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Adjuvant effect of liposome-encapsulated natural phosphodiester CpG-DNA

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Immunostimulatory CpG-DNA targeting TLR9 is one of the most extensively evaluated vaccine adjuvants. Previously, we found that a particular form of natural phosphodiester bond CpG-DNA (PO-ODN) encapsulated in a phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine (DOPE) : cholesterol hemisuccinate (CHEMS) (1 : 1 ratio) complex (Lipoplex(O)) is a potent adjuvant. Complexes containing peptide and Lipoplex(O) are extremely useful for B cell epitope screening and antibody production without carriers. Here, we showed that IL-12 production was increased in bone marrow derived dendritic cells in a CpG sequence-dependent manner when PO-ODN was encapsulated in Lipoplex(O), DOTAP or lipofectamine. However, the effects of Lipoplex(O) surpassed those of PO-ODN encapsulated in DOTAP or lipofectamine and also other various forms of liposome-encapsulated CpG-DNA in terms of potency for protein antigen-specific IgG production and Th1- associated IgG2a production. Therefore, Lipoplex(O) may have a unique potent immunoadjuvant activity which can be useful for various applications involving protein antigens as well as peptides. [BMB reports 2011; 44(11): 758-763]

INTRODUCTION

Adjuvants are substances added to or emulsified with a vaccine formulation to boost the potency and durability of antigen-specific immune response without toxicity. Aluminum salts have been commonly used as an adjuvant for -100 years; however, their mechanisms of action are only partially understood (1-3). Furthermore, currently approved vaccine adjuvants do not always elicit the desired protective and sustained immune response against different target pathogens. Therefore, identification and development of new adjuvants is still required.

Liposomes as vehicles for delivery have been widely investigated in developing vaccines to improve antibody production and immune responses (4-8). Investigators have shown that the pH-sensitive liposomes such as phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine (DOPE) : cholesterol hemisuccinate (CHEMS) improve antigen delivery to the cytosol and induction of cytotoxic T lymphocyte (CTL) responses (9). Furthermore, effective antigen-specific CTL responses are reported in mice immunized with CTL epitopes synthesized from Hantaan nucleocapsid protein (M6) or human papilloma virus E7 encapsulated in pH-sensitive liposomes (10). Cationic liposomes have been used as vehicles for delivery to improve uptake of antigens by macrophages and dendritic cells. The CTL response and antibody production were enhanced by encapsulated cationic liposomes consisting of molecules such as lipofectamine, 3β-[N- (N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride (Dc-Chol) and phosphatidylcholine stearylamine : cholesterol (Chol) (6, 11, 12).

Although liposomes are potent vehicles for delivering antigen to antigen presenting cells (APCs), they have not been investigated for the enhancement of immunogenicity and adjuvant effects. The immunostimulatory activities of CpG-DNA have received attention as a potentially useful approach for developing immunoadjuvants, when compared to other immune-stimulating agents such as flagella, lipid A, cytokines etc (7). Several investigators have shown that CpG-DNA upregulates antigen-presenting cell activity, Th1 immune response and immunoglobulin (Ig) isotype switching (13-15). The immunostimulatory activities as a potent adjuvant are enhanced by liposome-encapsulated CpG-DNA (16-18).

Previously, we have reported effective immunostimulatory natural phosphodiester CpG-DNA (PO-ODN) from chromosomal DNA sequences of *M. bovis* and *E. coli* (19, 20). The PO-DNA, specifically MB-ODN 4531(O) containing immunomodulatory CpG motifs, regulates the expression of Th1 cytokines such as IL-12 and IFN-γ in the innate immune system, without severe side effects (21). We found that the PO- ODN encapsulated in a DOPE : CHEMS complex (Lipoplex (O)) stimulated the immune response in cells from humans and mice (22). We also

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suggested that the Lipoplex(O) is a potent adjuvant and that complexes of peptide (or protein) and Lipoplex(O) can be applied for B cell epitope screening and antibody production without carriers (22). In this study, we report that Lipoplex(O) can effectively induce cytokine secretion in dendritic cells (DCs). We also compared the effect of liposome composition on the functional effects of Lipoplex(O) as an adjuvant for the induction of antigen-driven humoral immune responses.

