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Invited Review

Application of Surface-Linked Liposomal Antigens to the Development of Vaccines That Induce Both Humoral and Cellular Immunity

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CONTENTS:

SUMMARY: The first characteristic identified in surface-linked liposomal antigens was the ability to induce antigen-specific, IgE-selective unresponsiveness. These results remained consistent even when different coupling procedures were employed for antigens with liposomes or for liposomes with different lipid components. The potential usefulness of surface-linked liposomal antigens for application to vaccine development was further investigated. During this investigation, a significant difference was observed in the recognition of liposomal antigens by antigen-presenting cells between liposomes with different lipid components, and this difference correlated closely 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 observed between liposomes with different lipid components. Therefore, by utilizing the ability to induce cross-presentation, surface-linked liposomal antigens might be used to develop virus vaccines that would induce cytotoxic T lymphocyte (CTL) responses. We have successfully developed a liposome vaccine that is capable of inducing CTL responses against internal antigens of influenza viruses and thus removing virus-infected cells in the host. This CTL-based liposomal vaccine might be applicable to the development of vaccines against influenza and other viruses that frequently undergo changes in their surface antigenic molecules.

1. Introduction

Adjuvants are indispensable to vaccines, especially when weak immunogenic antigens are used. However, the currently used aluminum (alum) adjuvants are known to stimulate only humoral responses (1) and are known to induce IgE antibody production, which elicits allergic responses in some individuals following vaccination (2). Therefore, there is a need for improved adjuvants suitable for clinical use. Among the candidates for novel vaccine adjuvants, 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 liposomal vaccines proposed thus far have been prepared by entrapping antigen within the aqueous lumen of liposomes (7). However, it is known that encapsulated and surface-linked liposomal antigens induce differential humoral (8) and cellular (9) immune responses.

In general, extracellular antigens are presented via MHC class II molecules to $CD4^+$ T cells, whereas intracellular antigens are presented via MHC class I molecules to $CD8^+$ T cells. To induce antigen-specific cytotoxic T lymphocytes (CTLs), vaccine antigens must be loaded into the MHC class I processing pathway in antigen-presenting cells (APCs) via cross-presentation. In cross-presentation, exogenous proteins cross over to the endogenous pathway to gain access to MHC class I molecules (10). However, the currently approved alum adjuvant, which was first described by Glenny and Pope (11) in 1926, is known to be effective only for the induction of humoral immunity but not for the induction of cellular immunity (12). Consequently, the development of a novel vaccine adjuvant is essential to the production of $CD8^+$ T cell vaccines.

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In this review, data supporting an existing correlation 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 in the development of liposomal vaccines that would induce both humoral and cellular immunity has been discussed.

2. Surface-linked liposomal antigens induce IgE-selective unresponsiveness in an antigen-specific manner

Induction of antigen-specific, IgE-selective unresponsiveness was first observed in mice immunized with antigens coupled to the surfaces of red blood cells (RBCs) (13). When ovalbumin (OVA) was coupled with murine RBCs (MRBCs) using glutaraldehyde (GA) and inoculated into mice, the OVA-MRBC conjugate induced anti-OVA IgG antibody production at almost the same level as OVA in alum. However, no OVA-specific IgE antibody production was observed in the same mice. IgE-specific unresponsiveness was also observed in mice immunized with OVA coupled with sheep RBCs, suggesting that the induction of antigen-specific, IgE-selective unresponsiveness was not restricted to a property of MRBCs. These results demonstrate the potential ability of antigen-RBC conjugates in the development of vaccines that could induce sufficient IgG antibody production without inducing IgE synthesis. However, because the use of biological materials such as RBCs was avoided in vaccine development because of the possibility of infection, we decided to employ liposomes instead of RBCs.

The antibody responses in mice were investigated after immunization with surface-coupled OVA of liposomes (14). OVA was coupled to the liposomal surfaces via amino groups using GA. This OVA-liposome conjugate induced significant anti-OVA IgG antibody production in mice, whereas no OVA-specific IgE antibody production was observed. Therefore, the results demonstrate the potential of antigen-liposome conjugates with respect to the development of vaccines with least risk of allergic reaction as well as immunotherapy applications.

Coupling procedure of antigens with liposomes was further investigated (15). OVA-liposome conjugates were generated using four different coupling protocols: GA (Fig. 1A), N-(6-maleimidocaproyloxy) succinimide (EMCS; Fig. 1B), disuccinimidyl suberate (DSS; Fig. 1C), and N-succimidyl-3(2-pyridyldithio)propionate (SPDP; Fig. 1D), and the induction of antigen-specific IgG and IgE antibody production mediated by each was investigated. OVA-liposome conjugates coupled using GA or DSS induced substantial anti-OVA IgG antibody production but did not induce anti-OVA IgE antibody production. On the other hand, OVA-liposome conjugates coupled using EMCS or SPDP failed to completely induce anti-OVA IgE unresponsiveness.

To optimize the lipid component of liposomes, OVA-liposome conjugates were generated with liposomes comprising four different lipid components, including an unsaturated carrier lipid and three different saturated carrier lipids, and the abilities of these OVA-liposome conjugates to induce anti-OVA antibody production were investigated in mice. All OVA-liposome conjugates induced IgE-selective unresponsiveness. The membrane fluidity of liposomes, which was measured by detecting changes in the fluorescence polarization of a 1,6-diphenyl-1,3,5-hexatriene probe located in the lipid bilayers of liposomes, was significantly higher in liposomes consisting of unsaturated carrier lipids than in those consisting of saturated carrier lipids. The chain lengths of the three types of saturated fatty acids were 14, 16, and 18. In general, the shorter the acyl chain length, the higher the membrane mobility. As a result, the membrane mobility of liposomes correlated well with liposomal adjuvanticity in immunization with antigen-liposome conjugates (Fig. 2). The results suggest that the membrane fluidity of liposomes might affect the adjuvant effect of liposomes but not the induction of IgE-selective unresponsiveness in immunizations with surface-linked liposomal antigens (16).

To further examine whether the differential adjuvant effects displayed among liposomes with different lipid components were due to the differential recognition of liposomal antigens by APCs, antigen-liposome conjugates prepared using liposomes with differential lipid components were added to macrophage cultures and recognition and digestion of liposomal antigens were compared (17). The antigen-liposome conjugates that induced higher levels of antibody production in vivo were recognized more often by macrophages (Fig. 3A), and the liposome-coupled antigen was digested to a greater degree by macrophages than the antigen-liposome conjugates that induced lower levels of antibody production (Fig. 3B). These results correlated closely with those regarding antigen presentation by macrophages (Fig. 4); specifically, the macrophages cocultured with antigens coupled to liposomes with stronger adjuvant effect could more strongly activate antigen-specific T cells. Furthermore, 25-fold lesser antigen was required for antigen-specific T cell activation via antigen-liposome conjugates than via soluble antigens (Fig. 4B). These results demonstrated that the primary in vivo adjuvant activities of liposomes correlated closely with the recognition of antigen-liposome conjugates and the antigen presentation of liposome-coupled antigens by macrophages, thus suggesting that the adjuvant effects of these liposomes were exerted at the beginning of the immune response (i.e., antigen recognition by APCs).

