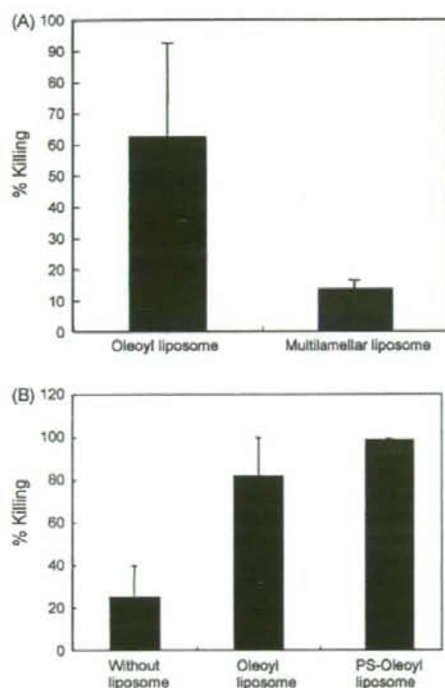

Fig. 1. Comparison of CTL activation capacities of various liposomes carrying peptides. (A) B6 mice were injected subcutaneously with either liposome with chemically bound $OVA_{257-264}$ on the surface or multilamellar liposome containing $OVA_{257-264}$ inside in the presence of CpG and anti-CD40 antibody. Composition of each liposome is described in Section 2. Seven days later, high CFSE-labeled target cells pulsed with $OVA_{257-264}$ and low CFSE-labeled target cells pulsed with $NP_{366-374}$ were injected intravenously as an *in vivo* CTL assay. Viability of the target cells in the spleen was examined at 12 h after injection. OVA specific killing was calculated as follows: %killing = [1 − {(number of cells carrying $OVA_{257-264}$ in immunized mice (CFSE high))/(number of cells carrying $NP_{366-374}$ in immunized mice (CFSE low))}/{(number of cells carrying $OVA_{257-264}$ in normal mice (CFSE high))/(number of cells carrying $NP_{366-374}$ in normal mice (CFSE low))}] × 100. Injection of liposome with chemically bound $OVA_{257-264}$ on the surface induced CTL activity more effectively than did multilamellar liposome containing $OVA_{257-264}$ inside ($p = 0.049$). (B) PS–Oleoyl liposome was generated by alteration of phosphatidyl choline to phosphatidyl serine of Oleoyl liposome. $OVA_{257-264}$–PS–Oleoyl liposome, $OVA_{257-264}$–Oleoyl liposome or $OVA_{257-264}$ unbound to liposome was inoculated with CpG and anti-CD40 antibody. Significant difference between CTL-activating capacities of $OVA_{257-264}$–PS–Oleoyl liposome and $OVA_{257-264}$–Oleoyl liposome was not observed ($p = 0.167$).

not detect a significant difference between CTL-activating capacities of between $OVA_{257-264}$–PS–Oleoyl liposome and $OVA_{257-264}$–Oleoyl liposome ($p = 0.167$), although $OVA_{257-264}$–Oleoyl liposome and $OVA_{257-264}$–PS–Oleoyl liposome induced significant CTL activity compared to that induced by peptide without liposome ($p = 0.013$ and $p < 0.001$, respectively) (Fig. 1B). On the other hand, Oleoyl liposome is preserved more easily than PS–Oleoyl liposome, because Oleoyl liposome is more stable than PS–Oleoyl liposome. Therefore, we used Oleoyl liposome in this study.

3.3. CTL activation by simultaneous inoculation of CpG and $OVA_{257-264}$ bound to Oleoyl liposome

We tested which combination among $OVA_{257-264}$–Oleoyl liposome, CpG and anti-CD40 antibody was critical for induction of CTL activity. Subcutaneous inoculation of a combination of CpG and $OVA_{257-264}$–Oleoyl liposome elicited CTL responses *in vivo*, while simultaneous inoculation of anti-CD40 and $OVA_{257-264}$–Oleoyl liposome, or $OVA_{257-264}$–Oleoyl liposome alone did not (Fig. 2). On the other hand, CTL activity elicited by CpG and $OVA_{257-264}$–Oleoyl liposome was not different from that elicited by CpG, anti-CD40 and $OVA_{257-264}$–Oleoyl liposome ($p = 0.12$). Therefore, inoculation of a combination of CpG and $OVA_{257-264}$–Oleoyl liposome was sufficient to elicit CTL responses *in vivo*.

3.4. Detection of immunoenhancers for eliciting CTL activity with peptide–Oleoyl liposome

In order to find effective immune enhancers eliciting CTL responses with peptide–Oleoyl liposome, we inoculated peptide–Oleoyl liposome simultaneously with various TLR ligands. $OVA_{257-264}$–Oleoyl liposome alone did not elicit CTL responses as shown in Fig. 2. CpG (10 μg/mouse) (TLR9 ligand) and Poly(I:C) (TLR3 ligand) (10 μg/mouse) showed sufficient adjuvant activity in combination with $OVA_{257-264}$–Oleoyl liposome compared with LPS (10 μg/mouse) (TLR4 ligand) ($p = 0.003$ and 0.012, respectively) (Fig. 3A). Administration of LPS

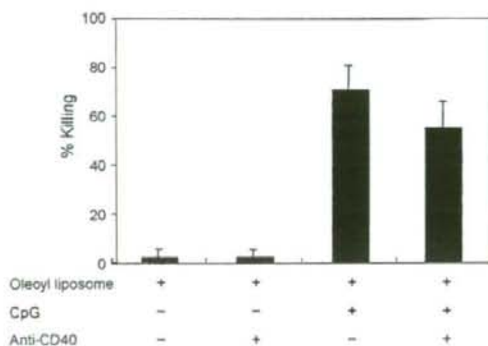

Fig. 2. CTL activation by simultaneous inoculation of CpG and $OVA_{257-264}$–Oleoyl liposome. Various combinations of CpG, anti-CD40 and $OVA_{257-264}$–Oleoyl liposome were inoculated subcutaneously into B6 mice for an *in vivo* CTL assay. Subcutaneous inoculation with CpG and $OVA_{257-264}$–Oleoyl liposome elicited CTL responses *in vivo*, but that with anti-CD40 and $OVA_{257-264}$–Oleoyl liposome ($p < 0.001$) or $OVA_{257-264}$–Oleoyl liposome alone ($p < 0.001$) did not. CTL activity elicited by CpG and $OVA_{257-264}$–Oleoyl liposome was not different from that elicited by CpG, anti-CD40 and $OVA_{257-264}$–Oleoyl liposome ($p = 0.12$).

