



Peptides coupled to the surface of a kind of liposome protect infection of influenza viruses

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Abstract

In our previous study, OVA conjugated on the surface of a liposome, we termed Oleoyl liposome, which consisted of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, dioleoyl phosphatidyl glycerol acid and cholesterol in a 4:3:7:2 molar ratio, induced OVA-specific IgG antibody production but not OVA-specific IgE antibody production that is detrimental to the host. Furthermore, OVA_{257–264}–Oleoyl liposome elicited CTL responses in the presence of CpG and rejected E.G7 tumors in mice. In this study we tested whether a peptide–Oleoyl liposome conjugates are capable of inducing protection against viral growth. Subcutaneous inoculation of NP_{366–374}–Oleoyl liposome with CpG inhibited growth of influenza viruses in lungs of mice. Thus, surface-linked liposomal peptide might serve as an effective vaccine without detrimental effects in the presence of immune potentiators.

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1. Introduction

Liposomes have been extensively used as delivery systems or adjuvant for vaccine antigens against bacterial [1,2]

and viral [3,4] infections and tumors [5,6]. The reason for such extensive usage is that liposomes protect antigens from degradation, and epitopic peptides encapsulated in liposomes can be easily phagocytosed by antigen-presenting cells and transferred into the classical MHC class I pathway via the phagosome-cytosol pathway [7–9]. Thus, liposomes are thought to provide peptide vaccines even in mucosal tissues.

Most of the liposomal vaccines proposed have been prepared by antigen entrapment within the aqueous lumen of liposomes. However, it is known that immune responses induced by encapsulated liposomal antigens are different from those induced by surface-linked liposomal antigens. We have demonstrated that surface-linked liposomal antigens

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte; CpG, cytosine-phosphate-guanine; OVA, ovalbumin; MDCK, Madin–Darby canine kidney; NP, nucleoprotein; PFU, plaque-forming units; TLR, toll-like receptor

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induce antigen-specific IgG antibody production but not antigen-specific IgE antibody production [10]. The greater the membrane mobility in the liposomes is, the greater is the antibody production induced by antigen–liposome conjugates, although the membrane fluidity of the liposomes might not affect the induction of IgE-selective unresponsiveness [11]. Indeed, ovalbumin (OVA) conjugated on the surface of a liposome, we termed Oleoyl liposome, which consisted of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, dioleoyl phosphatidyl glycerol acid and cholesterol in a 4:3:7:2 molar ratio, sufficiently induced OVA-specific IgG antibody production but not OVA-specific IgE antibody production. In addition, treatment with OVA–Oleoyl liposome 1 and 3 weeks after cholera toxin-combined OVA administration also suppressed IgE antibody responses. The suppression of anti-OVA IgE antibody production by OVA–Oleoyl liposome was accompanied by a simultaneous enhancement of specific IgA and IgG (IgG1 and especially IgG2a) antibody production [12]. Thus, surface-linked liposomal antigens are expected to serve as a novel protocol for the development of vaccines to induce minimal allergic response.

A synthetic peptide is expected to be a potential vaccine candidate because it has several advantages compared with other types of vaccines [13]. First, because of their simple and small molecular structures, synthetic peptides rarely cause serious complications. Second, small peptide fragments without mutation in microorganisms can be selected to serve as vaccines. By means of rapid and frequent mutations, microorganisms, especially influenza virus or HIV, can escape from the host immune system. Thus, the second advantage is critical to circumvent the limitation resulting from the use of native proteins of microorganisms as vaccines. However, such small peptide fragments do not always induce potent immune responses in the host. A peptide vaccine should be protected against degradation *in vivo* by certain carriers (vehicles).

In our previous study, an OVA peptide, OVA_{257–264}, conjugated on the surface of Oleoyl liposome elicited CTL responses in the presence of CpG and anti-IL10 in mice. Furthermore, this treatment rejected E.G7 tumors, generated by transducing the chicken OVA gene into the murine lymphoma cell line EL-4 [14]. In this study, we further examined the capacity for CTL activation of peptide–Oleoyl liposome in the presence of various immune potentiators (adjuvants) and compared the capacity for CTL activation with that of other liposomes carrying peptides. Subcutaneous inoculation of peptide–Oleoyl liposome in the presence of CpG elicited most effective CTL responses in mice. Furthermore, a nucleoprotein (NP) peptide derived from A/Aichi/2/68 (H3N2) influenza virus, NP_{366–374}, conjugated on Oleoyl liposome in the presence of CpG protected against infection of the influenza virus. Collectively, the results suggest that peptide–Oleoyl liposome in the presence of some sort of immune potentiators might serve as an effective vaccine without detrimental effects, such as allergic reactions.