3. Mechanism of IgE-selective unresponsiveness induced by antigen-liposome conjugates

Control of IgE antibody production is important for the prevention of IgE-related diseases. However, in contrast to the existing information regarding the induction of IgE production, little is known about the regulation of IgE production, with the exception of the well-documented mechanism involving T cell subsets and their cytokine products. During an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-coupled liposomal antigens, we discovered an alternative T cell-independent approach toward interfering with IgE production. In mice, immunization with OVA-liposome conjugates induced IgE-selective unresponsiveness without apparent Th1 polarization. Neither IL-12 (Fig. 5), IL-10, nor $CD8^+$ T

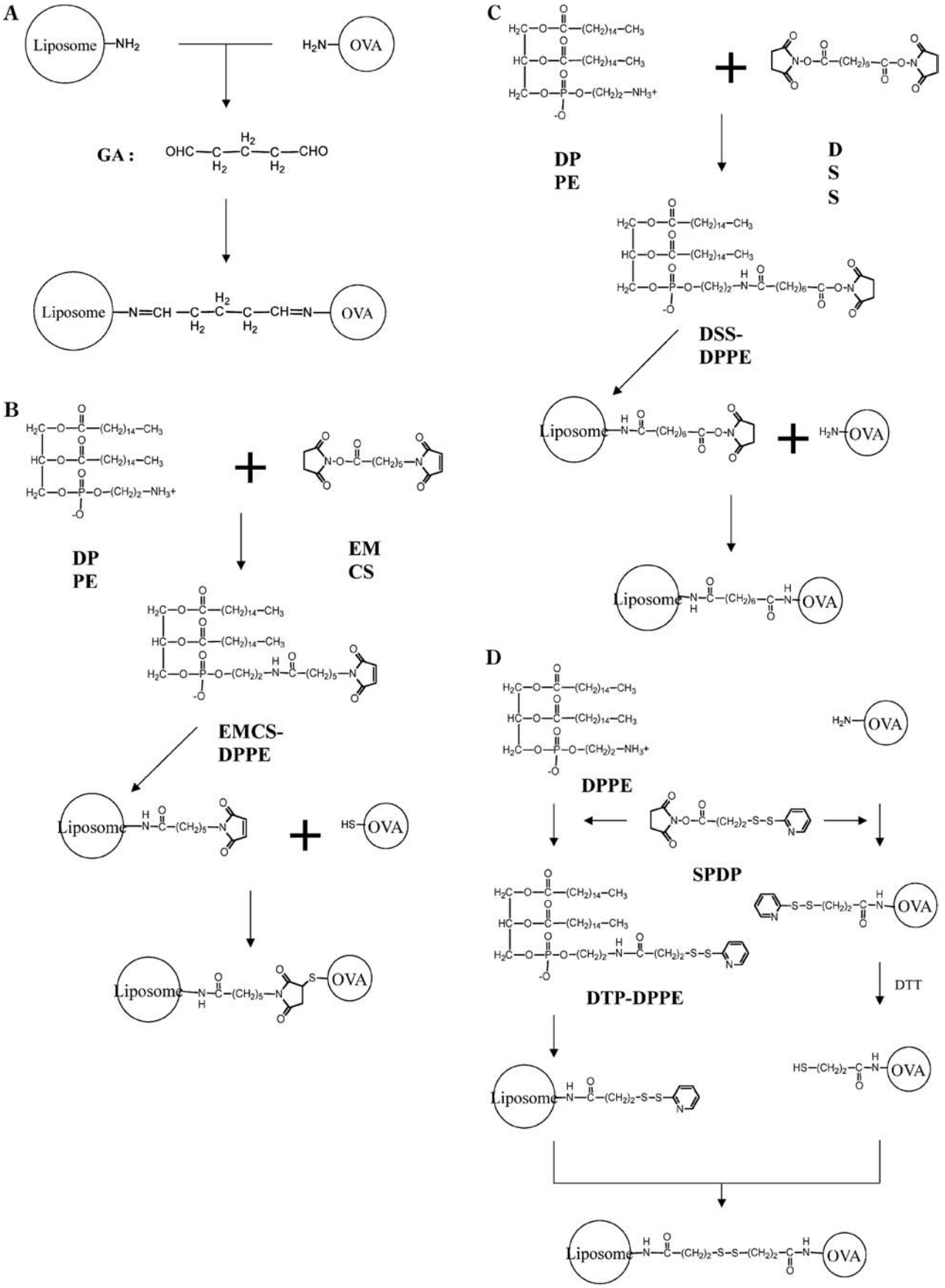

Fig. 1. Coupling procedure of antigen with liposomes. (A) Coupling of ovalbumin (OVA) to liposomes via glutaraldehyde (GA). (B) Coupling of OVA to liposomes via N-(6-maleimidocaproyloxy) succinimide (EMCS). (C) Coupling of OVA to liposomes via disuccimidyl suberate (DSS). (D) Coupling of OVA to liposomes via N-succimidyl 3-(2-pyridyldithio) propionate (SPDP).