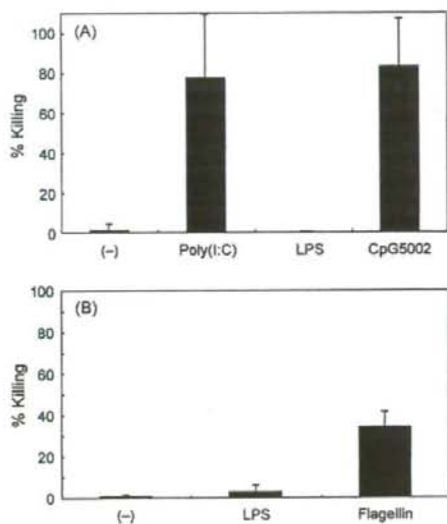

Fig. 3. Comparison of TLR ligands as adjuvants. We inoculated simultaneously with various TLR ligands and $OVA_{257-264}$– or $NP_{366-374}$–Oleoyl liposome. Seven days later, high CFSE-labeled target cells pulsed with $OVA_{257-264}$ or $NP_{366-374}$, and low CFSE-labeled target cells pulsed with VSV-NP_{52-59} (RGYVFQGL) or LCMV-$NP_{396-404}$ (FQPQNGQFI) were injected intravenously as an *in vivo* CTL assay. Viability of the target cells in the spleen was examined at 12 h after injection. Killing activity specific for $OVA_{257-264}$ or $NP_{366-374}$ was calculated as described in the legend of Fig. 1. (A) Inoculation dose of CpG, LPS or Poly(I:C) was 10 μg, respectively. CpG showed sufficient adjuvant activity in combination with $OVA_{257-264}$–Oleoyl liposome comparing with LPS (TLR4 ligand) ($p = 0.003$). Poly(I:C) (TLR3-ligand) worked as an enhancer similar to CpG ($p = 0.82$). (B) Flagellin (TLR5-ligand) (10 μg/mouse) or LPS (10 μg/mouse) was inoculated into mice with $NP_{366-374}$–Oleoyl liposome. In combination with the peptide–Oleoyl liposome, Flagellin showed significant adjuvant activity comparing with LPS ($p = 0.0027$).

(10 μg/mouse) and OVA protein induced *in vivo* CTL responses (around 40%killing) but that of OVA protein alone did not (data not shown). Thus, it seems that administration of 10 μg/mouse of LPS is quantitatively sufficient for working as adjuvant. Next, combination of another peptide–Oleoyl liposome and adjuvants was also tested. $NP_{366-374}$–Oleoyl liposome alone and with LPS did not elicit CTL responses, but Flagellin (TLR5 ligand) worked as an enhancer, although %killing was less than that by CpG or Poly(I:C) in combination with $OVA_{257-264}$–Oleoyl liposome (Fig. 3B).

3.5. Vaccination of NP366-374 bound to Oleoyl liposome in combination with CpG

In order to test whether peptide conjugated to Oleoyl liposome in combination with CpG works as a vaccine, we subcutaneously injected twice B6 mice with $NP_{366-374}$ conjugated to Oleoyl liposome in combination with CpG and measured virus titers in the lungs after intranasal challenge of influenza viruses. We detected CTL activity in spleens

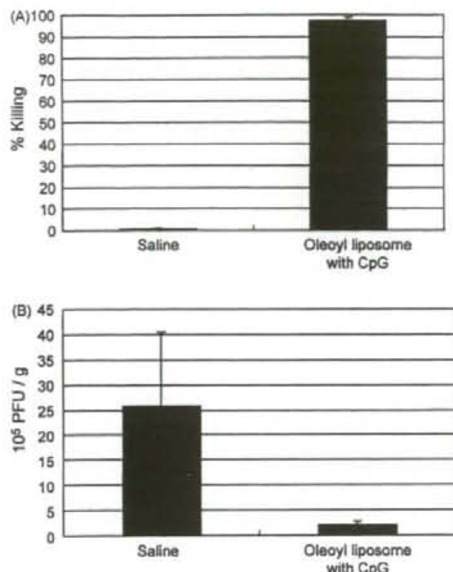

Fig. 4. Vaccination of $NP_{366-374}$ bound to Oleoyl liposome in combination with CpG. (A) B6 mice were injected twice subcutaneously with Oleoyl liposome with chemically bound $NP_{366-374}$ on the surface with CpG. Seven days later, high CFSE-labeled target cells pulsed with $NP_{366-374}$ and low CFSE-labeled target cells pulsed with $OVA_{257-264}$ were injected intravenously as an *in vivo* CTL assay. NP-specific killing was calculated as follows: %killing = [1 − {(number of cells carrying $NP_{366-374}$ in immunized mice (CFSE high))/(number of cells carrying $OVA_{257-264}$ in immunized mice (CFSE low))}/{(number of cells carrying $NP_{366-374}$ in normal mice (CFSE high))/(number of cells carrying $OVA_{257-264}$ in normal mice (CFSE low))}] × 100. Injection of $NP_{366-374}$–Oleoyl liposome with CpG induced CTL activity more effectively than that of saline ($p < 0.01$). (B) B6 mice were injected twice subcutaneously with Oleoyl liposome with chemically bound $NP_{366-374}$ on the surface with CpG. Seven days later, the immunized mice were challenged with Influenza virus, A/Aichi/2/68 (H3N2). At 5 days after challenge, lungs of the immunized mice were homogenized for use in a plaque-forming assay using MDCK cells as described in Section 2. Injection of $NP_{366-374}$–Oleoyl liposome with CpG reduced virus growth in the lungs more effectively than that of saline ($p = 0.047$).

of mice inoculated with a mixture of $NP_{366-374}$–Oleoyl liposome and CpG but not in spleens of mice inoculated with saline (Fig. 4A). Similarly, inoculation of a mixture of $NP_{366-374}$–Oleoyl liposome and CpG inhibited virus growth in mouse lungs, but saline did not (Fig. 4B).

We have demonstrated that surface-linked liposomal antigens induce antigen-specific IgG antibody production but not antigen-specific IgE antibody production [10]. Herein, we evaluated again whether $NP_{366-374}$–Oleoyl liposome and CpG induce IgE production, because IgE shows detrimental effects, such as allergy. $NP_{366-374}$–Oleoyl liposome and CpG induced IgG production specific for NP protein, although antibody titer was marginal (Fig. 5A). In contrast, $NP_{366-374}$–Oleoyl liposome and CpG did not elicit any IgE production against NP protein (Fig. 5B). In conclusion,

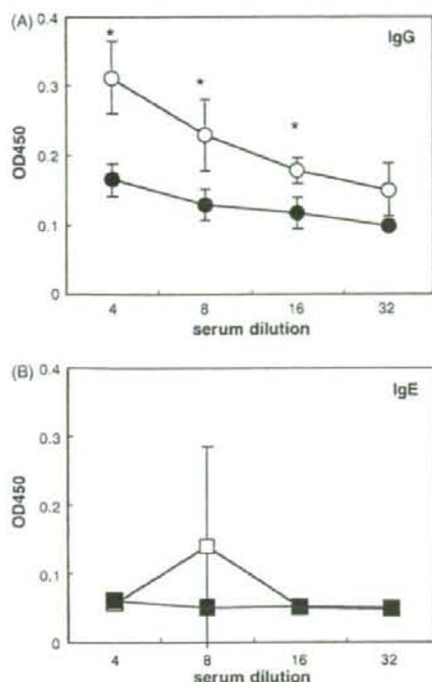

Fig. 5. Antibody production by simultaneous inoculation of CpG and $NP_{366-374}$–Oleoyl liposome. Sera were collected 5 days after infection following subcutaneous immunization twice with NP–liposome and CpG. (A) Total IgG against disrupted A/Aichi/2/68 (H3N2) in the serially diluted sera was measure with ELISA as described in the Section 2. IgG in the immunized sera (○) was subtle but significantly increased comparing control (●) (* indicates $p < 0.05$). (B) IgE against disrupted A/Aichi/2/68 (H3N2) in the immunized sera (□) or control (■) were measured with ELISA. Optical density was measured at 450 nm.

peptide–Oleoyl liposome is an effective vaccine candidate in the presence of CpG without detrimental effects in mice.

