be applicable for the development of tumor vaccines to present tumor antigens to antigen-presenting cells (APCs) and induce antitumor responses, and for the development of virus vaccines to induce cytotoxic T-cells (CTLs) to eliminate virus-infected host cells.

In this manuscript, data indicating the correlation existing between the lipid component of liposomes and the immune response induced by surface-linked liposomal antigens are summarized and the potential of surface-linked liposomal antigens for clinical application is discussed.

LIPOSOMES WITH DIFFERENTIAL LIPID COMPONENTS EXERT DIFFERENTIAL ADJUVANTICITY IN ANTIGEN-LIPOSOME CONJUGATES *VIA* DIFFERENTIAL RECOGNITION BY MACROPHAGES

Liposomes having differential lipid components were demonstrated to display differential adjuvant effects when antigen was coupled with liposomes *via* glutaraldehyde [12]. Antigen-liposome conjugates prepared using liposomes having differential lipid components were added to a macrophage culture, and phagocytosis and the digestion of liposome-coupled antigen by the macrophages were then investigated [17]. Mice were immunized with ovalbumin (OVA)-liposome conjugates which were made using "stearoyl" or

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"oleoyl" liposomes. Fig. (**1**) shows the serum anti-OVA IgG titers six weeks after the primary immunization with OVA-liposome conjugates or with plain OVA solution. The levels

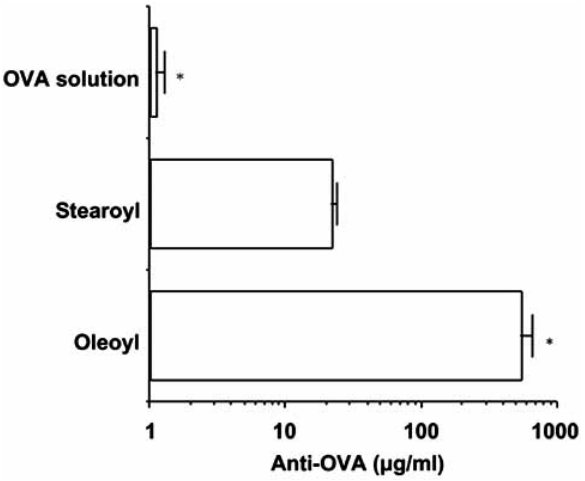

Fig. (1). Anti-OVA IgG antibody production in mice immunized with OVA-liposome conjugates. BALB/c mice were immunized with OVA-lipospome conjugates made using "stearoyl" or "oleoyl" liposomes or with plain OVA solution at 0 and 4 weeks. Six weeks after primary immunization, the mice were bled from the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

of anti-OVA IgG antibody production induced by two OVA-liposome conjugates were significantly different; OVA-liposome conjugates made using the "oleoyl" liposome induced a more than ten-fold higher level of anti-OVA IgG production compared with that induced by the OVA-liposome conjugates made using "stearoyl" liposomes. The same dose of plain OVA solution induced a far lower level of anti-OVA IgG antibody production as compared with that induced by OVA-liposome conjugates. In order to examine if the differential adjuvant effects between the two liposome preparations observed in the above experiment were due to differential recognition of liposomal antigens by antigen-presenting cells, phagocytosis of OVA-liposome conjugates by macrophages was investigated by adding fluorescence-labeled OVA coupled with "stearoyl" or "oleoyl" liposomes to the macrophage culture. Fig. (**2**) shows the fluorescence intensity of the 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. In order 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 liposomes to the macrophage culture. DQ^{TM}-OVA (Molecular Probes, Eugene, OR) exhibits green fluorescence upon proteolytic degradation. Fig. (**3**) shows the results of the FACS analysis at 60 min after the addition of OVA-lipospome conjugates to the culture. It appeared that the amount of OVA processed by macrophages in 60 min was greater when OVA was coupled to the "oleoyl" lipospomes than when OVA was coupled to the "stearoyl" lipospomes.

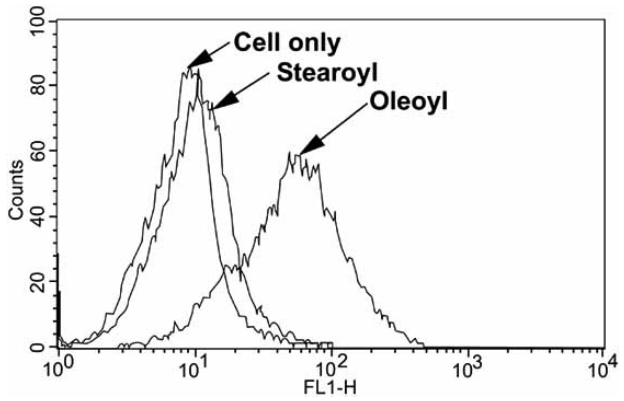

Fig. (2). Phagocytosis of OVA-liposome conjugates by macrophages. Fluorescence-labeled OVA was coupled to either "stearoyl" or "oleoyl" liposomes and added to the culture of macrophages. Macrophages recovered from the culture were analyzed using flow cytometry.