2. Materials and methods

2.1. Liposomes

Oleoyl liposomes, we termed, consisted of dioleoyl phosphatidyl choline, dioleoyl phosphatidyl ethanolamine, dioleoyl phosphatidyl glycerol acid, and cholesterol in a 4:3:2:7 molar ratio [11]. In some experiments, PS–Oleoyl liposomes were prepared by altering dioleoyl phosphatidyl choline to dioleoyl phosphatidyl serine in Oleoyl liposomes. The crude liposome solution was passed through a membrane filter (Nucleopore polycarbonate filter; Coster, Cambridge, MA) with a pore size of 0.2 μ m. Liposomal conjugates with peptides were prepared via disuccinimidyl suberate as described previously [15]. In addition, multilamellar liposome containing OVA peptide was prepared by mixing with peptide (100 nmol/mouse), 1.25 μ mol/mouse phosphatidylserine (Avanti Polar Lipids, Alabaster, AL), and 2.75 μ mol/mouse phosphatidylcholine (Avanti Polar Lipids) in chloroform as previously described [16]. The mixture in a glass tube was blown with N₂ gas, evaporated at 40 °C, and aspirated for 1 h. The peptides incorporated in the multilamellar liposome that adhered to the inside of the glass tube were dissolved in 50 μ l of PBS by vortex mixing.

2.2. Immunization

C57BL/6 mice (B6) (6–10 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). CpG5002 (5'-TCCATGACGTTCTGATGTT-3', Hokkaido System Science, Sapporo, Japan) (5–10 μ g/mouse) [17] and various peptides conjugated on Oleoyl liposome (0.6 mg/ml) in 100 μ l saline were subcutaneously inoculated into mice. Peptides used in this study were OVA_{257–264} (SIIN-FEKL), NP_{366–374} (ASNENMDAM) of an H3N2 influenza virus, NP_{366–374} (ASNENMETM) of an H1N1 influenza virus, VSV-NP_{52–59} (RGYVFQGL) or LCMV-NP_{396–404} (FQPQNGQFI). In some experiments, LPS (10 μ g/mouse) (Wako Pure Chemical Ind., Osaka, Japan), Flagellin (10 μ g/mouse) (Calbiochem, Darmstadt, Germany) or Poly(I:C) (10 μ g/mouse) (Invivogen, San Diego, CA) were used as an adjuvant instead of CpG. Since all experiments were performed with the approval of the Shiga University of Medical Science Animal Experiment Committee, we usually used three mice in each group of experiments to reduce number of experimental animals.

2.3. *In vivo* CTL assay

Spleen cells (2×10^7 cells/ml) from naïve B6 mice were incubated with 0.5 μ M peptide for 2 h at 37 °C. After washing twice with HBSS, the recovered cells (2×10^7 cells/ml) were labeled with different concentrations of carboxyfluorescein diacetate succinimidyl ester (CFSE) (0.25 μ M or 2.5 μ M, Molecular Probes, Eugene, OR) at room temperature for 10 min. Labeling was stopped with one

half volume of FCS followed by two additional washes. Five million cells carrying each peptide were mixed and injected intravenously into immunized mice. At 12 h after injection, the spleens were harvested and single cell suspensions were prepared. CFSE-positive cells were analyzed by a flow cytometer with exclusion of dead cells with propidium iodide. For example, NP-specific killing was calculated as follows: $\% \text{killing} = [1 - \{(\text{number of cells carrying NP}_{366-374} \text{ in immunized mice (CFSE high)}) / (\text{number of cells carrying OVA}_{257-264} \text{ in immunized mice (CFSE low)})\}] / \{(\text{number of cells carrying NP}_{366-374} \text{ in normal mice (CFSE high)}) / (\text{number of cells carrying OVA}_{257-264} \text{ in normal mice (CFSE low)})\}] \times 100$.

2.4. Influenza viruses

Influenza virus, A/Aichi/2/68 (H3N2), was propagated in the allantoic cavities of 10-day-old embryonated hen's eggs at 35 °C for 48 h. Then the viruses were purified by ultracentrifugation of allantoic fluid passed through a 10–50% sucrose density gradient. The viruses were suspended in PBS. The viruses were prepared from culture supernatant of Madin–Darby canine kidney (MDCK) cells for experimental infection [16].