4. Discussion

It has been shown that liposomes work as carriers of vaccines and supported immune responses by vaccines [19–26]. Furthermore, alteration of lipid composition has been reported to modulate immune responses [27–33]. Indeed, the inducibility of antigen-specific IgG antibody production by antigen–liposome has been shown to vary among the liposome preparations used for the production of antigen–liposome; the greater the membrane mobility in liposomes is, the greater is antibody production induced by antigen–liposome [11]. In this study we searched for a composition of liposome eliciting CTL activity more effectively by using an *in vivo* CTL assay, in which CTL activity was detected timely in immunized animals. Subcutaneous inoculation of CpG and OVA peptide, $OVA_{257-264}$, coupled on the surface of Oleoyl liposome, consisting of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, cholesterol, and dioleoyl phosphatidyl glycerol acid in a 4:3:7:2 molar ratio, elicited CTL responses more vigorously than did other liposomes in the presence of CpG. Furthermore, subcutaneous inoculation of a mixture of $NP_{366-374}$–Oleoyl liposome in combination with CpG protected against infection of influenza A virus.

In a previous study, we intranasally inoculated anti-CD40 antibody and multilamellar liposome containing $NP_{366-374}$ peptide, resulting in induction of protective CTL responses against influenza A virus in mouse lungs [18]. Indeed, in MHC class I deficient mice intranasal inoculation with anti-CD40 antibody and multilamellar liposome containing $NP_{366-374}$ peptide did not protect viral growth in lungs, therefore CTL responses were presumably involved in protective responses in lungs. Herein, however, subcutaneous inoculation of anti-CD40 antibody and multilamellar liposome containing $OVA_{257-264}$ peptide did not induce CTL responses in spleen. These data suggest that simultaneous inoculation of anti-CD40 antibody and multilamellar liposome containing $OVA_{257-264}$ peptide does not elicits systemic CTL responses, but when anti-CD40 antibody and multilamellar liposome containing $OVA_{257-264}$ peptide are locally inoculated, even subtle CTL responses are able to protect infection in the areas, such as lungs.

At present, a few adjuvants, such as aluminum hydroxide and MF59, are clinically in use, although a number of candidate adjuvants have been examined in preclinical studies. However, aluminum adjuvants and MF59 are known to be effective only for the induction of humoral immunity, not for the induction of cell-mediated immunity [34]. In addition, aluminum adjuvants are also known to induce the production of IgE antibodies, which is a cause of allergic response against the vaccine [35–37]. On the other hand, some candidate adjuvants activating innate immunity via TLR support cellular immunity. CpG oligonucleotides are known to enhance maturation and antigen-presenting capacity of dendritic cells via TLR9, resulting in activation of CTL responses. In this study, support of CpG was necessary for the induction of CTL response and protection against influenza infection by peptide–Oleoyl liposome. However, CpG are reported to possess toxicity against hepatocytes and induce immune suppression after daily high-dose administrations [38]. Similarly, Poly(I:C) supported the induction of CTL responses by peptide–Oleoyl liposome. In mice CpG and Poly(I:C) worked most effectively as adjuvant in combination with peptide–Oleoyl liposome, but in human usage CpG and Poly(I:C) will be converted to inhibit induction of detrimental effects, such as toxicity.

In previous studies, inoculation of CpG and antigens elicited CTL responses independent $CD4^+$ T cells [39,40]. Indeed, CTL responses were elicited by simultaneous inoculation of CpG and $OVA_{257-264}$–Oleoyl liposome in MHC class II-deficient mice in which $CD4^+$ T cells were deleted

(data not shown). Thus, this combination could be used as a vaccine in immunocompromised hosts, such as AIDS patients or aged people who have declined antibody production due to defective CD4$^+$ T cell help. Furthermore, it has been shown that pretreatment with OVA–liposomes suppressed IgE antibody responses to CT-combined OVA, with significantly high levels of production of both nasal IgA and serum IgG antibodies. Moreover, treatment with OVA–liposomes 1 and 3 weeks after CT-combined OVA administration also suppressed IgE antibody responses [12]. Taken together, the results in the present study suggest that peptide–Oleoyl liposome conjugates in combination of some sort of immuno-potentiators without toxicity, might serve as a vaccine candidate without detrimental effects, such as allergic responses, even in immunocompromised hosts.

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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

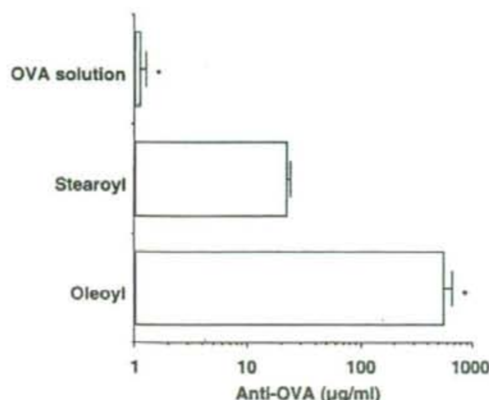

Fig. (1). Anti-OVA IgG antibody production in mice immunized with OVA-liposome conjugates. BALB/c mice were immunized with OVA-lipospome conjugates made using "stearoyl" or "oleoyl" liposomes or with plain OVA solution at 0 and 4 weeks. Six weeks after primary immunization, the mice were bled from the 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™-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.

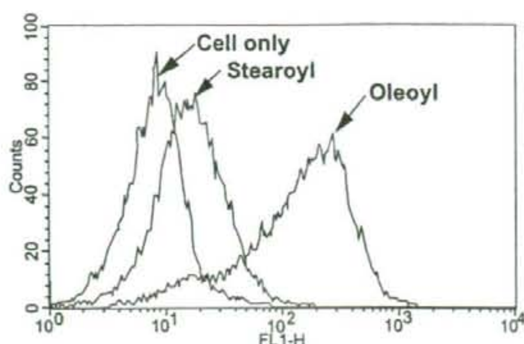

Fig. (2). Phagocytosis of OVA-liposome conjugates by macrophages. Fluorescence-labeled OVA was coupled to either "stearoyl" or "oleoyl" liposomes and added to the culture of macrophages. Macrophages recovered from the culture were analyzed using flow cytometry.