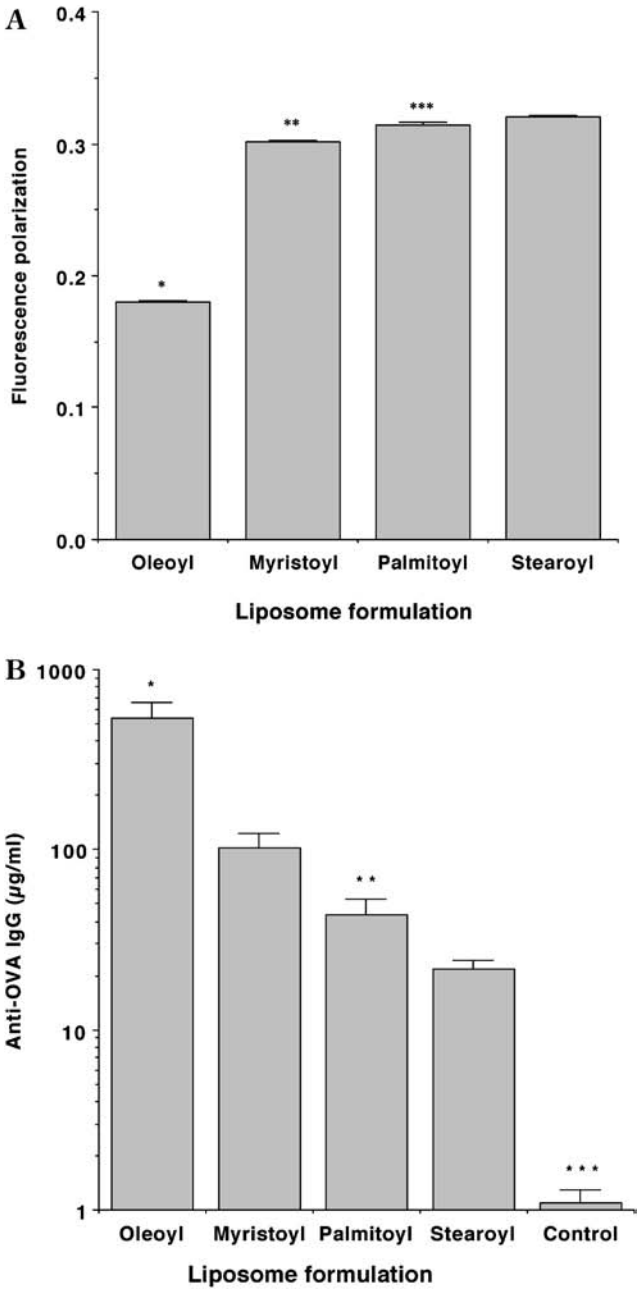

Fig. 2. Fluorescence polarization degree in liposomes having four different lipid formulations and antibody production in mice immunized with OVA-liposome conjugates. (A) Fluorescence polarization degree in liposomes. The degree of fluorescence polarization at 37°C is shown. Data represent mean and SE of triplicate measurements. Asterisk, $P < 0.000001$ compared with myristoyl group; two asterisks, $P < 0.001$ compared with palmitoyl group; three asterisks, $P < 0.01$ compared with stearoyl group. (B) Anti-OVA antibody production in mice. Six weeks after immunization, mice were bled from the tail vein and serum anti-OVA antibodies were measured. Data represents mean anti-OVA IgG and SE of 5 mice per group. Asterisk, $P < 0.005$ compared with myristoyl group; two asterisks, $P < 0.05$ compared with myristoyl group and stearoyl group; three asterisks, $P < 0.001$ compared with OVA-liposome-immune groups. Control group received injections with plain OVA solution at the same OVA dose as that given the OVA-liposome-immune group. Data represent mean and SE of 5 mice per group. Reprinted with permission from (16). Copyright (2001) American Chemical Society.

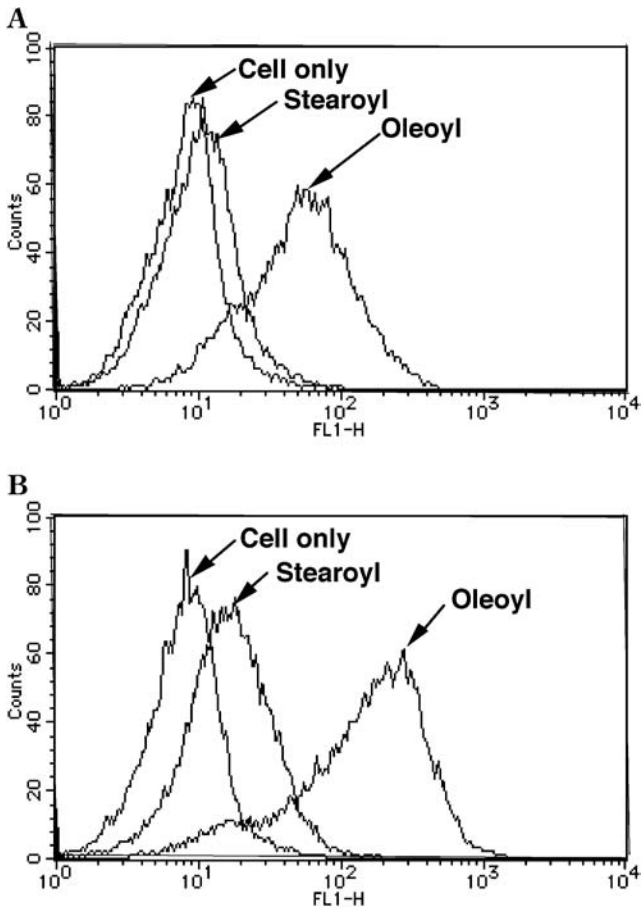

Fig. 3. Phagocytosis and digestion of OVA-liposome conjugates by macrophages. (A) Phagocytosis of OVA-liposome conjugates by macrophages. Fluorescence-labeled OVA was coupled to either stearoyl or oleoyl liposomes and added to the culture of macrophages. Macrophages recovered from the culture were analyzed using flow cytometry. (B) Digestion of liposome-coupled OVA by macrophages. Stearoyl or oleoyl liposomes coupled with DQ-OVA were added to the macrophage culture. Sixty minutes after the onset of the culture, macrophages were recovered and analyzed using flow cytometry. Reprinted with permission from (17). Copyright (2004) American Chemical Society.

cells (Table 1) participated in this regulatory process. Furthermore, $CD4^+$ T cells of mice immunized with OVA-liposome were capable of inducing antigen-specific IgE synthesis in athymic nude mice immunized with alum-adsorbed OVA. In contrast, immunization of the recipient mice with OVA-liposome conjugates did not induce anti-OVA IgE production, even when $CD4^+$ T cells of mice immunized with alum-adsorbed OVA were transferred. During the secondary immune response, OVA-liposome conjugates enhanced anti-OVA IgG antibody production but did not enhance ongoing IgE production, suggesting that the IgE-selective unresponsiveness induced by the liposomal antigen involved direct effects on IgE, but not IgG class switching, in vivo (Table 2). These results suggest the existence of an alternative T cell-independent mechanism for regulating IgE synthesis (18).