RESULTS

Effects of CpG-DNA encapsulated in liposomes on DCs

Adjuvant activity of CpG-DNA enhances the function of professional APCs and activates the production of cytokines that induce antigen-specific humoral immune responses (13). Dendritic cells (DCs), the most important APCs, constitute a major part of the innate immune system and have receptors such as Toll-like receptors (TLR). The recognition of CpG-DNA by DCs results in expression of Th1 cytokines such as IL-12 and IFN-γ. Therefore, we decided to evaluate the CpG-DNA effects of liposome encapsulation on production of IL-12 in DCs. We first examined whether backbone modification of CpG- DNA could induce IL-12 production in DCs derived from mouse bone marrow cells. In contrast to PO-ODN (MB-ODN 4531(O)), IL-12 production was potently induced by PS-ODN (MB-ODN 4531(S)) alone (Fig. 1). Interestingly, the cytokine expression in human peripheral blood mononuclear cells or mouse splenocytes is greatly enhanced when the PO-ODNs are encapsulated in a proper liposome complex (22). Therefore, we measured the IL-12 production in DCs to investigate the immunostimulatory activity of PO-ODN encapsulated in different liposome complexes. As expected, the IL-12 production was increased in a CpG sequence-dependent manner when PO-ODN was encapsulated in a DOPE : CHEMS (1 : 1 ratio) complex (Lipoplex(O)), *N*-[1-(2,3-dioleoyloxy)propyl]- *N,N,N*-trimethylammonium methylsulfate (DOTAP) or lipofectamine in DCs (Fig. 1).

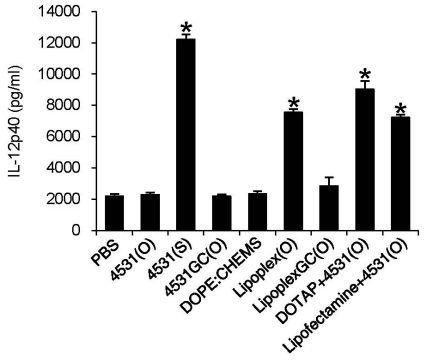

Fig. 1. Effect of PO-ODN encapsulated in liposomes on IL-12 production of DCs. The DCs were treated with the indicated CpG- DNA (5 μg/ml) or CpG-DNA (5 μg/ml) encapsulated in a DOPE : CHEMS complex, DOTAP, or Lipofectamine for 24 h, and the culture supernatants were harvested. The levels of IL-12 were measured using ELISA. Similar results were obtained in 3 independent sets of the experiments. (O), phosphodiester bond; (S), phosphorothioate backbone modification; 4531(O), MB-ODN 4531(O); 4531(S), MB-ODN 4531(S); 4531GC(O), MB-ODN 4531GC(O); Lipoplex(O), MB-ODN 4531(O) encapsulated in a DOPE : CHEMS complex; LipoplexGC(O), MB-ODN 4531GC(O) encapsulated in a DOPE : CHEMS complex; DOTAP + 4531(O), MB-ODN 4531 (O) encapsulated in DOTAP; Lipofectamine + 4531(O), MB-ODN 4531(O) encapsulated in Lipofectamine. *$P < 0.01$ (vs PBS control).

Comparison of adjuvant effects with PO-ODN encapsulated in different cationic liposome complexes

Previously, we have shown the effective adjuvant activity of Lipoplex(O) in production of antibodies specific to protein antigen and B cell epitopes without carriers (22). To compare the adjuvant activity of PO-ODN encapsulated in different cationic liposomes (DOTAP or lipofectamine) or DOPE : CHEMS complex, we immunized BALB/c mice with complexes containing hen egg lysozyme (HEL) and MB-ODN 4531(O) co-encapsulated in different liposome compositions (Fig. 2). When we injected mice with MB-ODN 4531(O) and HEL co-encapsulated in a DOPE : CHEMS complex (Lipoplex(O) + HEL), the BALB/c mice induced more HEL-specific total IgG production than the mice immunized with a complex consisting of MB-ODN 4531(O) and HEL co-encapsulated in the other cationic liposomes (DOTAP or lipofectamine) (Fig. 2A and B). We also validated the IgG isotype of the anti-HEL specific antibody in the sera collected from the immunized mice. As shown in Fig. 2C, the titer of the HEL-specific IgG1 is more likely to be enhanced in the mice injected with a complex of HEL and Lipoplex(O) than in the other liposome complex injected mice. Furthermore, immunization with a complex of HEL and Lipoplex(O) induced higher levels of IgG2a compared with IgG1 (Fig. 2A, C and D).