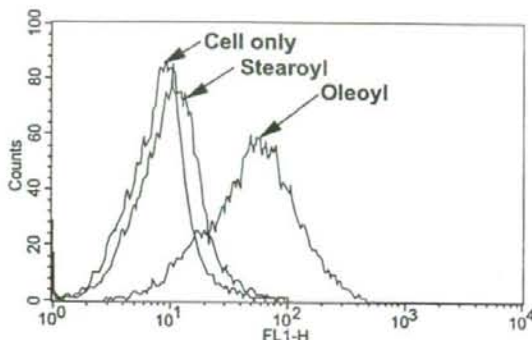

Fig. (3). Digestion of liposome-coupled OVA by macrophages. "stearoyl" or "oleoyl" liposomes coupled with DQ-OVA were added to the macrophage culture. Sixty minutes after the onset of the culture, the macrophages were recovered and analyzed using flow cytometry.

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.

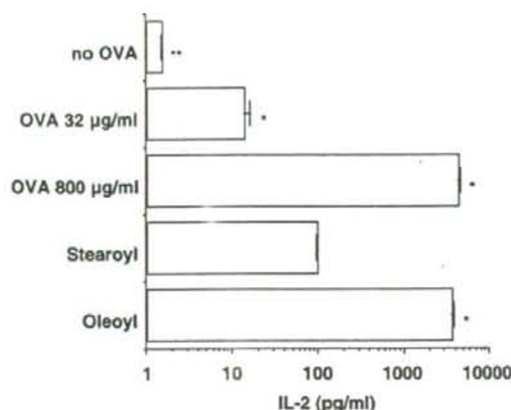

Fig. (4). Antigen presentation by macrophages pulsed with OVA-liposomes. Macrophages pre-incubated with OVA-liposomes were co-cultured with an OVA-specific T-cell clone. Data represent the mean IL-2 concentration and the SE of the culture supernatant in triplicate cultures. Asterisk, significant ($p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

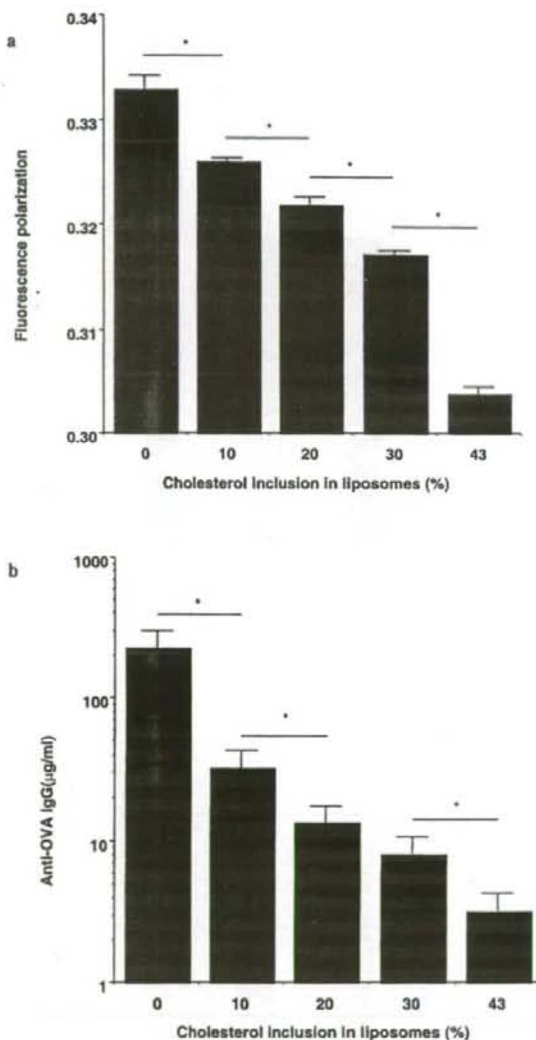

Fig. (5). **a**: Degree of fluorescence polarization in the liposomes with five different cholesterol inclusions. The degree of fluorescence polarization at 37°C was calculated. Data represent the mean and SE of the triplicate measurements. Asterisk, significant ($p<0.01$) difference.

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**).

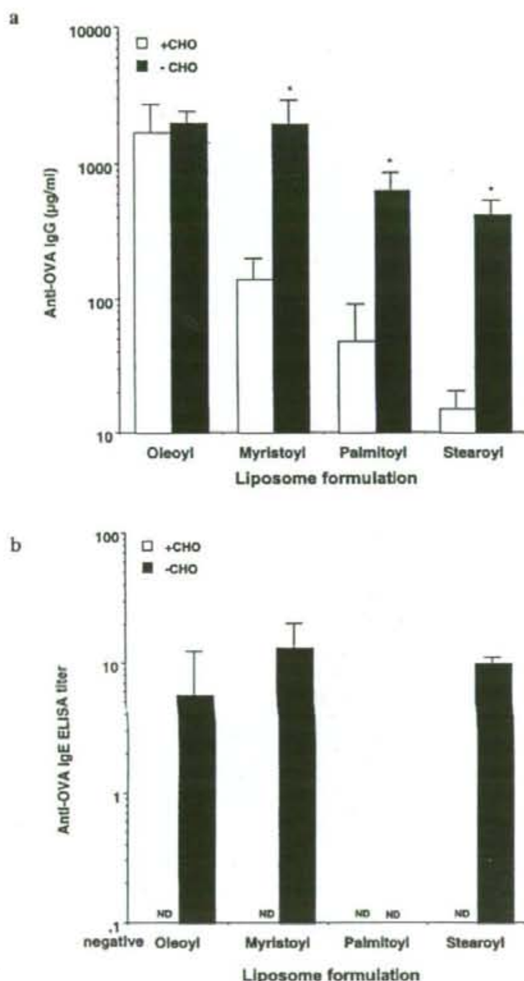

Fig. (6). Anti-OVA antibody production in mice immunized with OVA-liposome conjugates made using liposomes with four different lipid formulations with (□) or without (■) cholesterol. Six weeks after primary immunization, the mice were bled from the tail vein, and serum anti-OVA antibodies were measured. **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,

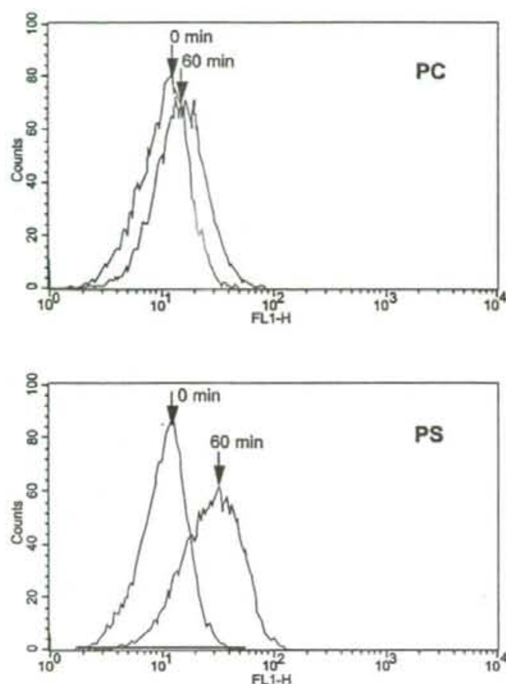

Fig. (7). Phagocytosis of OVA-liposome conjugates by macrophages. Fluorescence-labeled OVA was coupled to either PC-liposomes (**PC**) or PS-liposomes (**PS**) and added to a culture of macrophages. Macrophages recovered from the culture were analyzed using flow cytometry.