2.5. Virus challenge and protection tests

B6 mice were challenged intranasally with 15 μl of 10^4 plaque-forming units (PFU) of A/Aichi/2/68 (H3N2) under anesthesia. Since A/Aichi/2/68 (H3N2) did not kill mice without systemic infection, it was adequate for assessing virus growth in mouse lungs at 5 days after challenge. Lungs were collected from three mice of each group and used for the measurement of virus titers. In the measurement of virus titers, 10% suspensions of the tissue homogenates were prepared.

2.6. Titration of virus

MDCK cells were grown in minimal essential medium (MEM) supplemented with 10% bovine calf serum. Suspensions of the lungs serially diluted from 1:10 were inoculated into confluent MDCK cell monolayers on 6-well plates and incubated at room temperature for 1 h for adsorption. After 1 h, the inoculum was removed and cells were overlaid with MEM containing 1% bacto agar (BD Diagnostic Systems, Sparks, MD) and 5 $\mu\text{g}/\text{ml}$ of trypsin (BD Diagnostic Systems). After incubation at 35 °C for 2 days in 5% CO_2 , the plaques were counted. The limit of detection in this assay was 0.5×10^3 PFU/g [16].

2.7. ELISA

Enzyme-linked immunosorbent assay (ELISA). 96-Well plates were coated with 50 μl of purified A/Aichi/2/68 (H3N2) (20 $\mu\text{g}/\text{ml}$) disrupted with 0.05 M Tris–HCl (pH 7.8)

containing 0.5% Triton X-100 and 0.6 M KCl. After washing three times with PBS containing 0.05% Tween20, PBS with 3% BSA was added for blocking. Sera were collected 5 days after infection following subcutaneous immunization twice with NP–liposome and CpG. Serially diluted sera were incubated overnight. For measuring total IgG, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) (1/2000 $\times$ 50 μl) was added after washing 5 times. For measuring IgE, biotinylated anti-mouse IgE (clone 23G3) (eBioscience) (0.5 $\mu\text{g}/\text{ml}$ $\times$ 50 μl) was added. Horseradish peroxidase-conjugated Extravidin (Sigma–Aldrich) (1/3000 $\times$ 100 μl) was incubated for 3 h. Horseradish peroxidase activity was assessed by 3,3',5,5'-tetramethyl benzidine substrate (100 μl). The reaction was stopped by 1 N HCl (100 μl). Optical density was measured at 450 nm.

2.8. Statistical analyses

Statistical analyses were carried out using Student's *t*-test. *p* values <0.05 were considered significant.

3. Results

3.1. Comparison of CTL activation capacities of surface-linked liposomal OVA peptide and OVA peptide entrapped within the aqueous lumen of liposomes

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]. Thus, firstly we compared the capacity for activating CTL *in vivo* of liposome with chemically bound peptides on the surface (peptide–Oleoyl liposome) and that of the multilamellar liposome containing peptides inside. 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, and then 7 days later CFSE-labeled target cells pulsed with OVA_{257–264} were injected intravenously. Viability of the target cells in the spleen was examined at 12 h after injection. 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) (Fig. 1A).

3.2. Comparison of CTL activation capacities of liposomes consisting of different components of surface-linked liposomal OVA peptide

Some scavenger receptors recognize phosphatidyl serine and support phagocytosis. Thus, we altered phosphatidyl choline to phosphatidyl serine of Oleoyl liposome this liposome was termed PS–Oleoyl liposome. However, we could