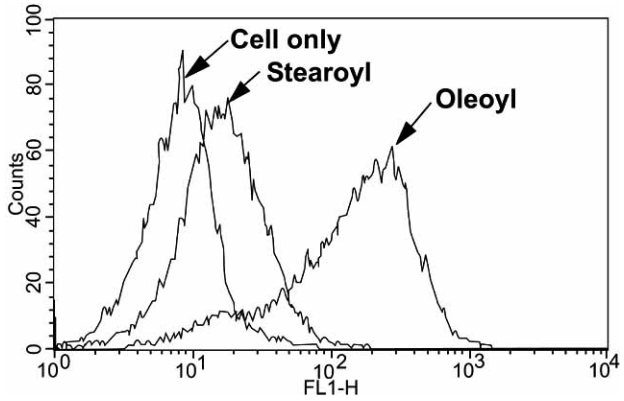

Fig. (3). Digestion of liposome-coupled OVA by macrophages. "stearoyl" or "oleoyl" liposomes coupled with DQ-OVA were added to the macrophage culture. Sixty minutes after the onset of the culture, the macrophages were recovered and analyzed using flow cytometry.

Antigen presentation by macrophages to an antigen-specific T-cell clone was further investigated using the same conjugates. Macrophages were cultured in the presence of OVA-liposome conjugates prior to the co-culture with the OVA-specific T-cell clone, 42-6A, and the IL-2 production by the T-cell clone was monitored. Fig. (**4**) shows the amount of IL-2 in the culture supernatant. A significantly higher level of IL-2 production was observed when the macrophages were pre-cultured with OVA-liposomes made using "oleoyl" liposomes. The amount of IL-2 was comparable to that when 800 μg/ml of plain OVA was added to the culture, although 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 substantial amount of IL-2 was produced when the macrophages were pre-cultured with OVA-liposome conjugates made using "stearoyl" liposomes, the IL-2 level was still more than ten-fold less than that in the "oleoyl" liposome group.

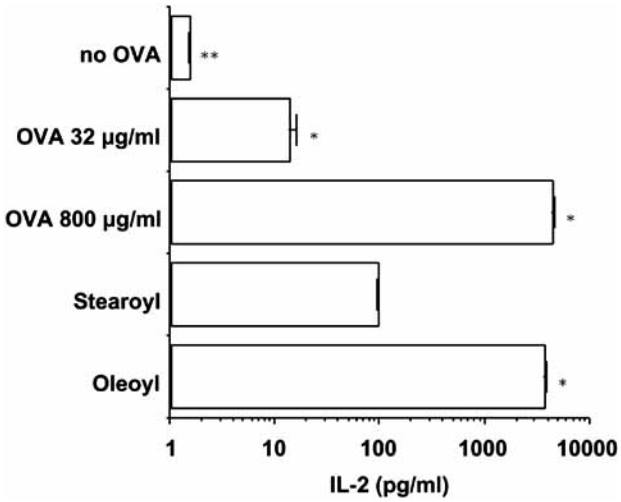

Fig. (4). Antigen presentation by macrophages pulsed with OVA-liposomes. Macrophages pre-incubated with OVA-liposomes were co-cultured with an OVA-specific T-cell clone. Data represent the mean IL-2 concentration and the SE of the culture supernatant in triplicate cultures. Asterisk, significant ($p<0.01$) difference as compared with the "stearoyl" group. Two asterisks, significant ($p<0.01$) difference as compared with the other groups.

Thus, these results clearly demonstrated that the adjuvant activity of liposomes observed primarily *in vivo* was closely correlated with the recognition of antigen-liposome conjugates and the 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., upon recognition of the antigen by antigen-presenting cells.

CHOLESTEROL INCLUSION IN LIPOSOMES AFFECTS INDUCTION OF ANTIGEN-SPECIFIC IGG AND IGE ANTIBODY PRODUCTION

In a study in which the induction of OVA-specific antibody production was investigated in mice by OVA-liposome conjugates made using four different lipid components [12], the highest titer of anti-OVA IgG was observed in mice immunized with OVA-liposomes made using liposomes with the highest membrane fluidity, suggesting that the membrane fluidity of liposomes affects their adjuvant effect. Here, liposomes with five different cholesterol inclusions, ranging from 0% to 43% of the total lipid, were made, and the induction of OVA-specific antibody production by OVA-liposome conjugates was compared among these liposome preparations [18].

In contrast to the results in the previous study [12], liposomes that contained no cholesterol and possessed the lowest membrane fluidity demonstrated the highest adjuvant effect for the induction of IgG antibody production. A significant difference was observed in the degree of fluorescence polarization among liposomes with different cholesterol inclusions (Fig. **5a**). The increase in fluorescence polarization values can be interpreted as the result of a decrease in mobility of the hydrophobic region of phospholipid bilayers in the membranes. The results suggest that the membrane fluidity of liposomes correlated well with the amount of cholesterol contained, and liposomes containing no cholesterol showed the lowest membrane fluidity. Liposomes in Fig. (**5a**) were then coupled with OVA and inoculated into mice. The level of anti-OVA IgG antibody production was correlated with the amount of cholesterol included in the liposomes (Fig. **5b**), and OVA-liposome conjugates prepared