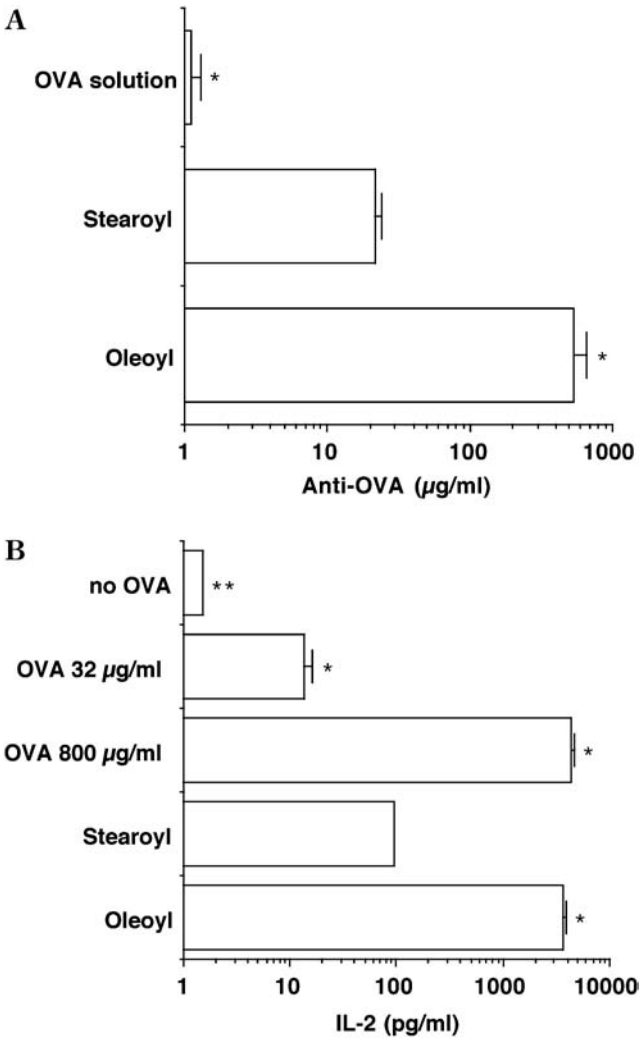

Fig. 4. Antibody production in mice immunized with OVA-liposome conjugates and antigen presentation by macrophages pulsed with OVA-liposomes. (A) Anti-OVA IgG antibody production. BALB/c mice were immunized with OVA-liposome made using stearoyl or oleoyl liposomes or with plain OVA solution at 0 and 4 weeks. Six weeks after primary immunization, mice were bled from the tail vein, and serum anti-OVA IgG was measured. Data represent mean and SE of five mice per group. Asterisk, significant ($P < 0.01$) difference as compared with stearoyl group. (B) Antigen presentation by macrophages pulsed with OVA-liposomes. Macrophages pre-incubated with OVA-liposomes were cocultured with OVA-specific T cell clone. Data represent mean IL-2 concentration and the SE of the culture supernatant in triplicate cultures. Asterisk, significant ($P < 0.01$) difference as compared with stearoyl group. Two asterisks, significant ($P < 0.01$) difference as compared with the other groups. Reprinted with permission from (17). Copyright (2004) American Chemical Society.

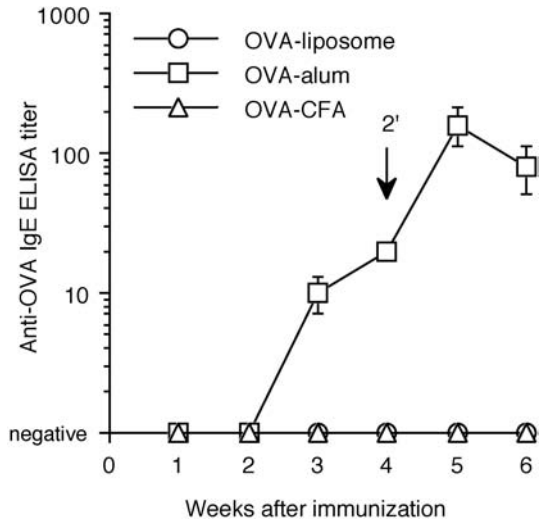

Fig. 5. Anti-OVA antibody production in IL-12 deficient mice immunized with OVA-liposome, OVA-alum, or OVA-CFA. IL-12 deficient mice were immunized with OVA-liposome (○), OVA-alum (□), or OVA-CFA (△) on days 0 and 28. Data represent mean anti-OVA IgE ELISA titers and SE of five mice per group. Reprinted with permission from (18). Originally published in the Journal of Immunology. Copyright© [2002] The American Associate of Immunologists, Inc.

Table 1. Effect of in vivo administration with mAbs on anti-OVA antibody production in mice immunized with OVA-liposome

	mAb	In vivo administration with mAb	Anti-OVA antibody	
			IgG (μg/ml)	IgE
Exp. 1	αIL-10	no	179.8 ± 22.0	N.D.
		yes	176.3 ± 18.7	N.D.
Exp. 2	αCD8	no	192.7 ± 28.3	N.D.
		yes	178.6 ± 23.8	N.D.

BALB/c mice were immunized with OVA-liposome at 0 and 4 weeks. During the immunization, mice received inoculation with monoclonal antibody (mAb). Six weeks after primary immunization, mice were bled and serum anti-OVA antibodies were monitored. Data represent the mean antibody titers and SE of five mice per group. N.D., not detected. Reprinted with permission from (18). Originally published in the Journal of Immunology. Copyright© [2002] The American Association of Immunologists, Inc.

4. Development of tetanus vaccines

Allergic symptoms such as local swelling and an immediate-type reaction are frequently observed phenomenon associated with human vaccination (19), and IgE formation upon vaccination is thought to contribute to these reactions. A diphtheria-pertussis-tetanus vaccine was shown to induce significant IgE antibody production in mice in addition to the induction of IgG antibody production (20). Therefore, a possible application for surface-linked liposomal antigens in tetanus vaccine development was investigated. Tetanus toxoid (Ttd) was coupled to liposomes using GA. Immunization of mice with these Ttd-liposomes induced substantial anti-Ttd IgG antibody production and an extremely low level of anti-Ttd IgE antibody production, whereas both alum-adsorbed Ttd (Ttd-alum) and a plain Ttd solution induced the production of both IgG and IgE antibodies against Ttd. In addition, Ttd-liposome immunized mice were successfully protected against a subsequent challenge with a lethal dose of tetanus toxin (Ttx). Moreover, secondary immunization with Ttd-liposome in mice, in which anti-Ttd IgG and IgE antibody production was induced by Ttd-alum, led to enhanced anti-Ttd IgG and limited anti-Ttd IgE antibody production. When this Ttd-liposome preparation was lyophilized, the efficacy of the Ttd-liposomes was maintained for up

Table 2. Anti-OVA antibody production in BALB/c nu/nu mice transferred splenic $CD4^+$ cells of BALB/c mice

Immunization of T cell donor	Immunization of recipient mice	Anti-OVA antibody	
		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

BALB/c mice were immunized with OVA-liposome, OVA-alum, or not at all, and splenic CD^4 cells of these mice were transfered intravenously into naive BALB/c nu/nu mice. Data represent the mean antibody titers and SE of five mice per group. N.D., not detected. Reprinted with permission from (18). Originally published in the Journal of Immunology. Copyright© [2002] The American Association of Immunologists, Inc.

to 6 months at 37°C, suggesting that this vaccine preparation would remain stable without refrigeration. These results demonstrate the potential ability of Ttd-liposome conjugates to produce a tetanus vaccine that could provide protection against Ttx while inducing the lowest level of anti-tetanus IgE antibody production (21).

The efficacy of these Ttd-liposome conjugates was further evaluated in SCID-hu-PBL mice. Severe combined immunodeficient (SCID) mice, in which human lymphoid cells can survive, were used to study the human anti-tetanus immune response. Human peripheral blood lymphocytes (hu-PBL) obtained from 88 healthy donors (age, 18–62 years) were transplanted into the SCID mice, and anti-Ttd antibody production and protection against lethal doses of Ttx were investigated in these SCID-hu-PBL mice. For 37.5% of the donors, hu-PBL transfer evoked significant human anti-Ttd IgG antibody production. Following in vivo immunization, the percentage of donors with PBL that exhibited positive anti-Ttd IgG production in the mice increased to 54.5%. The mean serum anti-Ttd IgG levels were also significantly elevated in response to immunization. The mean IgG titer in mice injected with PBL from donors <40 years of age was significantly higher than that in mice injected with PBL from donors ≥ 40 years. Four weeks after the cell transfer, the mice were challenged with Ttx. Induced protection against the Ttx challenge was observed primarily in mice that received PBL from donors aged <40 years. In vivo immunization in SCID mice with Ttd increased the number of Ttx-resistant cases. These results suggest that SCID-hu-PBL mice might serve as a tool for predicting the protective abilities of vaccines against pathogens in PBL donors and for evaluating vaccine efficacy (22).

