Activation of Toll-like receptor 9 and production of epitope specific antibody by liposome-encapsulated CpG-DNA

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Several investigators have shown that CpG-DNA has outstanding effects as a Th1-responsive adjuvant and that its potent adjuvant effects are enhanced by encapsulation with a liposome of proper composition. In this study, we showed that encapsulation with phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine (DOPE): cholesterol hemisuccinate (CHEMS) complex enhances the immunostimulatory activity of CpG DNA and the binding of CpG-DNA to TLR9. We also examined involvement of myeloid differentiation protein (MyD88) and NF-κB activation in liposome-encapsulated CpG-DNA-induced IL-8 promoter activation. In this manuscript, the natural phosphodiester bond CpG-DNA encapsulated by DOPE : CHEMS complex is designated as Lipoplex(O). Importantly, we successfully screened B cell epitopes of envelope protein (E protein) of hepatitis C virus (HCV-E) and attachment glycoprotein G of human respiratory syncytial virus (HRSV-G) by immunization with complexes of several peptides and Lipoplex(O) without carriers. Therefore, Lipoplex(O) is potentially applicable as a universal adjuvant for peptide-based epitope screening and antibody production. [BMB reports 2011; 44(9): 607-612]

INTRODUCTION

TLR9 in the innate immune system recognizes bacterial DNA and synthetic oligodeoxynucleotides that contain unmethylated CpG dinucleotides in the context of particular base sequences (CpG-DNA) as being non-self (1). CpG-DNA can be employed for regulation of the immune system, including stimulation of B cell proliferation, production of antibodies, and stimulation of macrophages, dendritic cells, and natural killer cells (2-4). Several investigators have shown that the CpG-DNA has an outstanding immunostimulatory activity for inducing a strong Th1 immune response, resulting in induction of Th1 cytokines such as IL-12, IFN-α and IFN-γ (3-5). The en-

hanced Th1 responses contribute to the protection of the host from infectious agents (6). CpG-DNA also improves antigen-presenting cell activity through upregulation of major histocompatibility (MHC) class II molecules and costimulatory molecules (CD40, CD80, and CD86) (7-9). Consequently, CpG-DNA has been widely applied as an effective vaccine adjuvant for infectious diseases and cancer (8, 9).

CpG-DNA can be divided into 2 classes based on the backbone type. First, the phosphorothioate backbone-modified CpG-DNA (PS ODN) includes the oligodeoxynucleotides stabilized by sulfurs replaced on the nonbridging oxygen atoms in the backbone. Although PS-ODN is able to dramatically enhance the CpG-DNA-induced immune responses by resistance to nuclease activity and improved the efficiency of CpG-DNA uptake into cells, it was reported that PS-ODN provokes severe side effects in a CG sequence-dependent and backbone modification-dependent manner (10-12). Second, the natural phosphodiester bond CpG-DNA (PO-ODN) is a regulator of the immune response and an immunoadjuvant for the induction of Ag-driven Th1 responses without severe side effects. However, the PO-ODN is susceptible to nuclease activity, and the immunostimulatory activity of PO-ODN is relatively lower than that of PS-ODN (4).

Previously, we developed potent immunomodulatory PO-ODN from chromosomal DNA sequences of *M. bovis* and *E. coli* (15, 16). The PO-ODN, specifically MB-ODN 4531(O) containing immunomodulatory CpG motifs, regulates expression of Th1 cytokines and does not have severe side effects. We also found that the PO-ODN encapsulated in a phosphatidyl-β-oleoyl-γ-palmitoyl ethanolamine (DOPE): cholesterol hemisuccinate (CHEMS) complex (Lipoplex(O)) is able to enhance the stimulation of immune responses in the cells of humans and mice (15). Here, we investigated the effects of liposome-encapsulated PO-ODN or PS-ODN on TLR9-mediated signal transduction pathways and performed screening of B cell epitopes using several peptides and Lipoplex(O).