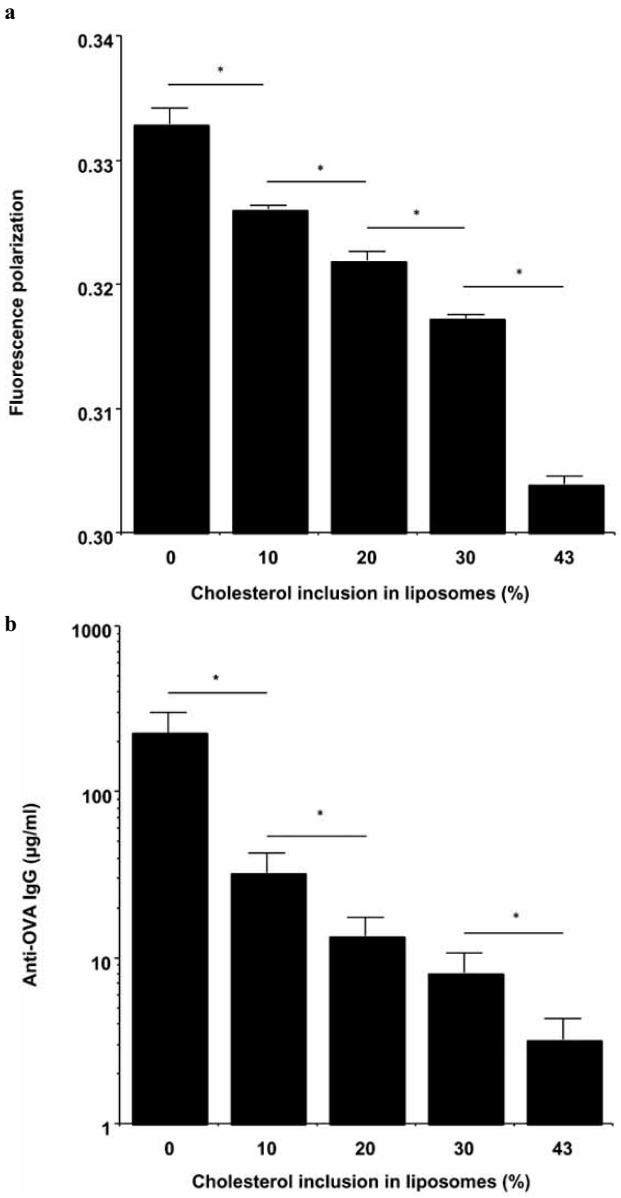

Fig. (5). a: Degree of fluorescence polarization in the liposomes with five different cholesterol inclusions. The degree of fluorescence polarization at 37°C was calculated. Data represent the mean and SE of the triplicate measurements. Asterisk, significant ($p<0.01$) difference.

b: Anti-OVA antibody production in mice immunized with OVA-liposome conjugates made using liposomes with five different cholesterol inclusions. Six weeks after primary immunization, the mice were bled from the tail vein, and the serum anti-OVA IgG were measured. Data represent the mean and SE of five mice per group. Asterisk, significant ($p<0.01$) difference.

using liposomes that contained no cholesterol induced the highest level of anti-OVA IgG antibody production. In addition, when the liposomes with four different lipid compositions were used, OVA-liposome conjugates made using liposomes that did not contain cholesterol induced significantly higher levels of anti-OVA IgG antibody production than did those made using liposomes that contained cholesterol (Fig. **6a**), and, furthermore, the conjugates with no cholesterol induced production of a substantial amount of anti-OVA IgE, except in the case of "palmitoyl" liposomes (Fig. **6b**).

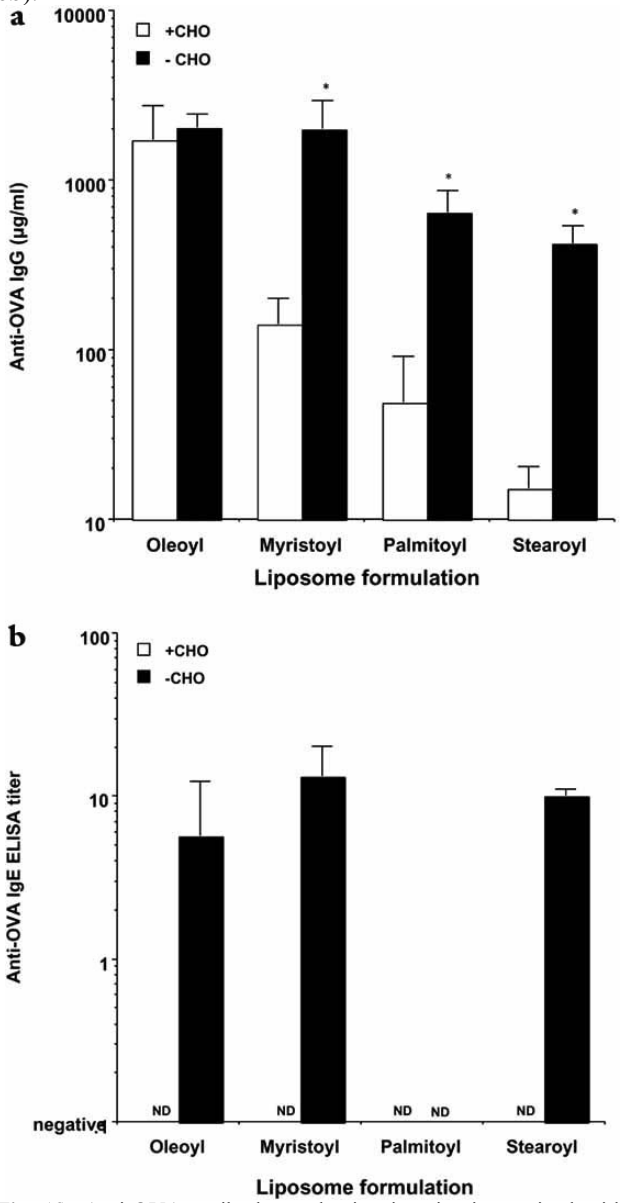

Fig. (6). Anti-OVA antibody production in mice immunized with OVA-liposome conjugates made using liposomes with four different lipid formulations with (□) or without (■) cholesterol. Six weeks after primary immunization, the mice were bled from the tail vein, and serum anti-OVA antibodies were measured. **a**, IgG. **b**, IgE. Data represent the mean and SE of five mice per group. Asterisk, significant ($p<0.01$) difference as compared with liposomes of the same formulation containing cholesterol. ND, not detected.