5. Development of Shiga toxin-producing *Escherichia coli* (STEC) vaccines

STEC infection is a current and serious public health concern because it causes two life-threatening complications; hemorrhagic colitis and hemolytic uremic syndrome. For the prevention of STEC infection and protection against systemic complications, immunization and therapeutic strategies have been considered. Immunization strategies to reduce the incidence and effects of STEC would include the following: vaccination of livestock to reduce the STEC load; vaccination of humans to prevent gut colonization by STEC; and vaccination of humans to prevent the serious systemic complications of STEC diseases. Vaccines directed against colonization factors would effectively prevent both individual STEC infections and infection spread within the community. However, the current information regarding the factors responsible for gut colonization by STEC is limited. Because active immunization with a detoxified Shiga toxin (Stx) to induce the production of neutralizing antibodies against Stx was an attractive option, we evaluated the efficacy of Stx-liposome conjugates preventing life-threatening and systemic complications caused by STEC infection.

Purified Stxs, Stx1 and Stx2, were coupled to liposomes using GA. Both Stx1 and Stx2 were detoxified during the coupling procedure. Immunization of mice with Stx1-liposome or Stx2-liposome induced substantial anti-Stx1 or anti-Stx2 IgG antibody production, respectively. Mice immunized with the Stx2-liposome conjugates were protected against intravenous challenge with a lethal dose of Stx2, and the degree of protection correlated well with the amount of IgG induced against Stx2. Although the Stx1-liposome conjugates failed to induce protection against Stx1, the reduction in body weight observed after the toxin challenge correlated inversely with the amount of anti-Stx1 IgG induced, suggesting that Stx1 neutralizing antibody was present in Stx1-liposome conjugate-immunized mice. In addition, the Stx-liposome conjugates induced no detectable anti-Stx IgE antibody production (23).

The efficacy of the Stx-liposome conjugates was further evaluated in mice infected orally with cytotoxin-producing *E. coli* O157:H7. The mice were immunized with a mixture of Stx1-liposome and Stx2-liposome conjugates, followed by oral *E. coli* O157:H7 infection. Eight weeks after immunization, the mice were inoculated intragastrically with 10^9 CFU of *E. coli* O157:H7 strain 96-60. All Stx-liposome conjugate-immunized mice survived without any apparent symptoms, whereas the non-immunized control mice died within 5 days. In addition, as shown by other antigen-liposome con-

jugates, Stx-liposome conjugates induced undetectable levels of anti-Stx IgE antibody but substantial amounts of anti-Stx IgG antibodies (24).

As the Stx-liposome conjugate-immunized mice were successfully protected against oral STEC infection, the induction of protection against Stx2 by Stx2-liposome was further evaluated in monkeys. Stx2-liposome conjugates induced substantial anti-Stx2 IgG and Stx2 neutralizing antibody production in monkeys. The test monkeys were successfully protected against challenges with lethal doses of Stx2. Moreover, these monkeys exhibited no apparent symptoms, whereas nonimmunized control monkeys died within 4 days after presenting with hemorrhagic gastroenteritis and renal disorder. In addition, as shown in other cases involving antigen-liposome conjugates, the Stx2-liposome conjugates did not induce anti-Stx2 IgE antibody production, although it stimulated the production of a substantial amount of anti-Stx2 IgG antibodies (25).

These results suggest that Stx-liposome conjugates might serve as candidate vaccines for inducing protection against STEC infection-related death.

6. Antigens coupled to liposome induce cellular immunity

Liposomes with different lipid components were found to exhibit different adjuvant effects when antigens were chemically coupled to their surfaces (17). The presentation of liposome-coupled OVA was further investigated in vitro, and it was found that OVA coupled to liposomes comprising unsaturated fatty acid was presented to both $CD4^+$ and $CD8^+$ T cells, whereas OVA coupled to liposomes made using saturated fatty acid was presented only to $CD4^+$ T cells. Confocal laser scanning microscopy analysis demonstrated that a fraction of OVA coupled to liposomes comprising unsaturated, but not saturated fatty acid, underwent processing beyond the MHC class II compartment (Fig. 6), suggesting that OVA might be degraded in the cytosol and that the peptides generated in this manner would subsequently be presented to $CD8^+$ T cells via MHC class I (26).

The ability to induce the cross-presentation of an antigen coupled to unsaturated fatty acid-containing liposomes was further confirmed by the in vivo induction of CTL and tumor eradication in mice; specifically, E.G7 tumors were completely eradicated in $OVA_{257-264}$-liposome conjugate-immunized mice (Fig. 7). In those mice, the frequencies of $CD8^+$ T cells that reacted to $OVA_{257-264}$ peptides in the context of $H\text{-}2K^b$ were significantly increased. These results suggested that the selected liposomal lipid components might render surface-coupled liposomal antigens applicable for the development of tumor vaccines to present tumor antigens to APCs and induce antitumor responses (27).

Given the above results, the mechanism by which liposome-coupled antigens were cross-presented by APCs to $CD8^+$ T cells was further investigated. Flow cytometric analysis demonstrated that antigens coupled to the surfaces of unsaturated fatty-acid-liposomes were taken up by APCs even at 4°C; however, this was not true of saturated fatty-acid-liposomes. When the two inhibitors, dimethylamiloride (DMA) and cytochalasin B,