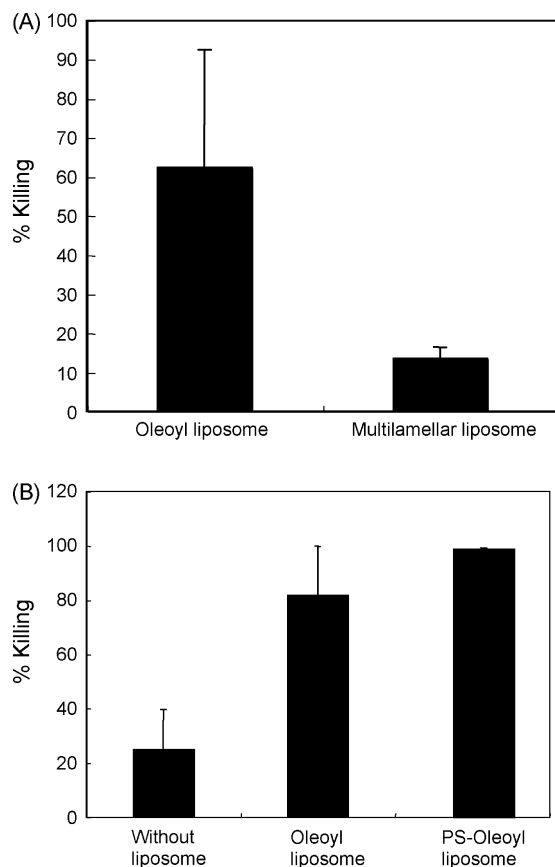


Fig. 1. Comparison of CTL activation capacities of various liposomes carrying peptides. (A) B6 mice were injected subcutaneously with either liposome with chemically bound OVA_{257–264} on the surface or multilamellar liposome containing OVA_{257–264} inside in the presence of CpG and anti-CD40 antibody. 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 = $[1 - \{(\text{number of cells carrying OVA}_{257-264} \text{ in immunized mice (CFSE high)}) / (\text{number of cells carrying NP}_{366-374} \text{ in immunized mice (CFSE low)})\}] / \{(\text{number of cells carrying OVA}_{257-264} \text{ in normal mice (CFSE high)}) / (\text{number of cells carrying NP}_{366-374} \text{ in normal mice (CFSE low)})\}] \times 100$. 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 $\mu\text{g}/\text{mouse}$) (TLR9 ligand) and Poly(I:C) (TLR3 ligand) (10 $\mu\text{g}/\text{mouse}$) showed sufficient adjuvant activity in combination with OVA_{257–264}–Oleoyl liposome compared with LPS (10 $\mu\text{g}/\text{mouse}$) (TLR4 ligand) ($p = 0.003$ and 0.012 , respectively) (Fig. 3A). Administration of LPS

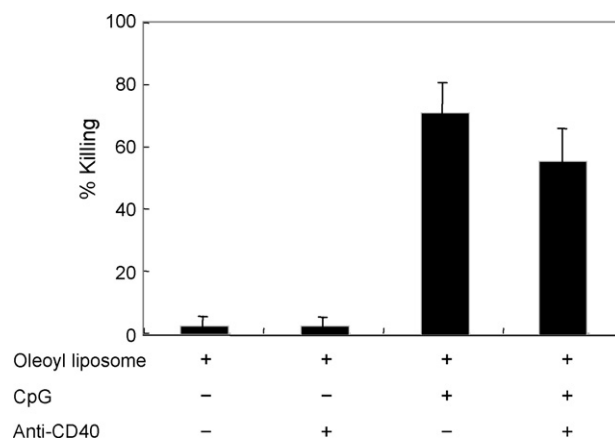


Fig. 2. CTL activation by simultaneous inoculation of CpG and OVA_{257–264}–Oleoyl liposome. Various combinations of CpG, anti-CD40 and OVA_{257–264}–Oleoyl liposome were inoculated subcutaneously into B6 mice for an *in vivo* CTL assay. Subcutaneous inoculation with CpG and OVA_{257–264}–Oleoyl liposome 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 ($p = 0.12$).

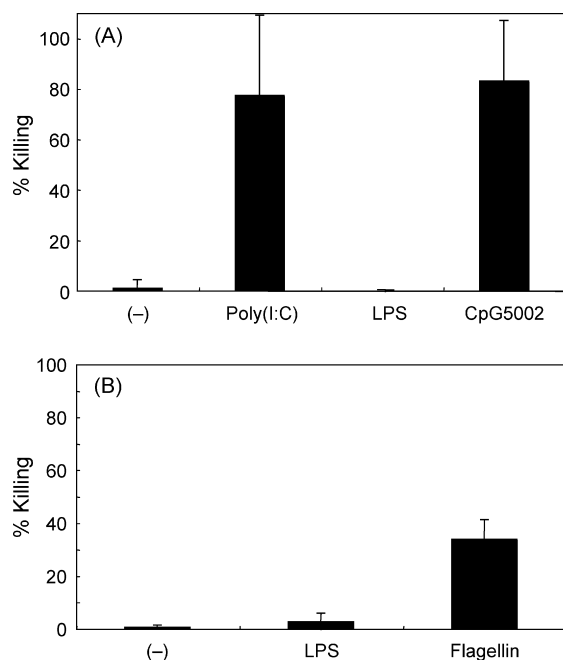


Fig. 3. Comparison of TLR ligands as adjuvants. We inoculated simultaneously with various TLR ligands and OVA_{257–264}– or NP_{366–374}–Oleoyl liposome. Seven days later, high CFSE-labeled target cells pulsed with OVA_{257–264} or NP_{366–374}, and low CFSE-labeled target cells pulsed with VSV-NP_{52–59} (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 μ g, 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 μ g/mouse) or LPS (10 μ g/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 μ g/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 μ g/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 NP_{366–374} 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