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.

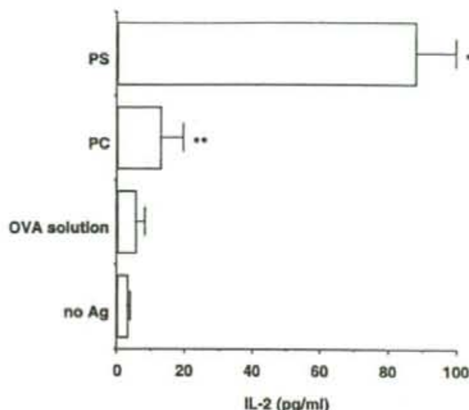

Fig. (8). Antigen presentation by macrophages pulsed with OVA-liposomes. Macrophages pre-incubated with OVA-liposomes were co-cultured with OVA-specific T-cell clones. Data represent the mean IL-2 concentration and the SE of the culture supernatant in triplicate cultures. Asterisk, significant ($p<0.01$)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

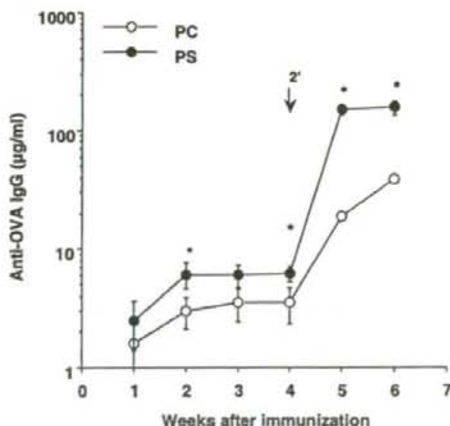

Fig. (9). Anti-OVA IgG antibody production in mice immunized with OVA-liposome conjugates. BALB/c mice were immunized with OVA-liposome conjugates made using PC- liposomes (PC) or PS-liposomes (PS) at 0 and 4 weeks. The mice were bled weekly from the tail vein, and serum anti-OVA IgG was measured. Data 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-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).

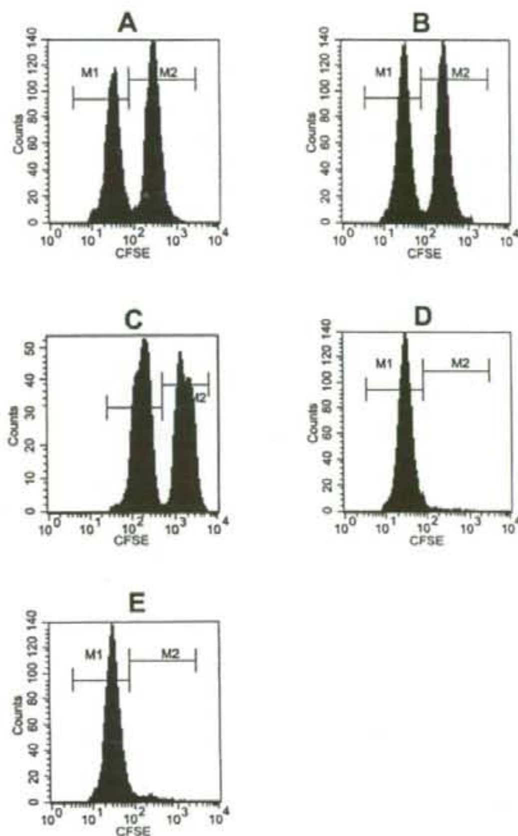

Fig. (11). *In vivo* CTL induction in mice immunized with OVA-liposome conjugates. Mice were injected with 100 μg of anti-IL-10 monoclonal antibodies and 5 μg of CpG with PBS (**A**), a mixture of $OVA_{257-264}$ and "unsaturated" liposomes (**B**), $OVA_{257-264}$-"saturated" liposome conjugates (**C**), $OVA_{257-264}$-"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.

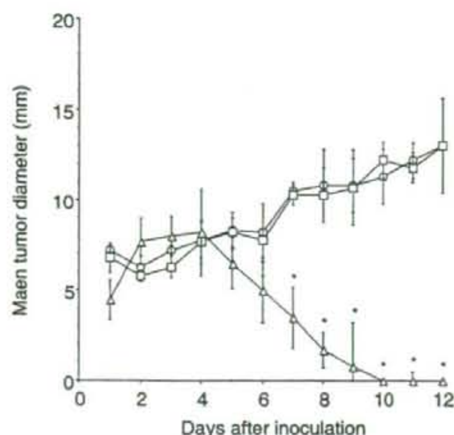

Fig. (12). Effect of peptide-liposome conjugates on the growth of the E.G7 tumor in mice. The tumor was established, and a mixture of CpG and anti-IL-10 was inoculated around the tumor mass in conjunction with liposome-coupled peptide (△), peptide solution containing the same amount of peptide as liposome-coupled 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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論文-8

研究動向　ワクチン製造

リポソーム表面結合抗原を用いたワクチン創製

内田哲也　種市麻衣子

Application of surface－linked liposomal antigens for the development of vaccines

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Abstract

The potential ability of surface-linked liposomal antigens for application to vaccine development was investigated. During the course of this investigation, 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. In addition to this "quantitative" difference between liposomes with differential lipid components, a "qualitative" difference (i.e., the differential ability to induce cross-presentation) was observed among 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 are potentially applicable for the development of vaccines with the least allergic side effects and for a novel protocol of allergen immunotherapy. In addition, by the utilization of their ability to induce cross-presentation, surface-linked liposomal antigen might potentially serve as a candidate protocol for virus vaccines which induce CTL response, and for tumor vaccine preparation to present tumor antigens to APCs and induce effective antitumor responses.