Unexpectedly, the OVA-liposome conjugates made using liposomes containing no cholesterol induced production of a substantial amount of anti-OVA IgE, except in the "palmitoyl" liposome group. It is unlikely that the level of IgE production in those groups was related to the titer of IgG antibody production, since in mice immunized with OVA-liposome conjugates prepared using "oleoyl" liposomes, IgE antibody production was induced only in the group of no-cholesterol liposomes, although a similar level of IgG antibody production was induced in both the "oleoyl" and "stearoyl" liposome groups regardless of the presence or absence of cholesterol in liposomes. By the inclusion of cholesterol, liposomes might be made more resistant to disintegration and biological degradation [19]. Perhaps the change in the stability of liposomes caused by cholesterol inclusion affects both adjuvanticity and the capacity to induce IgE-selective unresponsiveness by antigen-coupled liposomes. Both IL-4 and IL-13 are known to play a key role in the induction of IgE antibody production [20]. However, since the $CD4^+$ T cells of mice immunized with OVA-liposome conjugates produced IL-4 upon *in vitro* stimulation with OVA (data not shown), regardless of *in vivo* IgE production, antigen-specific IL-4 production by T cells did not participate in the regulation of IgE production in mice immunized with OVA-liposome conjugates.

Thus, these results suggest that cholesterol inclusion in liposomes affects both the adjuvanticity for IgG production and the regulatory effects on IgE synthesis by the coupling of antigens to the surface of liposomes.

INCLUSION OF PHOSPHATIDYLSERINE IN LIPOSOMES INCREASES THEIR ADJUVANTICITY

Exposure of phosphatidyl serine on apoptotic cells is known to result in the enhanced recognition of apoptotic cells by phagocytes [21]. By the inclusion of phosphatidyl serine in the lipid component of liposomes, increased liposome-immune adjuvant activity was expected. Two different liposome preparations containing either phosphatidyl serine (PS-liposome) or phosphatidyl choline (PC-liposome) were made, and macrophage recognition, processing, and antigen presentation of surface-coupled liposomal antigen were compared between them [22].

When OVA-liposome conjugates were added to a culture of macrophages, enhanced recognition and processing of OVA by the macrophages were observed by the inclusion of phosphatidyl serine in the liposomes. The phagocytosis of OVA-liposome conjugates 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. Fig. (**7**) shows the fluorescence intensity of macrophages cultured for 60 min with the OVA-liposome conjugates. More OVA was incorporated into the macrophages when the OVA was coupled to PS-liposomes (OVA-PS-liposome) than when OVA was coupled to PC-liposomes (OVA-PC-liposome). The results correlated well with those regarding the macrophage antigen presentation of liposome-coupled OVA. Macrophages were cultured overnight in the presence of OVA-lipospome conjugates prior to co-culture with the OVA-specific T-cell clone,

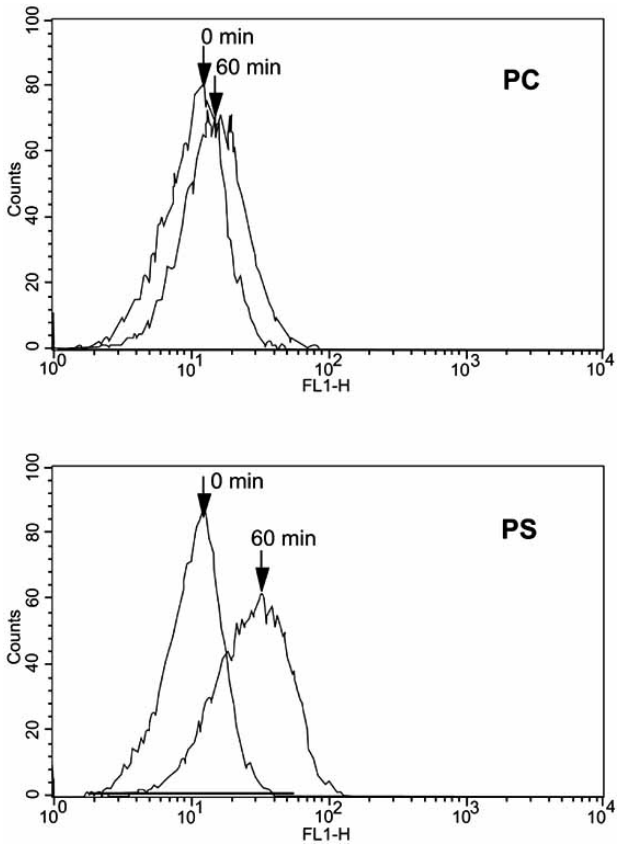

Fig. (7). Phagocytosis of OVA-liposome conjugates by macrophages. Fluorescence-labeled OVA was coupled to either PC-liposomes (**PC**) or PS-liposomes (**PS**) and added to a culture of macrophages. Macrophages recovered from the culture were analyzed using flow cytometry.

42-6A. IL-2 production by the T-cell clone was then monitored. Fig. (**8**) 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-PS-liposomes. The amount of OVA in the culture to which the OVA-liposome conjugates were added was 32 μg/ml. However, the addition of 32 μg/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 pre-cultured with OVA-PC-liposomes, the amount of IL-2 was more than three-fold lower than that produced when PS-liposomes were added to the culture.