To further define the contribution of PO-ODN encapsulated in different cationic liposomes to protein antigen, we performed the same experiments with another antigen - ovalbumin (OVA). The amount of the OVA-specific total IgG was higher in the mice injected with a complex of OVA and Lipoplex(O) than in mice injected with the other liposome complex (Fig. 3A). Importantly, immunization with a complex of OVA and Lipoplex(O) produced a higher level of IgG2a than immunization with OVA and other liposome complexes (Fig. 3B and C).

These results indicate that encapsulation of PO-ODN and protein antigen with a DOPE : CHEMS complex rather than another liposome significantly enhanced the production of protein antigen-specific IgG.

Adjuvant effects of PO-ODN encapsulated in combination with other various liposomes

To further investigate the superior adjuvant activity of PO-ODN when encapsulated in DOPE : CHEMS complex, we prepared different liposome complexes consisting of HEL and MB-ODN 4531(O) co-encapsulated with DOPE : CHEMS (1 : 1 ratio),

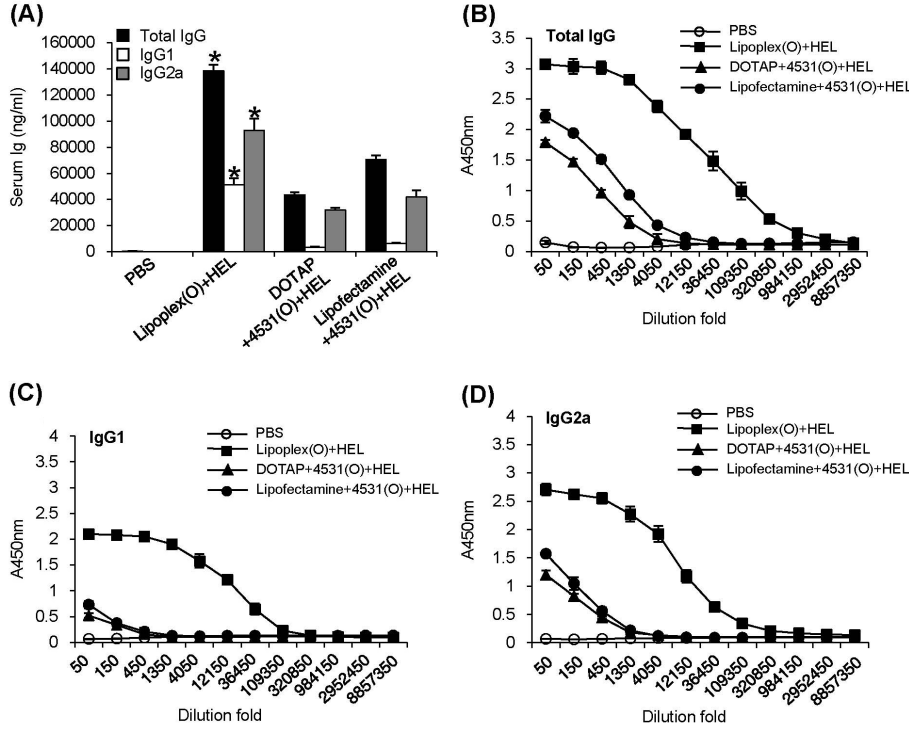

Fig. 2. Production of Th1-associated HEL-specific IgG induced by Lipoplex(O). Three BALB/c mice were immunized 3 times with one of the following combinations : HEL (50 μg/mouse) and MB-ODN 4531(O) co-encapsulated in DOPE : CHEMS complex (Lipoplex(O) + HEL), DOTAP (DOTAP + 4531(O) + HEL) or lipofectamine (Lipofectamine + 4531(O) + HEL). Serum was collected, and the total IgG and IgG isotypes were assayed using an ELISA kit. (A) Amounts of anti-HEL-specific total IgG and IgG isotypes. *$P < 0.01$. (vs other liposome complexes). (B) Titers of anti-HEL-specific total IgG. (C) Titers of anti-HEL-specific IgG1. (D) Titers of anti-HEL-specific IgG2a. Each bar and graph represents Mean ± SD values obtained from 3 individual experiments.