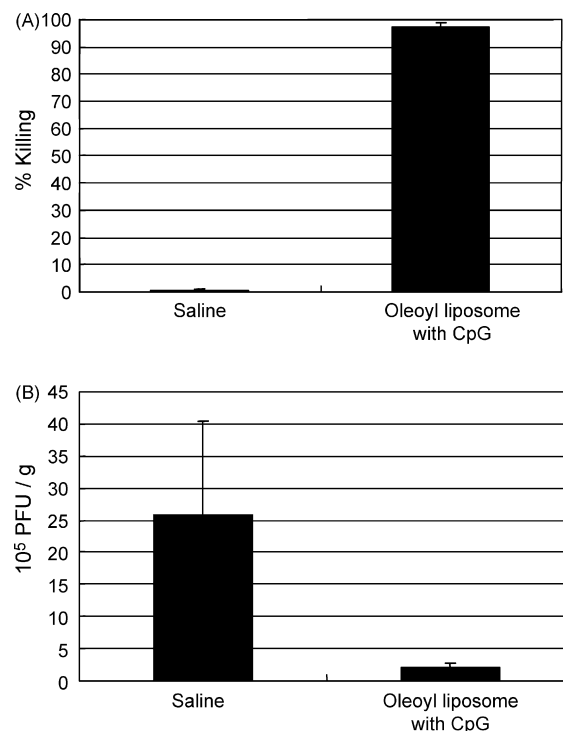


Fig. 4. Vaccination of NP_{366–374} bound to Oleoyl liposome in combination with CpG. (A) B6 mice were injected twice subcutaneously with Oleoyl liposome with chemically bound NP_{366–374} on the surface with CpG. Seven days later, high CFSE-labeled target cells pulsed with NP_{366–374} and low CFSE-labeled target cells pulsed with OVA_{257–264} were injected intravenously as an *in vivo* CTL assay. NP-specific killing was calculated as follows: %killing = $[1 - \{(\text{number of cells carrying NP}_{366-374} \text{ in immunized mice (CFSE high)})/(\text{number of cells carrying OVA}_{257-264} \text{ in immunized mice (CFSE low)})\}]/\{(\text{number of cells carrying NP}_{366-374} \text{ in normal mice (CFSE high)})/(\text{number of cells carrying OVA}_{257-264} \text{ in normal mice (CFSE low)})\}] \times 100$. 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,

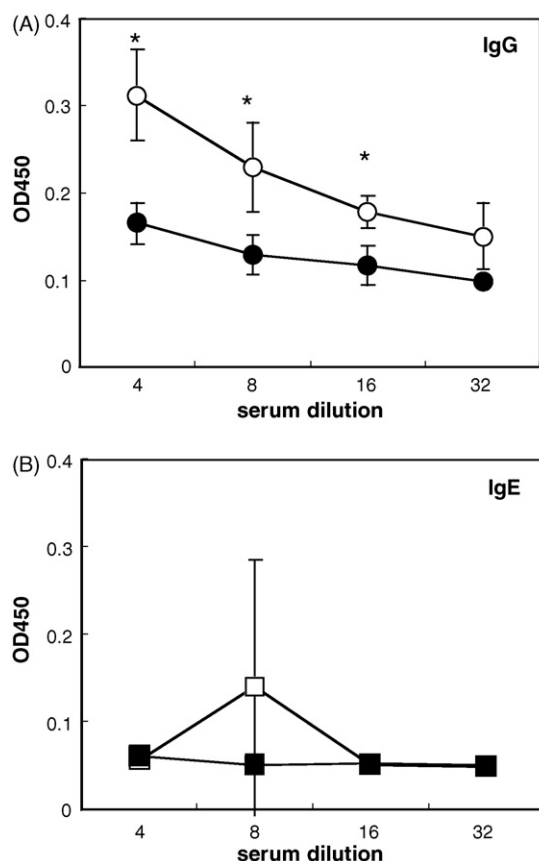


Fig. 5. Antibody production by simultaneous inoculation of CpG and NP_{366–374}–Oleoyl liposome. Sera were collected 5 days after infection following subcutaneous immunization twice with NP–liposome and CpG. (A) Total IgG against disrupted A/Aichi/2/68 (H3N2) in the serially diluted sera was measure 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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