Furthermore, *in vivo* immunization in mice with OVA-liposome conjugates made using PS-liposomes induced a significantly higher level of anti-OVA IgG antibody production than was induced by OVA-liposome conjugates made using PC-liposomes (Fig. **9**). IgE-selective unresponsiveness was induced by OVA-liposome conjugates regardless of the lipid components of liposomes (data not shown). The levels of anti-OVA IgG antibody production induced by the two different types of OVA-liposome conjugate were significantly different; OVA-PS-liposomes induced significantly higher levels of anti-OVA IgG antibody production than that observed in association with OVA-PC-liposomes at 2, 4, 5, and 6 weeks after primary immunization.

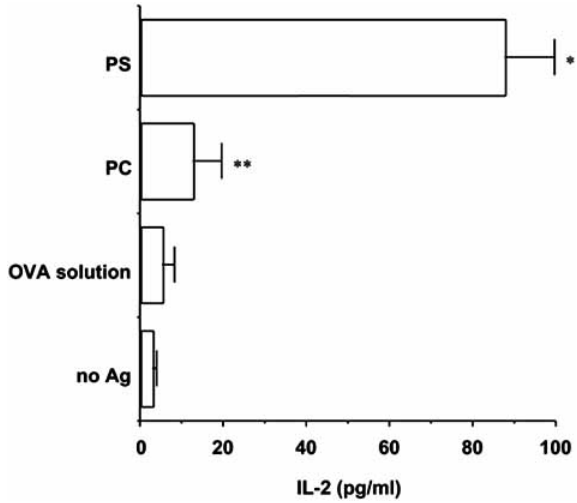

Fig. (8). Antigen presentation by macrophages pulsed with OVA-liposomes. Macrophages pre-incubated with OVA-liposomes were co-cultured with OVA-specific T-cell clones. Data represent the mean IL-2 concentration and the SE of the culture supernatant in triplicate cultures. Asterisk, significant ($p<0.01$) difference as compared with the other groups. Two asterisks, significant ($p<0.01$) difference as compared with the OVA solution.

A variety of cell surface molecules (e.g., lectin-like molecules [23], CD14 [24], scavenger receptor antagonists [25], and PS [21]), 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 [21], 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

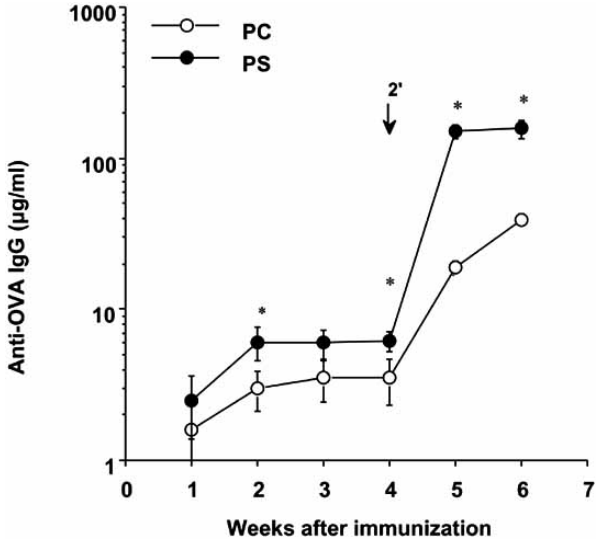

Fig. (9). Anti-OVA IgG antibody production in mice immunized with OVA-liposome conjugates. BALB/c mice were immunized with OVA-liposome conjugates made using PC- liposomes (PC) or PS-liposomes (PS) at 0 and 4 weeks. The mice were bled weekly from the tail vein, and serum anti-OVA IgG was measured. Data represent the mean and SE of five mice per group. Asterisk, significant ($p<0.01$) difference between the PC- and PS-liposome groups.

hand, a number of reports have shown that PS possesses immunosuppressive properties, such as an inhibition of the T-cell mitogen response [26], a reduction of macrophage NO synthesis [27], the inhibition of tumor cytotoxicity by macrophages [28], and the suppression of antigen-specific antibody production when PS was orally administered [29]. 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.

Thus, these results suggest that the inclusion of phosphatidyl serine in liposomes enhances the recognition and processing of surface-coupled liposomal antigen by macrophages and increases liposome-immune adjuvant activity.

ANTIGENS COUPLED TO THE SURFACE OF LIPOSOMES MADE USING UNSATURATED FATTY ACID ARE CROSS-PRESENTED TO $CD8^+$ T CELLS AND INDUCE POTENT ANTITUMOR IMMUNITY

Liposomes with differential lipid components were demonstrated to display differential adjuvant effects when antigens were chemically coupled to their surfaces [12, 17]. Here, the antigen presentation of liposome-coupled OVA was investigated *in vitro*, and it was found that OVA coupled to liposomes made using unsaturated fatty acid was presented to both $CD4^+$ and $CD8^+$ T-cells while OVA coupled to liposomes made using saturated fatty acid was presented only to $CD4^+$ T cells [16]. Splenic adherent cells of BALB/c mice were co-cultured with OVA-liposome conjugates made using liposomes with two different lipid components for 2 h, and subsequently cultured with splenic $CD4^+$ or $CD8^+$ T cells of OVA-immune BALB/c mice. As shown in Table (**1**), OVA-liposome conjugates made using liposomes with two different lipid components induced the production of comparable levels of IL-5 and IFN-γ by $CD4^+$ T cells, while OVA solution with the same antigen concentration as OVA-lipospome conjugates induced a much lower level of IL-5 production and no IFN-γ. However, OVA-liposome conjugates made using "saturated" liposomes did not induce either IL-5 or IFN-γ production by $CD8^+$ T cells, while OVA-liposome conjugates made using "unsaturated" liposomes induced a significant production of both IL-5 and IFN-γ.