1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DSPC) : Chol (55 : 45 ratio), DSPC : CHEMS : phosphatidylethanolamine-poly(ethylene glycol) (PEG-PE) (6 : 4 : 0.3 ratio), Chol : DOPE : PEG-PE (4 : 6 : 0.06 ratio) or Dc-Chol : DOPE : PEG-PE (4 : 6 : 0.06 ratio). Then, we immunized BALB/c mice with the different formulations. The BALB/c mice immunized with the various combinations with liposomes produced large amounts of -specific total IgG (Fig. 4A). Especially, Lipoplex(O) induced a marked increase in the production of HEL-specific IgG2a than IgG1, whereas the other liposome complexes predominantly enhanced the production of HEL-specific IgG1 (Fig. 4B and C).

DISCUSSION

Chemically inactivated vaccines are widely used in clinics; however, these vaccines have disadvantages such as a risk of pathogen reactivation, cost for maintenance of vaccine stability, and induction of autoimmune diseases (23-25). Therefore, new vaccine technologies have been developed to improve safety and efficacious target-specific immune response by using highly purified specific antigens such as recombinant proteins and peptides. However, these refined antigens often have clear limitations for inducing an effective immune response. Furthermore, there are significant challenges in the development of effective vaccines for complex pathogens such as malaria and HIV etc, especially for elderly or immunocompromised people. Therefore, development of new efficacious adjuvants that can boost immunogenicity is still required.

New types of adjuvants for stimulating the TLR pathway have been extensively studied to develop effective and safe vaccines (26). Especially, immunostimulatory CpG-DNA targeting TLR9 was extensively evaluated as an adjuvant (13). The phosphorothioate-modified type of CpG-DNA (PS-ODN) has functional adjuvant effects inducing Th1-response and immunoglobulin (Ig) isotype switching (13). The potent adjuvant effects were enhanced by liposome encapsulation (16-18). However, it was reported that PS-ODN induces backbone- related side effects, such as induction of transient lymphadenopathy (27), lymphoid follicle destruction (28) and arthritis (29). Previously, we have shown that a particular form of natural phosphodiester bond CpG-DNA (MB-ODN-4531(O)) encapsulated in a specific liposome complex (Lipoplex(O)) induces potent immunomodulatory activity (22). The Lipoplex(O) improves the production of IgG2a specific to antigenic protein in mice. In contrast, incomplete Freund's adjuvant induced more production of antigen specific IgG1 than IgG2a. Furthermore, immunization of mice with several B cell epitopes co-encapsulated with Lipoplex(O) without carriers induces each peptide-specific IgG2a production in a CG sequence-dependent manner. The IgG production by a complex of B cell epitope and Lipoplex(O) was dependent on TLR9.

In this report, we found that IL-12 was released from bone marrow derived dendritic cells (BMDCs) in a CpG sequence-dependent manner when the DCs were stimulated with PO-ODN encapsulated in a DOPE : CHEMS (1 : 1 ratio) complex (Lipoplex(O)), DOTAP or Lipofectamine (Fig. 1). This re-