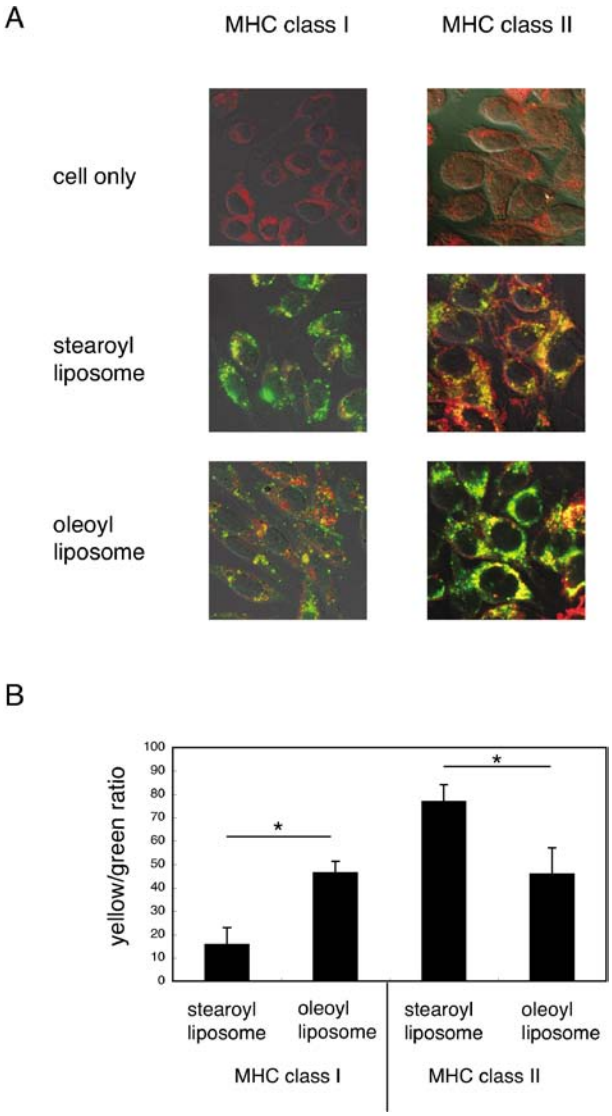

Fig. 6. (Color online) Confocal laser scanning microscopic analysis of macrophages co-cultured with DQ-OVA-liposome conjugates. (A) DQ-OVA was coupled to either stearoyl or oleoyl liposomes and added to the culture of cloned macrophages expressing DM-DsRed (class II) or labeled with red fluorescein (class I). Two hours after the onset of the culture, macrophages were recovered and analyzed using confocal laser scanning microscopy. These optically merged images are representative of most cells examined by confocal microscopy. Yellow, co-localization of green (DQ-OVA after proteolytic degradation) and red (macrophage DM or class I); cell only, macrophages without coculture with DQ-OVA-coupled liposomes. (B) Green- and yellow-color compartments in the immunofluorescent pictures were quantified by the image analysis software MetaMorph. Ratios of the yellow to green compartments are shown. Data represent the mean values ± SD of the images shown in Fig. 7A. Asterisk, significant ($P < 0.01$) difference of samples. Reprinted with permission from (26).

which inhibit pinocytosis and phagocytosis by APCs, respectively, were added to the APC cultures prior to antigen pulsing, DMA but not cytochalasin B significantly reduced the uptake of liposome-coupled antigens. Further analysis of the intracellular trafficking of liposomal antigens using confocal laser scanning microscopy revealed that in APCs, a fraction of the liposome-coupled antigens taken up was delivered to the

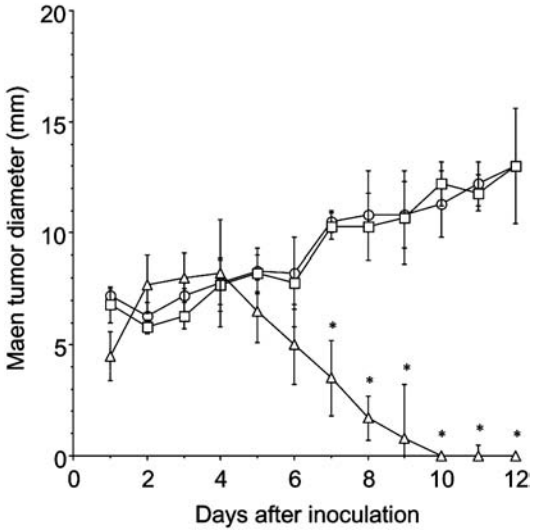

Fig. 7. Effect of peptide-liposome conjugates on the growth of E.G7 tumor in mice. A mixture of CpG and anti-IL-10 were inoculated around the tumor mass with liposome-coupled peptide (△), peptide solution containing the same amount of peptide as liposome-coupled peptide (□), or with nothing (○). *, $P < 0.001$ as compared with the mean diameter of mice without inoculation of liposome-coupled peptides. Data represent the mean and SE of four mice per group. Reprinted with permission from (27). Originally published in the Journal of Immunology. Copyright© [2006] The American Associate of Immunologists, Inc.

lysosomal compartment. In agreement with the reduced antigen uptake by APCs, antigen presentation by APCs was also significantly inhibited by DMA, and resulted in reduced interferon (IFN)-γ production by antigen-specific $CD8^+$ T cells. These results suggest that antigens coupled to the surfaces of liposomes consisting of unsaturated fatty acids might be pinocytosed by APCs, loaded into the MHC class I processing pathway, and presented to $CD8^+$ T cells. Therefore, these liposome-coupled antigens are expected to be applicable for vaccine development to induce cellular immunity (26).

$CD8^+$ T cells provide broad immunity against viruses because they are able to recognize all types of viral proteins. Therefore, the development of vaccines capable of inducing long-lived memory $CD8^+$ T cells is desired to prevent diseases, especially those for which no vaccines exist currently. However, when designing $CD8^+$ T cell vaccines, the role of $CD4^+$ T cells in the induction and maintenance of memory $CD8^+$ T cells remains uncertain. Therefore, the requirement for $CD4^+$ T cells in the induction and maintenance of memory $CD8^+$ T cells was investigated in liposome-coupled CTL epitope peptides-immunized mice. When OVA-derived CTL epitope peptides were chemically coupled to the surfaces of liposomes and inoculated into mice, both primary and secondary CTL responses were induced successfully. These results were further confirmed in mice in which the $CD4^+$ T cells had been eliminated, suggesting that $CD4^+$ T cells were not required for the generation of memory $CD8^+$ T cells in response to immunization with liposome-coupled peptides. Therefore, surface-linked liposomal antigens capable of inducing long-lived memory $CD8^+$ T cells without the contribution of $CD4^+$ T cells might be applicable to vaccine development to prevent viral infection (28).

7. Development of a CTL-based universal influenza vaccine

The current vaccination strategy against influenza is to elicit neutralizing antibody responses against hemagglutinin (HA), a surface glycoprotein of influenza viruses. Although antibody-mediated immunity against viral surfaces reduces the likelihood of infection and morbidity, an antibody against one influenza virus type or subtype can provide limited or no protection against another type. Moreover, an antibody against one antigenic variant of an influenza virus might not protect against a new antigenic variant of the same type or subtype because of the frequent development of antigenic variants through antigenic drift. Therefore, although vaccines designed to induce antibodies against HA provide reasonable protection against homologous viruses, it is feared that the currently produced vaccines may have HA sequences that differ so significantly from those of any pandemic strain that the vaccines would have little or no efficacy because of the high rate of viral diversification (29). During natural infection, it is known that the host responds by inducing humoral and cellular immune responses against the pathogen. Humoral immune responses are represented by the production of antibodies that bind to the surfaces of bacteria and viruses, whereas cellular immune responses mediate immunity against intracellular pathogens. Since Effros et al. (30) reported in 1977 that influenza A virus-specific CTLs were broadly cross-reactive against cells of the same MHC class I type that had been infected with serologically distinct H1N1 and H3N2 influenza viruses, it has been known that CTLs specific for internal proteins exhibit high cross-reactivity between strains and subtypes, reflecting an internal viral protein conservation rate of $>90\%$ (31). Lee et al. (32) reported that memory T cells established in response to seasonal human influenza A infection cross-reacted with H5N1 in healthy individuals who had not been exposed to H5N1 viruses. $CD8^+$ T cells of the participants recognized multiple synthesized influenza peptides, including peptides from the H5N1 strain. Therefore, vaccine formulas that include heterosubtypic T cell-mediated immunity might confer broad protection against both avian and human influenza A viruses.