RESULTS AND DISCUSSION

Effect of PS-ODN encapsulated in liposomes on IL-8 promoter activation

Although investigators developed the natural PO-ODN to

overcome severe side effects of PS-ODN, the immunostimulatory activities of PO-ODN in human cells are limited (16, 17). To enhance the immunostimulatory activity of PO-ODN, several liposomes were applied as a vehicle for delivery. When PO-ODN was encapsulated in DOTAP, the uptake of PO-ODN and the production of IFN-α were enhanced in hPBMCs (18, 19). Previously, we screened a natural immunostimulatory PO-ODN from bacterial chromosomal DNA, but the PO-ODN was much less potent in inducing HLA-DRA and hBD-2 promoter activation in RPMI 8226 cells than in mice (13, 14, 20). However, PO-ODN encapsulated in DOTAP can elicit HLA-DRA and hBD-2 promoter activation and gene expression in RPMI 8226 cells in a CG sequence-dependent manner (20). We also compared the effects of liposome-encapsulated PO-ODN on the IL-8 promoter activation and cytokine production in mouse cells and human cells (15). After stimulation with PO-ODN encapsulated in liposomes such as DOTAP, the DOPE : CHEMS complex, and the DSPC : CHEMS : PEG-PE complex, the IL-8 promoter was significantly activated in both human cells and mouse cells. The most potent activation was produced by PO-ODN encapsulated in DOPE : CHEMS (1 : 1 ratio) complex. We refer to MB-ODN 4531(O) encapsulated in a DOPE : CHEMS) complex as Lipoplex(O). Lipoplex(O) induced the production of cytokines such as IL-6, IL-12, and IFN-γ in mouse splenocytes and hPBMCs, and also induced secretion of IL-12 and IFN-γ for much longer time periods than MB-ODN 4531(O) did by in vivo application, in a TLR9-dependent manner (15).

In this study, we determined the effects of liposome- encapsulated MB-ODN 4531(S) (PS-ODN) on IL-8 promoter activity

in a human B cell line (RPMI 8226) and the mouse macrophage cell line, RAW 264.7. The MB-ODN 4531(S) encapsulated in a DOPE : CHEMS complex (Lipoplex(S)) was more effective in eliciting IL-8 promoter activation than free MB-ODN 4531(S) in the human B cell line (Fig. 1A). In agreement with the results from PO-ODN, PS-ODN encapsulated in DOPE : CHEMS (1 : 1 ratio) complex produced the largest improvement of IL-8 promoter activation in human and mouse cells (Fig. 1B). Furthermore, Lipoplex(S) more potently activated the IL-8 promoter in human RPMI 8226 cells than did MB-ODN 4531(S) encapsulated in other liposomes such as DOTAP, lipofectamine, or 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine (DSPC) : CHEMS : phosphatidylethanolamine-poly(ethylene glycol) (PEG-PE) (6 : 4 : 0.3 ratio) complex. To investigate the contribution of CG dinucleotide sequences to the ability of Lipoplex(S) to activate IL-8 promoter, we synthesized MB-ODN 4531GC(S) in which one CG dinucleotide sequence was reversed to GC. When the cells were treated with MB-ODN 4531GC(S) encapsulated in a DOPE : CHEMS complex, and the IL-8 promoter activation was significantly reduced, but still observed in the mouse and human cell lines (Fig. 1B), showing the strong immunostimulatory effect of the phosphorothiate modification. Taken together, encapsulation with DOPE : CHEMS (1 : 1 ratio) complex greatly enhanced the immunostimulatory activity of CpG-DNA in human cell lines in a backbone modification-independent manner.

Enhanced interaction of CpG-DNA and TLR9 by encapsulation in a DOPE : CHEMS complex

Previously, we have shown that the Lipoplex(O) can elicit en-
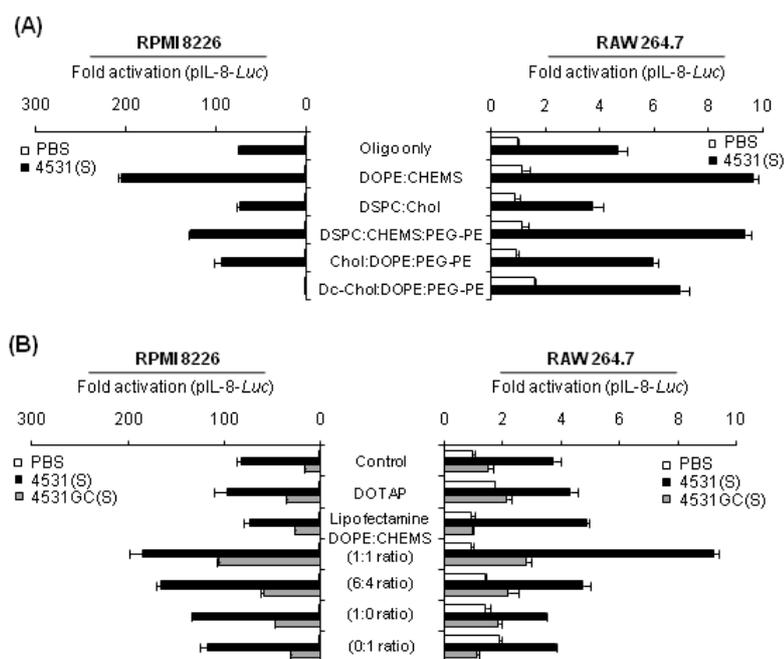
Fig. 1. Effect of PS-ODN encapsulated in different liposome complexes on IL-8 promoter activation. (A) The activation of IL-8 promoter enhanced by PS-ODN encapsulated in the liposome complex. (B) The significance of CpG motif in activation of IL-8 promoter induced by PS-ODN. RPMI 8226 cells and RAW 264.7 cells were treated with MB-ODN 4531(S) or MB-ODN 4531GC(S) encapsulated in the indicated liposome complex. The luciferase activity of the cells was measured in relative light units, and this value was normalized to Renilla activity. The results are represented as fold activation and compared to the PBS-treated control. Each bar represents Mean ± SD values of 3 individual experiments. (S), phosphorothioate backbone modification; 4531(S), MB-ODN 4531(S); 4531 GC(S), MB-ODN 4531GC(S).