Key words: liposome, vaccine, tumor, virus, cross-presentation

はじめに

著者らはこれまで，リポソーム表面結合抗原をワクチンの創製に応用することを目標とした検討を行ってきた．従来，リポソームを抗原のキャリアとして用いるための検討には主として抗原を内包させたリポソームが用いられてきたが，著者らは抗原をリポソームの表面に化学結合したものをマウスに投与すると抗原に特異的なIgE抗体の産生が選択的に抑制されることを見いだした．現行のワクチンにはアジュバントとして水酸化アルミニウムが用いられているが，アルミニウムアジュバントはIgE抗体産生をよく誘導することが知られており，IgE抗体が関与していると考えられるワクチン接種後の副反応が例年多数報告されている．そこで，リポソ

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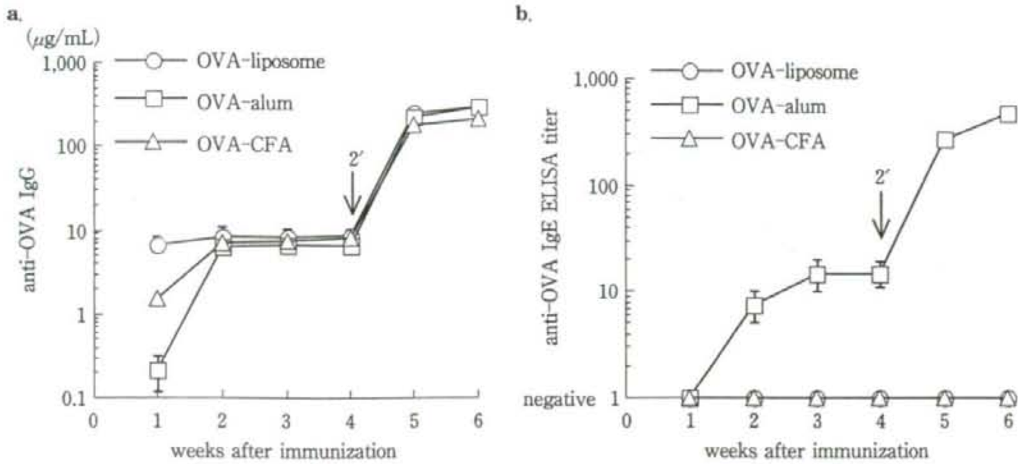

図1-a　OVA免疫マウスにおける抗OVA IgG抗体産生
BALB/cマウスに3種類の異なるアジュバントを用いてOVAを0週，4週の2回免疫し，血清中の抗OVA IgG抗体をELISAを用いて測定した.

-b　OVA免疫BALB/cマウスにおける抗OVA IgE抗体産生
図1-aの血清について抗OVA IgE抗体をELISAを用いて測定した.

ーム表面結合抗原をアレルギー反応を惹起しにくいワクチンの創製に応用することが期待された．更に，ワクチンの創製に使用するリポソームの最適な脂質組成を検討する過程で，ある種の脂質を用いてリポソームを作製し，その表面に抗原を化学結合させると，抗原がMHCクラスIを介して抗原提供細胞からCD8陽性T細胞に呈示され，抗原特異的な細胞傷害性T細胞が誘導されることが近年明らかになった．このことから，リポソーム表面結合抗原を細胞性免疫の誘導を目標とするウイルスワクチン，および腫瘍治療薬の創製に応用することが期待された．

本稿では，リポソーム表面結合抗原を用いてこれまでに行われた検討の経緯および今後の臨床応用可能性について解説を行う．

1．リポソーム表面結合抗原によって誘導されるIgE選択的無反応

BALB/cマウスにリポソーム，alum，完全フロイントアジュバント(CFA)の3種の異なるアジュバントを用いて卵白アルブミン(OVA)を免疫すると，抗OVA IgG抗体産生は同程度に誘導された(**図1-a**)が，抗OVA IgE抗体産生は

表1　OVA免疫マウスにおける抗OVA IgG1/IgG2a産生

immunization	anti-OVA antibodies (mg/mL)	
	IgG1	IgG2a
OVA-liposome	260.2±72.7	239.4±80.8
OVA-alum	252.8±70.4	32.6±5.4*
OVA-CFA	29.2±11.5	132.7±21.7*

図1の実験における初回免疫6週後の血清について，抗OVA Igサブクラスを測定した.
*有意差($p<0.01$)あり

OVA-alum免疫群でのみ顕著に観察され，OVA-リポソーム，OVA-CFA免疫群では観察されなかった(**図1-b**)．初回免疫6週後における抗OVA IgサブクラスはOVA-alum免疫群でIgG1がIgG2aと比較して有意に高く，これとは反対にOVA-CFA免疫群でIgG2aがIgG1と比較して有意に高かったが，OVA-リポソーム免疫群では両者の間に有意な差がみられなかった(**表1**)．

これらの結果から，OVA-alumおよびOVA-CFAはそれぞれTh2およびTh1タイプの免疫応答を誘導するがOVA-リポソームはどちらとも

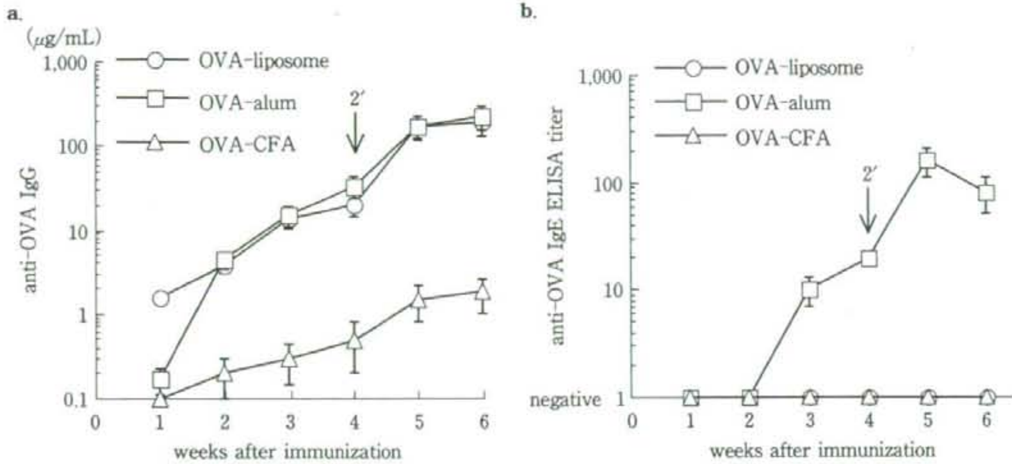

図2-a OVA免疫IL-12ノックアウトマウスにおける抗OVA IgG抗体産生
IL-12ノックアウトマウスに3種類の異なるアジュバントを用いてOVAを0週，4週の2回免疫し，血清中の抗OVA IgG抗体をELISAを用いて測定した．

-b OVA免疫IL-12ノックアウトマウスにおける抗OVA IgE抗体産生
図2-aの血清について抗OVA IgE抗体をELISAを用いて測定した．

いえないことを示唆している．これらのマウスのCD4陽性T細胞を試験管内で抗原刺激してサイトカイン産生を検討した結果，OVA-リポソーム免疫群，OVA-alum免疫群においては検討したすべてのサイトカインの顕著な産生が認められた．抗OVA IgE産生を誘導しなかったOVA-リポソーム免疫群においてもTh1サイトカイン(IL-2およびIFN-γ)だけでなくTh2サイトカイン(IL-4およびIL-5)の産生が観察され，IgE産生の抑制効果とT細胞サイトカイン産生のプロファイルが相関しない結果となった．これに対して，OVA-CFA免疫群ではIL-4およびIL-5の産生がみられず，典型的なタイプI免疫応答が誘導されていることが示唆された．