Because the liposomal conjugates efficiently induced CTLs when the CTL epitope peptides were coupled to the liposomal surfaces, these conjugates were expected to be applicable to the development of CTL-based vaccines. The efficacy of a CTL-based influenza vaccine was first evaluated in a C57BL/6 mouse model with an $H\text{-}2^b$-restricted CTL epitope, $NP_{366\text{–}374}$, which was derived from nucleoprotein (NP) of H3N2 influenza virus (33). The liposome-coupled peptide $NP_{366\text{–}374}$ induced antigen-specific CTLs and successfully suppressed replication of H3N2 influenza virus in the lungs of the C57BL/6 mice. Then we further evaluated the possible application of liposome-coupled peptides to the development of an influenza vaccine using HLA-A*0201-restricted CTL epitope peptides in HLA-A*0201-transgenic mice.

Liposomal conjugates formed with CTL epitope pep-

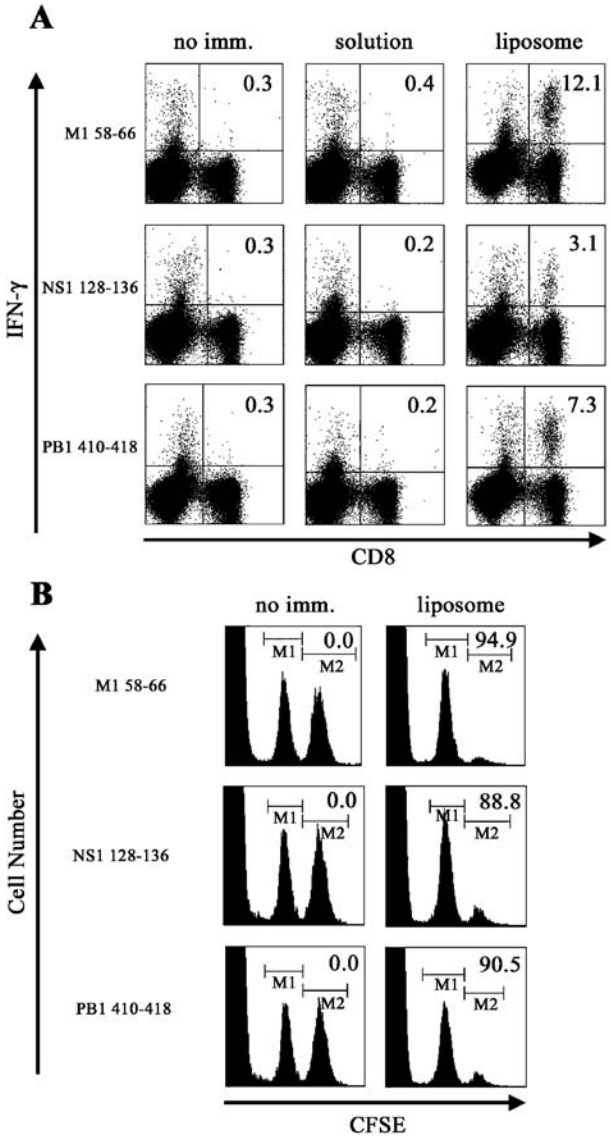

Fig. 8. Induction of antigen-specific $CD8^+$ T cell and CTL responses. Mice received immunization with the liposome-coupled peptides M1 58-66, NS1 128-136, and PB1 410-418 (liposome) or liquefied peptides in the same amounts as liposome conjugates (solution) in the presence of CpG. Control mice received no immunization (no imm.). One week after the immunization, ICS (A) and in vivo CTL assay (B) were performed. (A) ICS of antigen-specific $CD8^+$ T-cells among spleen cells of A2Tg mice. Cells were stained for their surface expression of CD8 (x axis) and their intracellular expression of IFN-γ (y axis). The numbers shown indicate the percentage of $CD8^+$ cells that were positive for intracellular IFN-γ. (B) Induction of CTLs by liposome-coupled peptides. The numbers shown indicate the percentage of total cells killed. The data shown are representative of three independent experiments. Reprinted with permission from (34).

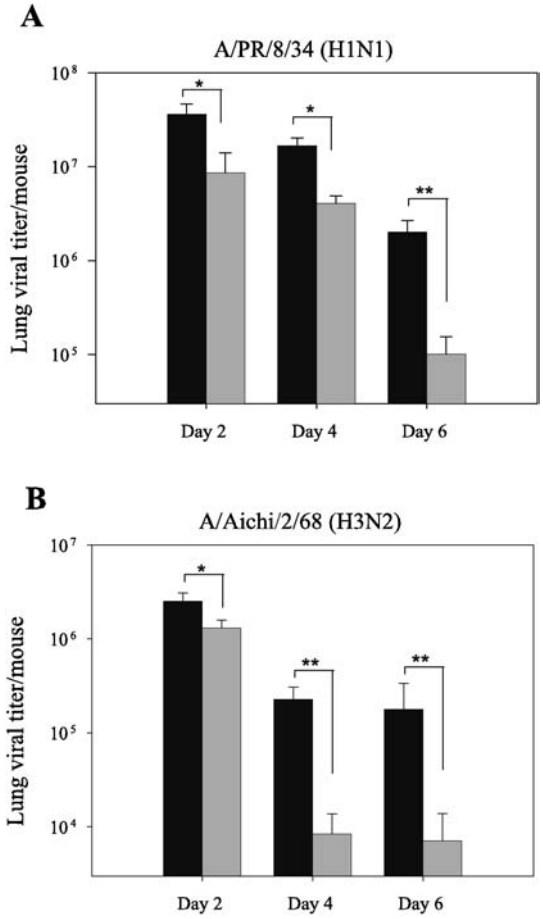

Fig. 9. Viral titers in lungs of mice following challenge with H1N1 or H3N2 influenza virus. Mice received immunization with either liposome-coupled peptide M1 58-66 liposome conjugates in the presence of CpG (gray bars) or none (black bars). One week later, mice were challenged intranasal administration with either the H1N1 (A) or the H3N2 influenza virus (B). On day 2, 4, or 6 post-infection, viral titers in the lungs were determined by calculating $TCID_{50}$ using MDCK cells. Data represent mean and SE of five mice per group. *, $P < 0.05$; **, $P < 0.01$. Reprinted with permission from (34).

tides from highly conserved internal antigens of influenza viruses were evaluated for their ability to protect against influenza virus infection. The liposome-coupled CTL epitope peptides successfully induced antigen-specific $CD8^+$ T cells and CTLs in HLA-A*0201-transgenic mice (Fig. 8). In addition, after nasal infection with the H1N1 or H3N2 virus, viral replication in the lung was significantly inhibited in the immunized mice (Fig. 9). These protective activities lasted for at least 6 months after immunization. Therefore, these results suggest that liposome-coupled CTL epitope peptides derived from highly conserved internal antigens of influenza viruses might facilitate the development of vaccines that would induce protection against heterosubtypic influenza virus infection (34).