hanced intracellular uptake, endosomal localization, and biological activity of CpG-DNA (15). As CpG-DNA-mediated immune activation was initiated by recognition of TLR9 (1), we decided to evaluate the effects of DOPE : CHEMS complex encapsulation on binding of CpG-DNA to TLR9 and established HEK 293 cells stably expressing TLR9. After the cells were incubated with biotin-labeled MB-ODN 4531(O) or MB-ODN 4531(S) for 1 h, immunoprecipitation assays were performed for identification of the TLR9 and CpG-DNA interaction using strepavidin-conjugated beads in the cell lysates (Fig. 2). When the HEK 293 cells were incubated with biotinylated-MB-ODN 4531(S), TLR9 was co-immunoprecipitated with MB-ODN 4531(S). A stronger band, indicating enhanced interaction of TLR9 and MB-ODN 4531(S), appeared in the Lipolex(S)-treated cells. Similar results were obtained when interaction of TLR9 and MB-ODN 4531(O) was analyzed. When the HEK 293 cells were incubated with Lipopex(O), binding of MB-ODN 4531(O) to TLR9 increased more than with non-encapsulated free MB-ODN 4531(O), but less than with MB-ODN 4531(S) or Lipoplex(S). These results show that encapsulation of CpG-DNA (PO-ODN and PS-ODN) in a DOPE:CHEMS complex increases the interaction with TLR9.

Effects of CpG-DNA encapsulated in a DOPE : CHEMS complex on MyD88- and NF-κB-mediated IL-8 promoter activation

It was well-known that myeloid differentiation protein (MyD88) is engaged in TLR9-mediated intracellular signaling. Interaction of TLR9 with CpG-DNA induces activation of the MyD88/IL-1R-associated kinase (IRAK) pathway, leading to nuclear localization of several transcription factors such as NF-κB for cytokine gene expression (7). Therefore, we used an expression plasmid that encodes a dominant negative version of MyD88 (△MyD88) and a mutant IκBα protein (IκBα Super Repressor, IκBαSR) which cannot be phosphorylated on serines 32 and 36 to investigate the involvement of MyD88 and NF-κB activation in Lipoplex(O)- or Lipoplex(S)-induced IL-8
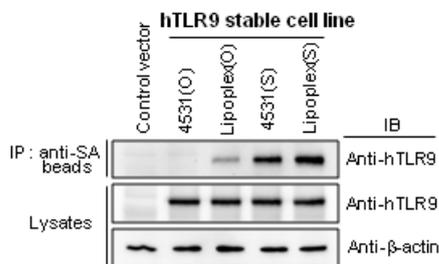
Fig. 2. Effect of DOPE : CHEMS encapsulation on CpG-DNA- TLR9 interaction. The immunocomplexes were prepared with streptavidin-conjugated beads (anti-SA beads) and the interaction of biotin-labeled CpG-DNA with hTLR9 was analyzed by immunoblotting with anti-hTLR9 antibody. 4531(O or S), biotin-labeled MB-ODN 4531(O or S); Lipoplex(O or S), biotin-labeled MB-ODN 4531(O or S) encapsulated in DOPE : CHEMS complex.
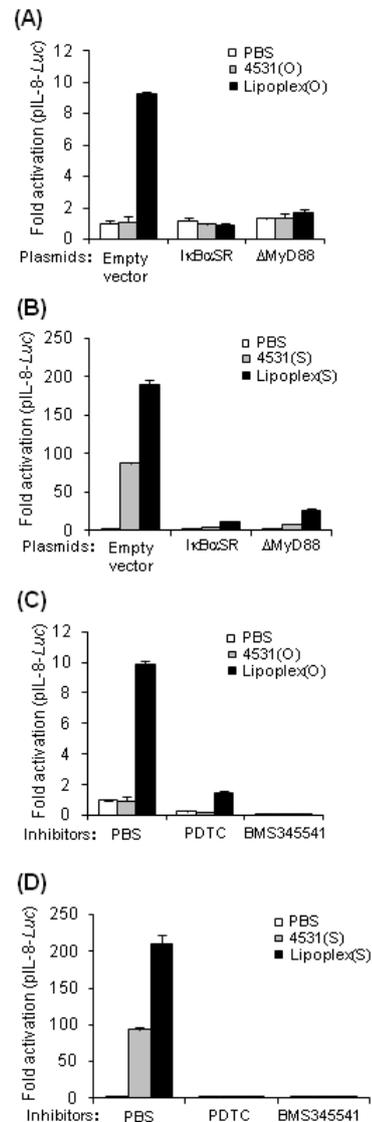
Fig. 3. Effect of CpG-DNA encapsulated in DOPE : CHEMS on TLR9/MyD88-mediated cellular activation. Typical MyD88-dependent NF-κB activation was required for IL-8 promoter activation by CpG-DNA encapsulated in DOPE:CHEMS complex in RPMI 8226 cells. (A, B) RPMI 8226 cells transfected with empty vector, IκBαSR expression vector, or △MyD88 expression vector were treated with 4531(O), Lipoplex(O), 4531(S) or Lipoplex(S) for 12 h. (C, D) RPMI 8226 cells were pretreated with PDTC or BMS345541 and then treated with 4531(O), Lipoplex(O), 4531(S), or Lipoplex(S) for 12 h. Luciferase activity was measured in relative light units, and the value was normalized to *Renilla* activity. The results are represented in terms of fold activation and are compared to the PBS-treated control. Each bar represents Mean ± SD values obtained from 3 individual experiments. Lipoplex, MB-ODN encapsulated in DOPE : CHEMS (1 : 1 ratio) complex; (O), phosphodiester backbone linkage; (S), phosphorothioate backbone modification; 4531(O), MB-ODN 4531(O); 4531(S), MB- ODN 4531(S); Lipoplex(O), MB-ODN 4531(O) encapsulated in a DOPE : CHEMS (1:1 ratio) complex; Lipoplex(S), MB-ODN 4531 (S) encapsulated in a DOPE : CHEMS (1 : 1 ratio) complex.