Confocal laser scanning microscopic analysis demonstrated that a portion of the OVA coupled to liposomes made using unsaturated-, but not saturated fatty acid, received processing beyond the MHC class II compartment, suggesting that degradation of OVA might occur in the cytosol, and that the peptides generated in this manner would be presented to $CD8^+$ T cells *via* MHC class I. Macrophages expressed DM-DsRed ("Mϕ alone" in Fig. **10**). The yellow spots in the "saturated" panel in Fig. (**10**) show that DQ-OVA coupled to liposomes received processing in the class II compartment. In contrast, in the "Unsaturated" panel in Fig. (**10**), both green and yellow spots were observed, suggesting that a portion of the DQ-OVA coupled to "unsaturated" liposomes did not receive processing in the class II compartment.

The ability to induce cross-presentation of an antigen coupled to liposomes consisting of unsaturated fatty acid was further confirmed by the *in vivo* induction of cytotoxic T lymphocytes. The cross-presentation of OVA coupled to "unsaturated" liposomes was further confirmed utilizing experiments of *in vivo* CTL induction. As shown in Fig. (**11**), both $OVA_{257\text{-}264}$ (D) and whole OVA (E) coupled to "unsaturated" liposomes successfully induced CTLs against target cells pulsed with $OVA_{257\text{-}264}$ but not against target cells pulsed with control $NP_{366\text{-}374}$. On the other hand, a mixture of $OVA_{257\text{-}264}$ and "unsaturated" liposomes (B), and $OVA_{257\text{-}264}$ coupled to "saturated" liposomes (C) failed to induce CTLs against target cells pulsed with $OVA_{257\text{-}264}$.

The ability to induce cross-presentation of an antigen coupled to liposomes consisting of unsaturated fatty acid was also confirmed by the induction of tumor eradication in mice. E.G7 tumors in mice that received combined inoculation with $OVA_{257\text{-}264}$-liposome conjugates, CpG, and anti-IL-10 monoclonal antibodies were completely eradicated. In those mice, the frequency of $CD8^+$ T cells reactive with $OVA_{257\text{-}264}$ peptides in the context of $H\text{-}2K^b$ was significantly increased. In order to examine the effectiveness of liposome-coupled peptides *in vivo*, we performed tumor-rejecting experiments. B6 mice were injected subcutaneously with E.G7 cells transfected with OVA DNA, and solid tumors with a diameter of more than 5 mm were established around 7 to 10 days after the injection. Liposome-coupled peptides, $OVA_{257\text{-}264}$, with CpG and the anti-IL-10 antibody were injected twice around the tumor mass. As shown in Fig. (**12**), a significant ($p<0.001$) decrease of the mean tumor diameter was observed as early as 7 days after inoculation of

Table 1. Cytokine Production by Splenic CD4/CD8 T-Cells of Mice Immunized with OVA After Co-Culture with OVA-Pulsed SAC

in vitro Ag	Liposomes	CD4		CD8	
		IL-5	IFN-γ	IL-5	IFN-γ
none		ND	ND	ND	ND
OVA solution		96.2±12.5	ND	ND	ND
OVA-liposome	Saturated	910.2±23.0	88.7±45.0	ND	ND
OVA-liposome	Unsaturated	1065.5±31.9	115.1±28.6	163.3±99.1	149.9±83.8

Splenic CD4/CD8 T-cells were taken from mice immunized with OVA and were cultured with OVA-pulsed SAC. Data represent the mean cytokine concentration (pg/ml) in the culture supernatants and SE of triplicate culture. ND, not detected.

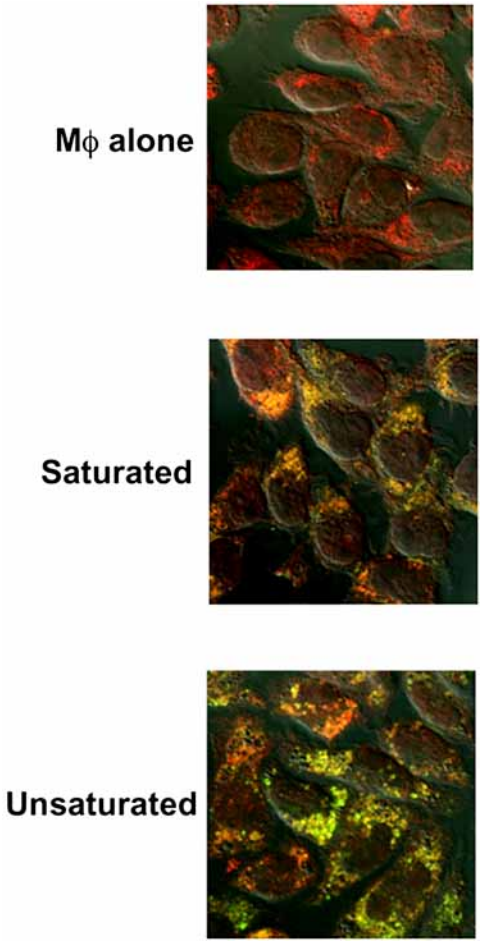

Fig. (10). Confocal laser scanning microscopic analysis of macrophages co-cultured with DQ-OVA-liposome conjugates. DM-DsRed-expressing macrophages were co-cultured with DQ-OVA-liposome conjugates made using "saturated" or "unsaturated" liposomes. Two hours after the onset of the culture, the macrophages were recovered and analyzed using confocal laser scanning microscopy. Macrophage alone, macrophages without co-culture with DQ-OVA-liposome conjugates. These optically merged images are representative of most cells examined by confocal microscopy. Yellow, co-localization of green (DQ-OVA received processing) and red (macrophage DM).