8. Conclusions

Surface-linked liposomal antigens are expected to be applicable for the vaccine development against viruses, in particular those viruses that evade humoral immunity by varying their surface proteins such as influenza virus, coronaviruses (severe acute respiratory syndrome and Middle East acute respiratory syndrome), hepatitis C virus, human immunodeficiency virus, and noroviruses.

Moreover, as described above, surface-linked liposomal antigens were initially found to effectively induce antigen-specific IgG antibody production without inducing IgE synthesis. Taken together, liposome-coupled antigens are potentially applicable to the development

of vaccines that would induce both humoral and cellular immunity.

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Conflict of interest None to declare.

REFERENCES

1. Audibert FM, Lise D. Adjuvants: current status, clinical perspectives and future prospects. Immunol Today. 1993;14:281-4.
2. Mark A, Bjorksten B, Granstrom M. Immunoglobulin E responses to diphtheria and tetanus toxoids after booster with aluminum-adsorbed and fluid DT-vaccines. Vaccine. 1995;13: 669-73.
3. Gregoriadis G. Immunological adjuvants: a role for liposomes. Immunol Today. 1990;11:89-97.
4. Alving CR, Koulchin V, Glenn GM, et al. Liposomes as carriers of peptide antigens: induction of antibodies and cytotoxic T lymphocytes to conjugated and unconjugated peptides. Immunol Rev. 1995;145:5-31.
5. De Haan A, Tomee JFC, Huchshorn JP, et al. Liposomes as an immunoadjuvant system for stimulation of mucosal and systemic antibody responses against inactivated measles virus administered intranasally to mice. Vaccine. 1995;13:1320-4.
6. Gluck R. Liposomal presentation of antigens for human vaccines. Pharm Biotechnol. 1995;6:325-45.
7. Gregoriadis G. Liposomes as immunoadjuvants and vaccine carriers: antigen entrapment. Immunometh. 1994;4:210-6.
8. Shahum E, Therien HM. Immunopotentiation of the humoral response by liposomes: encapsulation versus covalent linkage. Immunology. 1988;65:315-7.
9. Fortin A, Shaum E, Krzystyniak K, et al. Differential activation of cell-mediated immune functions by encapsulated and surface-linked liposomal antigens. Cell Immunol. 1996;169:208-17.
10. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. Annu Rev Immunol. 2001;19:47-64.
11. Glenny AT, Pope CG. Immunology notes. XXIII. The antigenic value of toxoid precipitated by potassium alum. J Pathol Bacteriol. 1926;29:31-40.
12. Aggrebeck H, Wantzin J, Heon I. Booster vaccination against diphtheria and tetanus in man: comparison of three different vaccine formations-III. Vaccine. 1996;14:1265-72.
13. Uchida T, Horino A, Naito S, et al. IgE-specific unresponsiveness in mice induced by OVA coupled with murine red blood cells. Int Arch Allergy Immunol. 1994;104:405-8.
14. Naito S, Horino A, Nakayama M, et al. Ovalbumin-liposome conjugate induced IgG but not IgE antibody production. Int Arch Allergy Immunol. 1996;109:223-8.
15. Nakano Y, Mori M, Nishinohara S, et al. Antigen-specific, IgE-selective unresponsiveness induced by antigen-liposome conjugates: comparison of four different conjugation methods. Int Arch Allergy Immunol. 1999;120:199-208.
16. Nakano Y, Mori M, Nishinohara S, et al. Surface-linked liposomal antigen induces IgE-selective unresponsiveness regardless of the lipid components of liposomes. Bioconj Chem. 2001;12: 391-5.
17. Tanaka Y, Kasai M, Taneichi M, et al. Liposomes with differential lipid components exert differential adjuvanticity in antigen-liposome conjugates via differential recognition by macrophages. Bioconj Chem. 2004;15:35-40.
18. Taneichi M, Naito S, Kato H, et al. T cell-independent regulation of IgE antibody production induced by surface-linked liposomal antigen. J Immunol. 2002;169:4246-52.
19. Ellenberg SS, Chen RT. The complicated task of monitoring vaccine safety. Public Health Rep. 1997;112:10-20.
20. Naito S, Takahashi M, Ishida S, et al. Induction of IgE antibody production in mice with different DPT-vaccine preparations. Jpn J Med Sci Biol. 1995;48:117-22.
21. Naito S, Horino A, Komiya T, et al. Induction of protection against tetanus toxin in mice by the administration with tetanus toxin-liposome conjugate. Int Arch Allergy Immunol. 1998;116: 215-9.
22. Naito S, Okada Y, Kato H, et al. Anti-tetanus toxoid antibody production and protection against lethal doses of tetanus toxin in SCID-hu-PBL mice. Int Arch Allergy Immunol. 2000;123: 149-54.
23. Naito S, Horino A, Komiya T, et al. Protection against verocytotoxin (VT) in mice induced by liposome-coupled VT. Int Arch Allergy Immunol. 1997;114:293-7.
24. Fukuda T, Komiya T, Takahashi M, et al. Induction of protection against oral infection with verotoxin-producing *Escherichia coli* O157:H7 in mice by verotoxin-liposome conjugate. Int Arch Allergy Immunol. 1998;116:313-7.
25. Suzaki Y, Ami Y, Nagata N, et al. Protection of monkeys against Shiga toxin induced by Shiga toxin-liposome conjugates. Int Arch Allergy Immunol. 2002;127:294-8.
26. Tanaka Y, Taneichi M, Kasai M, et al. Liposome-coupled antigens are internalized by antigen-presenting cells via pinocytosis and cross-presented to $CD8^+$ T cells. PLoS One. 2010;5(12): e15225.
27. Taneichi M, Ishida H, Kajino K, et al. Antigens chemically coupled to the surface of liposomes are cross-presented to $CD8^+$ T cells and induce potent antitumor immunity. J Immunol. 2006; 177:2324-30.
28. Taneichi M, Tanaka Y, Kakiuchi T, et al. Liposome-coupled peptides induce long-lived memory $CD8^+$ T cells without $CD4^+$ T cells. PLoS One. 2010;5(11):e15091.
29. Olsen B, Munster VJ, Wallensten A, et al. Global patterns of influenza A virus in wild birds. Science. 2006;312:384-8.
30. Effros RB, Doherty PC, Gerhard W, et al. Generation of both cross-reactive and virus-specific T-cell populations after immunization with serologically distinct influenza A viruses. J Exp Med. 1977;145:557-68.
31. Kreijtz JH, de Mutsert G, van Baalen CA, et al. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J Virol. 2008;82:5161-6.
32. Lee LY, Ha do LA, Simmons C, et al. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J Clin Invest. 2008; 118:3478-90.
33. Nagata T, Toyota T, Ishigaki H, et al. Peptides coupled to the surface of a kind of liposome protect infection of influenza viruses. Vaccine. 2007;25:4914-21.
34. Uchida T. Development of a cytotoxic T-lymphocyte-based, broadly protective influenza vaccine. Microbiol Immunol. 2011; 55:19-27.