OVA-リポソームによって誘導されるIgE産生の選択的抑制がIL-12に依存的であるか否かをIL-12ノックアウトマウスを用いて検討したところ，**図2-a**に示すように，OVA-リポソームおよびOVA-alum免疫群では同等の抗OVA IgG産生が誘導されたがOVA-CFA免疫群では他の2群と比較して顕著に低値であったことから，OVA-CFAによって誘導される抗OVA IgG産生はIL-12に依存的であることが示唆された．抗OVA IgE抗体産生はOVA-alum免疫群で顕著に観察されたが，OVA-リポソーム免疫群では観察されなかった(**図2-b**)．このことから，OVA-リポソームによるIgE産生の選択的抑制効果はIL-12に非依存的な機序によるものであることが示唆された．

OVA-リポソームとOVA-CFAはともにIgE産生を選択的に抑制するという点で共通しているが，OVA-リポソーム免疫マウスのCD4陽性T細胞は顕著にTh2サイトカインを産生するのに対してOVA-CFA免疫マウスのCD4陽性T細胞はTh2サイトカインを産生しなかったことから，OVA-CFAは典型的なTh1タイプ免疫応答の誘導を介してIgE産生の選択的抑制を誘導するがOVA-リポソームは必ずしもそうではないことが示唆された．このことは，Igサブクラスの検討と，IL-12欠損マウスを用いた検討によって裏付けられた．IL-12はTh1の誘導において中心的役割を担うことが知られており，OVA-CFAによって誘導される抗OVA IgG産生はIL-12欠損マウスにおいて正常マウスと比較して低レベルであったがOVA-リポソームは正常マウスと同レベルの抗OVA IgG産生を誘

導した.

2. IgE選択的無反応は抗原とリポソームとの結合方法によらず誘導される

当初，抗原とリポソームとの結合はグルタールアルデヒド(GA)を用いて行ったが[1]，この方法ではGAによって重合した抗原がリポソームに結合する．抗原-リポソーム結合物によって誘導されるIgE抗体産生の選択的抑制効果が，抗原とリポソームとの結合によるものであるか，またはGAによる抗原の重合によるものであるかを確認する目的で，GAのほかにN-(6-maleimidocaproyloxy) succinimide(EMCS), disuccinimidyl suberate(DSS), N-succimidyl 3-(2-pyridyldithio) propionate(SPDP)の3種類の架橋試薬を使用してOVA結合リポソームを作製し，比較検討を行った[2]．その結果，いずれの結合方法でOVA結合リポソームを作製した場合においても抗OVA IgE抗体産生は誘導されず，抗OVA IgG抗体産生は同程度に誘導された．一方，OVAとリポソームとの混合溶液，あるいはラテックス粒子にOVAを物理吸着させたものは顕著な抗OVA IgE抗体産生を誘導したことから，IgE抗体産生の選択的抑制を誘導するためには抗原とリポソームとの化学結合が必須であるが，抗原とリポソームとの架橋方法にはよらないことが示唆された.

3. IgG抗体産生の誘導におけるアジュバント効果はリポソームの脂質組成によって異なる

不飽和脂肪酸(オレイン酸)，およびアシル鎖長の異なる飽和脂肪酸(ミリスチン酸，パルミチン酸，ステアリン酸)を用いて脂質組成の異なる4種類のリポソームを作製し，それぞれについて表面にOVAを結合したものをマウスに投与して抗体産生誘導能の比較を行った[3]．その結果，**図3**に示すように，抗原特異的IgG抗体産生は不飽和脂肪酸を組成にもつリポソームを使用した群において最も高く，飽和脂肪酸の中ではアシル鎖長(ミリスチン酸=14，パルミチン酸=16，ステアリン酸=18)の短いほどIgG抗体産生の誘導能が高かった．IgG抗体産生の誘導能はリポソームの膜流動性と相関したことから，リポソームの膜流動性とアジュバント効果との間に密接な関連があると考えられた．また，いずれの場合にもIgE抗体産生は誘導されなかったことから，リポソームのアジュバント効果とリポソーム表面結合抗原によるIgE抗体産生の選択的抑制効果とは独立していることが示唆された.

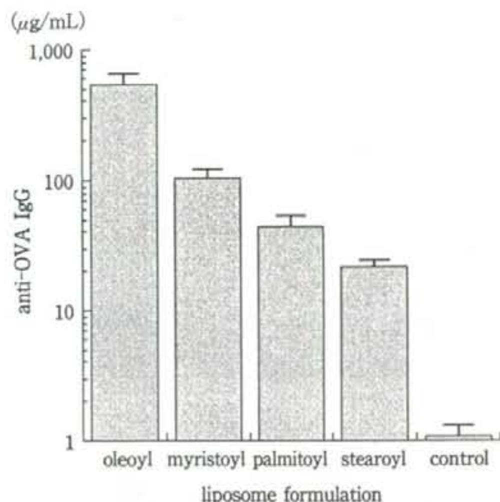

図3　脂質組成の異なるリポソームと結合したOVAによって誘導される抗OVA IgG抗体産生

オレイン酸，ミリスチン酸，パルミチン酸，ステアリン酸からなるリポソームにOVAを結合させ，BALB/cマウスに0週，4週の2回免疫した．初回免疫後6週における血清中抗OVA IgG抗体価を示す.

更に，蛍光標識したOVAあるいは消化を受けると蛍光を発するOVAをリポソームと結合させたものをマクロファージ培養中に添加して共焦点蛍光顕微鏡を用いた解析を行ったところ，オレイン酸からなるリポソームに結合したOVAはステアリン酸からなるリポソームに結合したOVAと比べて同一時間内により多くマクロファージに貪食され(**図4-a**)，消化を受ける(**図4-b**)ことが示された[4]．このことから，いわゆるアジュバント効果は抗原提供細胞による認識され易さと相関することが示唆された.