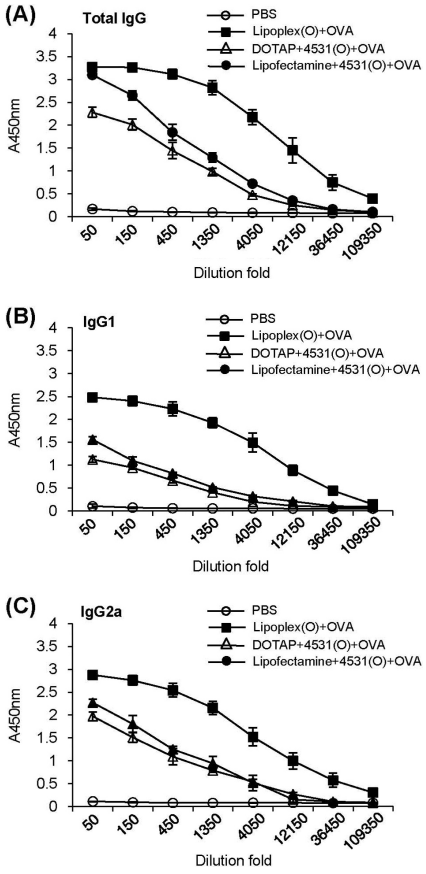

Fig. 3. Production of OVA-specific IgG induced by Lipoplex (O). (A-C) C57BL/6 mice (n = 3/group) were immunized with one of the following : OVA (50 μg/mouse) and MB-ODN 4531(O) co-encapsulated in DOPE : CHEMS complex (Lipoplex(O) + OVA), DOTAP (DOTAP + 4531(O) + OVA) or lipofectamine (Lipofectamine + 4531(O) + OVA). Serum was collected, and titers of total IgG and IgG isotypes were assayed using an ELISA kit. (A) Titers of total IgG. (B) Titers of IgG1. (C) Titers of IgG2a. Bar graphs represent Mean ± SD values obtained from 3 individual mice.

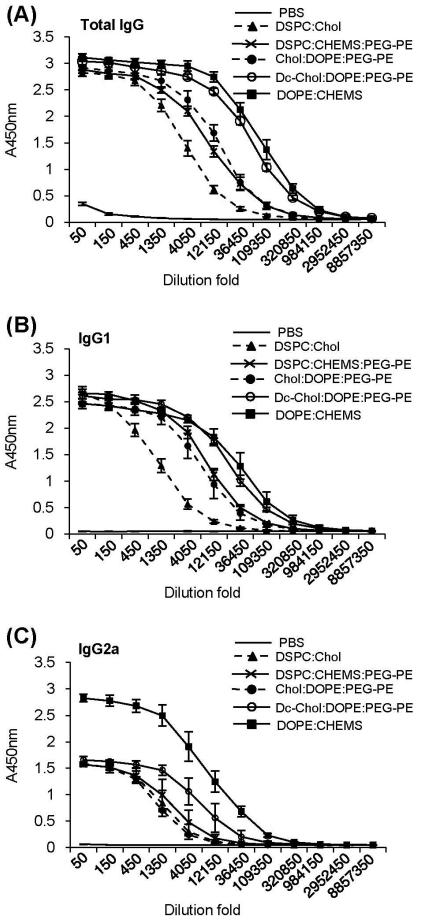

Fig. 4. Induction of HEL-specific IgG production by immunization with MB-ODN 4531(O) and HEL encapsulated in combination with various types of liposomes. The titers of anti-HEL-specific IgG induced by MB-ODN 4531(O) and HEL encapsulated in the indicated liposome complexes were assayed using an ELISA kit. (A) Titers of anti-HEL-specific total IgG. (B) Titers of anti-HEL-specific IgG1. (C) Titers of anti-HEL-specific IgG2a. Bar graphs represent Mean ± SD values obtained from 3 individual mice.

sult directly shows that DCs, as professional APCs, may be equally activated by Lipoplex(O) or PO-ODN encapsulated with DOTAP or Lipofectamine. However, Lipoplex(O) showed greater functional effects than the other 2 liposome complexes as a Th1-responsive adjuvant, by inducing more IgG2a than IgG1 specific to antigenic proteins such as HEL and OVA (Fig. 2 and 3). Furthermore, Lipoplex(O) had more potent effects than other various liposome complexes (Fig. 4). Therefore, we can conclude that Lipoplex(O) is more effective than other forms of liposome-encapsulated CpG-DNA in terms of the adjuvant potency for protein antigen-specific IgG production and Th1-associated IgG2a production. The exact mechanisms or unique properties of Lipoplex(O) involved in this phenomenon remain to be determined in the future studies.