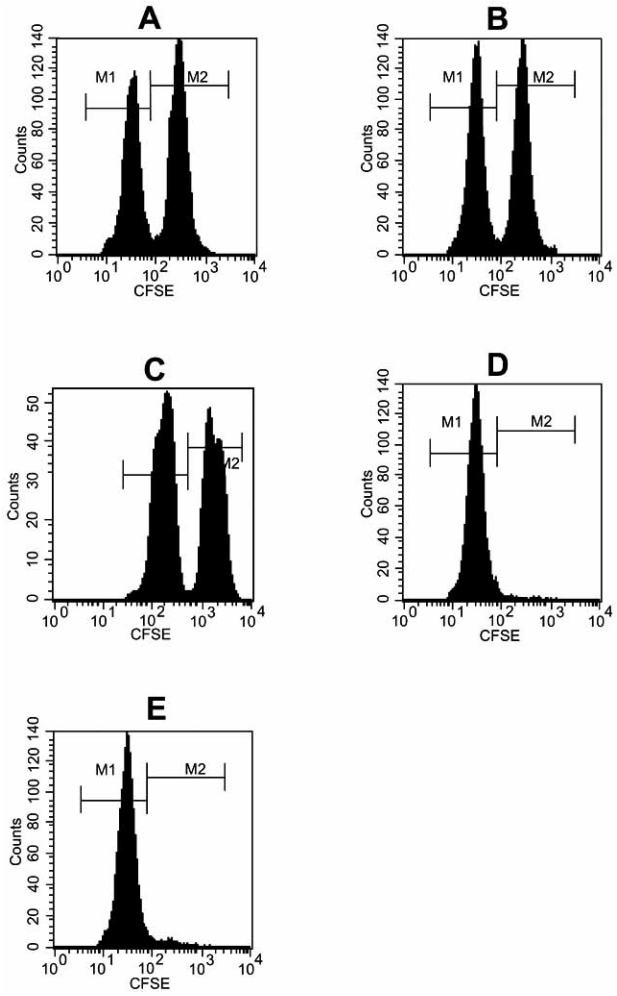

Fig. (11). *In vivo* CTL induction in mice immunized with OVA-liposome conjugates. Mice were injected with 100 μg of anti-IL-10 monoclonal antibodies and 5 μg of CpG with PBS (**A**), a mixture of $OVA_{257-264}$ and "unsaturated" liposomes (**B**), $OVA_{257-264}$-"saturated" liposome conjugates (**C**), $OVA_{257-264}$-"unsaturated" liposome conjugates (**D**), and OVA-"unsaturated" liposome conjugates (**E**). CFSE bright cells (M2) were pulsed with $OVA_{257-264}$, and CFSE dull cells (M1) were pulsed with $NP_{366-374}$ peptide as a control. Data represent the results of flow cytometric analysis for splenocytes from each mouse.

liposome-coupled $OVA_{257-264}$ with CpG and the anti-IL-10 antibody, and the tumors were completely eradicated in 12 days. In contrast, injection of CpG and the anti-IL-10 antibody with peptide solution containing the same amount of $OVA_{257-264}$ as liposome-coupled $OVA_{257-264}$ did not eradicate the established tumors. These results suggested that the liposome-coupled $OVA_{257-264}$ might effectively present $OVA_{257-264}$ to CTLs, resulting in tumor rejection.

In most APCs, exogenous antigens cannot be presented by the MHC class I pathway because the exogenous antigens are unable to gain access to the cytosolic compartment. This segregation of exogenous antigens from the class I pathway is important in preventing CTLs from killing healthy cells that have been exposed to foreign antigens but are not infected [30]. Consequently, in general, exogenous antigens do not prime CTL responses *in vivo*. However, there are several exceptions to this rule, reflecting an ability of the antigens to be delivered into the cytosolic compartments [31-35]. In this study, antigens coupled to liposomes consisting of unsaturated fatty acid were presented to both $CD4^+$ and $CD8^+$ T cells. Confocal laser scanning microscopic analysis demonstrated that a portion of the OVA coupled to liposomes received processing beyond the MHC class II compartment, suggesting that the degradation of OVA occurs in the cytosol, and that peptides generated in this manner would be presented to $CD8^+$ T cells *via* MHC class I. Cross-presentation induced by OVA coupled to liposomes consisting of unsaturated fatty acids was further confirmed in the *in vivo* CTL induction experiments. CTLs were successfully induced *in vivo* only when OVA or $OVA_{257-264}$ chemically coupled to "unsaturated" liposomes was inoculated into mice.