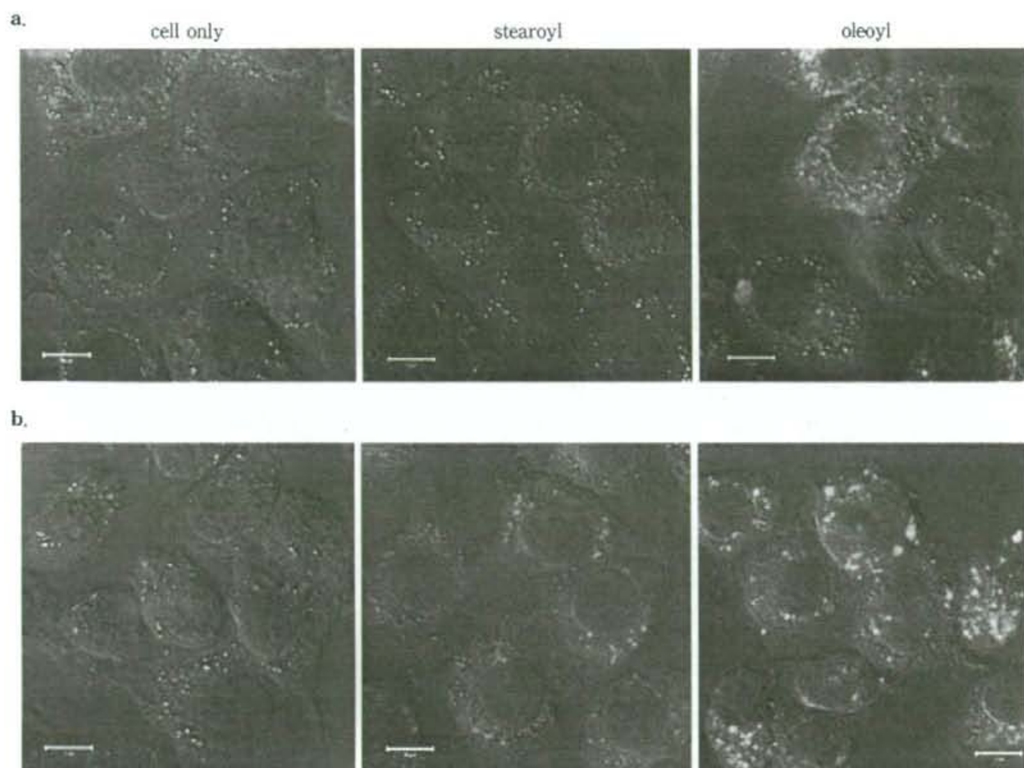

図4-a 2種類の異なる脂質組成のリポソームに結合したOVAのマクロファージによる貪食
蛍光標識したOVAをステアリン酸(stearoyl), オレイン酸(oleoyl)からなるリポソームと結合させ, マクロファージ培養中に添加した. 培養開始後60分における共焦点蛍光顕微鏡像を示す.

-b 2種類の異なる脂質組成のリポソームに結合したOVAのマクロファージによる消化
消化を受けると蛍光を発するOVA(DQ-OVA)をステアリン酸(stearoyl), オレイン酸(oleoyl)からなるリポソームと結合させ, マクロファージ培養中に添加した. 培養開始後60分における共焦点蛍光顕微鏡像を示す.

4. リポソーム表面結合抗原によって誘導されるIgE選択的無反応はT細胞に非依存的である

OVA-リポソームおよびOVA-alum免疫マウス由来のCD4陽性T細胞について, T細胞欠損マウス(ヌードマウス)において抗OVA抗体産生を誘導する能力を比較検討した[5]. **表2**に示すように, OVA-リポソーム免疫マウス由来CD4陽性T細胞はOVA-alumを免疫したヌードマウスにおいて抗OVA IgE産生を誘導した. これに対して, OVA-alum免疫マウス由来CD4陽性T細胞はヌードマウスをOVA-リポソームで免疫したとき抗OVA IgE産生を誘導しなかった. このように, IgE産生の選択的抑制はヌードマウスをOVA-リポソームで免疫したときに誘導され, T細胞ドナーの免疫方法との関係はみられなかった. また, IL-10およびCD8陽性T細胞がIgE産生の調節に関与していることが知られているが, IL-10の活性を中和する抗体, およびCD8陽性T細胞を除去する抗体の*in vivo*投与はOVA-リポソームによるIgE産生の選択的抑制に影響しなかった.

T細胞欠損マウスへのCD4陽性T細胞移入実

表2　T細胞欠損マウスにおける抗OVA IgG/IgE抗体産生

immunization of T-cell donor	immunization of recipient mice	anti-OVA antibodies	
		IgG(μg/mL)	IgE ELISA titer
no immunization	OVA-liposome	12.3±8.7	N.D.
	OVA-alum	17.7±5.2	N.D.
OVA-liposome	OVA-liposome	144.7±24.3	N.D.
	OVA-alum	124.3±12.8	105.6±10.7
OVA-alum	OVA-liposome	178.0±28.3	N.D.
	OVA-alum	246.8±29.4	139.3±12.3

OVA-liposome, OVA-alumで免疫したBALB/cマウス由来のCD4陽性T細胞をT細胞欠損マウスに静脈投与した後, OVA-liposomeあるいはOVA-alumで免疫して抗OVA IgGおよびIgE抗体産生を観察した.

験によってOVA-リポソーム免疫マウス由来CD4陽性T細胞はヌードマウスにおける抗OVA IgE産生を誘導することが示唆され, OVA-alum免疫マウス由来CD4陽性T細胞はヌードマウスをOVA-リポソームで免疫すると抗OVA IgE産生を誘導しないことが示されたことから, OVA-リポソームによるIgE産生の抑制機構にCD4陽性T細胞は関与していないことが示唆された. CD4陽性T細胞の代わりに脾細胞からB細胞のみを除去したものを用いたときにも同様の結果が得られたことから, OVA-リポソームによって誘導されるIgE産生の選択的抑制においてはB細胞が重要な役割を担っていることが示唆された.

これらの結果は, 同一の抗原に対して異なるアジュバントを使用することにより異なるパターンの免疫応答が誘導されることを示している. OVAは元来アレルゲン性を有しているが, リポソームあるいはCFAを使用することによりIgE産生が選択的に抑制される. OVA-CFAによるIgE産生の抑制にはTh1が関与しているがOVA-リポソームによるIgE産生の抑制にはTh1は関与していない. このことはTh1およびTh2のバランスによらないIgE産生調節機構の存在を示唆している[6].

5. 不飽和脂肪酸からなるリポソームを用いて作製されたリポソーム表面結合抗原は細胞傷害性T細胞(CTL)を誘導し, 腫瘍拒絶を誘導する

上述のようにリポソームの脂質組成を変えることによりアジュバント効果が変化することが示されたが, このような量的な変化のほかに, リポソームの脂質組成を変えることによってリポソーム表面結合抗原が液性免疫(抗体産生), あるいは細胞性免疫(CTL活性化)を誘導する, という質的変化がもたらされることが近年の検討の結果, 明らかになった[7]. マクロファージのクラスII compartmentを赤色に蛍光標識しておき, リポソームに結合したOVAを緑色に蛍光標識したマクロファージ培養中に添加すると, 飽和脂肪酸からなるリポソームに結合したOVAは貪食を受けた後すべてマクロファージのクラスII compartmentに集結するが, 不飽和脂肪酸からなるリポソームに結合したOVAの一部は細胞質にとどまることがわかった. 更に, 消化を受けて蛍光を発するOVAをリポソームに結合させて同様の検討を行ったところ, 不飽和脂肪酸からなるリポソームに結合したOVAの一部は細胞質中で消化を受けることがわかった. この結果は試験管内でのCD4およびCD8陽性T細胞への抗原提供を検討した結果ともよく一致した. すなわち, 飽和脂肪酸からなるリポソームに結合したOVAをOVA免疫マウス由来のT細胞および抗原提供細胞の培養中に添加