MATERIALS AND METHODS

ODNs

ODNs were synthesized by ST Pharm Co., Ltd (Seoul, Korea) and GenoTech (Daejeon, Korea). MB-ODN 4531 consisted of 20 bases containing 3 CpG motifs (underlined) : AGCAGCGTTCGTGTCGGCCT. The sequence of MB-ODN 4531 used in this study was either phosphodiester (MB-ODN 4531(O)) or phosphorothioate-modified (MB-ODN 4531(S)). MB-ODN 4531GC(O) is a derivative of MB-ODN 4531(O) with one of its CG sequences reversed to GC (underlined) : AGCAGGCTTGCTGTGCGCCT. The endotoxin content of the ODNs was <1 ng/mg of ODN, as measured by a *Limulus amebocyte* as-

say (Whittaker Bioproducts, Walkersville, MD, USA).

Preparation of CpG-DNA encapsulated in liposome complexes

Liposome encapsulation of CpG-DNA was performed as previously described (22). The liposomes CHEMS, Chol, DOPE, and DSPC were purchased from Sigma-Aldrich (St. Louis, MO, USA). DC-Chol and PEG-PE were obtained from Avanti-Polar Lipids (Alabaster, AL, USA). Complexes of CpG-DNA with DOTAP (Roche, Indianapolis, IN, USA) or lipofectamine (Invitrogen, Carlsbad, CA, USA) were prepared in accordance with the manufacturer's specifications. Liposome complexes consisting of CpG-DNA encapsulated with DOPE : CHEMS were prepared as previously reported (30), with minor modifications (22).

Preparation of protein and CpG-DNA co-encapsulated in liposome complexes

Complexes of CpG-DNA and protein encapsulated with DOTAP (Roche) or lipofectamine (Invitrogen) were prepared in accordance with the manufacturer's specifications. Liposome complexes consisting of protein and CpG-DNA co-encapsulated with DOPE : CHEMS (1 : 1), DSPC : Chol (55 : 45 ratio), DSPC : CHEMS : PEG-PE (6 : 4 : 0.3 ratio), Chol : DOPE : PEG-PE (4 : 6 : 0.06 ratio), or Dc-Chol : DOPE : PEG-PE (4 : 6 : 0.06 ratio) were prepared as previously reported (30) with minor modifications (22). Briefly, DOPE and CHEMS were mixed in ethanol at a molar ratio of 1 : 1, evaporated with nitrogen gas to produce a solvent-free lipid film, and resuspended in a mixture containing equal volumes of water-soluble CpG-DNA and protein, followed by vigorous stirring at room temperature for 30 min. After adjusting the pH to 7.0, the Lipoplex solution was sonicated for 30 s. The solution was filtered with a 0.22 μm filter and freeze-thawed 3 times using liquid nitrogen.

Generation of bone marrow-derived dendritic cells (BMDCs)

BMDCs were generated as previously reported (31) with minor modifications (32). The culture supernatants were collected, and the IL-12 levels were measured using commercially available ELISA kits (R&D Systems) in accordance with the manufacturer's specifications.

Mice and immunization

Mice were maintained under specific-pathogen-free conditions. Four-week-old male BALB/c (H-2^b) mice were purchased from Central Lab. Animal, Inc. (Seoul, Korea). All animal procedures performed in this study were conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research & Quarantine Service of Korea. The protocol was approved by the Institutional Animal Care and Use Committee of Hallym University (Permit Number : Hallym 2009-47). The mice were sacrificed under Zoletil 50+Rompun anesthesia, and all efforts were made to minimize pain. On 3 occasions at 10 day intervals, the mice were injected intraperitoneally with a complex consisting of 50 μg of HEL (or OVA) and 50 μg of MB-ODN 4531(O) encapsulated in the indicated liposome.

Antigen-specific Ig ELISA

Serum was collected from the blood samples obtained by a heart punch method 10 days after the final injection. To determine the amounts of total IgG, IgG1, and IgG2a, we coated 96-well immunoplates (Nalgen Nunc International, Rochester, NY, USA) with 5 μg/ml of HEL (or OVA) and then blocked them with 0.05% of Tween-20 in PBS (PBST) containing 1% BSA. Total IgG, IgG1, and IgG2a levels were measured as previously described (22).

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