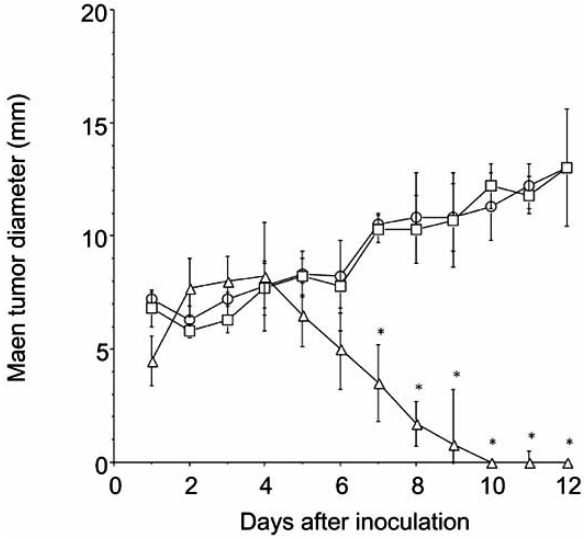

Fig. (12). Effect of peptide-liposome conjugates on the growth of the E.G7 tumor in mice. The tumor was established, and a mixture of CpG and anti-IL-10 was inoculated around the tumor mass in conjunction with liposome-coupled peptide (△), peptide solution containing the same amount of peptide as liposome-coupled peptide (□), or with nothing (○). Asterisk, $P<0.001$ as compared with the mean diameter of the mice without inoculation of liposome-coupled peptides. Data represent the mean and SE of four mice per group.

We next investigated the ability of antigen-liposome conjugates to induce antitumor immunity. The aim of cancer vaccination is to generate an immune-mediated anti-tumor associated antigen (TAA) response resulting in the elimination of the tumor. The antigen of choice may be the whole protein alone or with immune stimulatory components, or defined epitopes (e.g., peptides) of the target antigen [36]. Recent preclinical studies have demonstrated that combined therapies involving the use of vaccines with cytokines, activators of DCs such as TLR ligands or mAb to CD40, or recombinant vectors that provide a stimulus to the innate immune system resulted in enhanced antitumor responses [37]. In the present study, antigenic peptides were chemically coupled to the surface of liposomes and inoculated into tumor-bearing mice in combination with CpG and anti-IL-10 monoclonal antibodies. This treatment successfully induced eradication of the tumor mass, whereas the inoculation of mice with CpG and anti-IL-10 monoclonal antibodies with peptide solution containing the same amount of $OVA_{257-264}$ as liposome-coupled $OVA_{257-264}$ did not affect E.G7 tumor growth (Fig. **12**). It has been reported that CpG and the anti-IL-10 receptor antibody reverse tumor-induced DC paralysis, resulting in tumor rejection by CTL activated by the DC [38].

Thus, the results obtained in these experiments suggest that, by choosing lipid components for liposomes, surface-coupled liposomal antigens might be applicable for the development of tumor vaccines to present tumor antigens to APCs and induce antitumor responses.

CONCLUSIONS AND FUTURE PERSPECTIVES

We have investigated the potential of surface-linked liposomal antigens for application to vaccine development, in contrast to previous investigations on liposome-based drug-delivery systems, which have focused on the encapsulation of antigens into liposomes [7, 39]. During the course of this investigation, several advantages of the liposome-coupled antigens over the liposome-encapsulated antigens became apparent. (i) A predominant coupling efficiency of antigens to liposomes; following our previously reported procedure [11] for coupling antigens to liposomes, approximately 50% of the antigens bound to the surface of liposomes, whereas in the antigen-encapsulation, a 60-fold higher volume of antigens was required to obtain the same amount of conjugates (unpublished observation). (ii) Antigen-specific and IgE-selective unresponsiveness induced by surface-linked liposomal antigens; antigens chemically coupled to the surface of liposomes induced antigen-specific IgG but not IgE antibody production in mice [10] and also in monkeys [40], suggesting the potential of surface-linked liposomal antigens for application to the development of vaccines with minimal allergic side-effects. In addition, during the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-linked liposomal antigen, we found the existence of an alternative mechanism, not involving T cells, in the regulation of IgE synthesis [13]. (iii) An enhanced recognition of liposomal antigens by APCs; since liposomes basically consist of immunologically inert fatty acid, they are hardly recognized by APCs. Therefore, some contrivance, such as the introduction of mannose on the surface of liposomes [41], is required in antigen-encapsulated liposomes to enhance the recognition of liposomes by APCs. On the other hand, in surface-linked liposomal antigens, antigens expressed on the surface of liposomes might be recognized more efficiently by APCs, which might result in an enhanced presentation to T cells. In fact, surface-linked liposomal antigens induced a significantly higher level of antigen-specific IgG production than that by liposome-encapsulated antigens in mice (unpublished observation). In addition, a significant difference, which correlated closely with the adjuvant activity of liposomes, was observed in the recognition of liposomal antigens by APCs between liposomes with different lipid components; more antigens coupled to the "unsaturated" liposomes were engulfed by macrophages *in vitro* and a higher level of antigen-specific antibody production was induced *in vivo* than when "saturated" liposomes were used, suggesting that the adjuvant effects of liposomes are exerted at the beginning of the immune response, i.e., upon the recognition of antigens by APCs [17]. In addition to this "quantitative" difference between liposomes with differential lipid components, a "qualitative" difference (i.e., a differential ability to induce cross-presentation) was also observed between "saturated" and "unsaturated" liposomes. Although the precise mechanism underlying this difference is currently unclear, the significant difference in membrane mobility observed between these liposomes [12] might affect their ability to induce cross-presentation. These data suggest that differential lipid components in liposomes lead to differential processing and presentation of liposomal antigens in APCs.

Taken together, these results indicate that surface-linked liposomal antigens may be applicable for the development of vaccines which induce minimal allergic reaction and virus

vaccines which induce CTL responses, and for the development of a drug which induces potent antitumor immunity.

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