promoter activation (Fig. 3A and 3B). Cotransfection of △MyD88 or IκBαSR with the promoter-reporter construct markedly inhibited the IL-8 promoter activation in RPMI 8226 cells treated with Lipoplex(O) or Lipoplex(S). Furthermore, IL-8 promoter activation after stimulation with Lipoplex(O) or Lipoplex(S) was markedly decreased by the pretreatment with an IKK-2 inhibitor BMS-345541 or an NF-κB inhibitor PDTC (Fig. 3C and 3D). Based on these results, it is evident that typical MyD88-dependent NF-κB activation is also required for IL-8 promoter activation by CpG-DNA encapsulated in a DOPE : CHEMS complex in human cell lines in a backbone modification-independent manner.

IgG production by peptide epitope and CpG-DNA encapsulated in DOPE : CHEMS complex

Previously, we have shown the potent adjuvant effect of Lipoplex(O) on production of antibodies specific to B cell epitopes without carriers (15). Specifically, we successfully produced antibody against human hepatocellular carcinoma-specific transmembrane 4 superfamily member 5 (TM4SF5) protein by immunization with a complex of synthetic peptide and Lipoplex(O) without carriers. Therefore, we used this system for screening of B cell epitopes against proteins of infectious pathogens. Specifically, we aimed to screen B cell epitopes originating from envelope protein (E protein) of hepatitis C virus (HCV-E), attachment glycoprotein G and fusion protein F of the human respiratory syncytial virus (HRSV-G and HRSV-F). Candidate peptide sequences of pathogen were selected and synthesized on the basis of their hydrophilicity, hydrophobicity, secondary structure, antigenicity index, and amphipathicity.

We screened B cell epitopes of HCV E protein, HRSV G protein, and F protein by immunizing BALB/c mice with each peptide and Lipoplex(O). As a result, the mice showed a dramatic increase in the production of HCV-E2 202 peptide-specific IgG (Fig. 4A-C) and HRSV-G1 peptide-specific IgG (Fig. 4E and F). The production of IgG was significantly higher when the peptides and CpG-DNA were coencapsulated with DOPE : CHEMS complex than with other liposomes, indicating the prominent adjuvant activity of Lipoplex(O) (Fig. 4D). We also found that the complex of peptide and Lipoplex(O) induced significantly higher levels of IgG2a than IgG1, indicating a predominant Th1 response.

Taken together, these results confirm that Lipoplex(O) can be used as a universal adjuvant for peptide-based B cell epitope screening and antibody production through enhanced
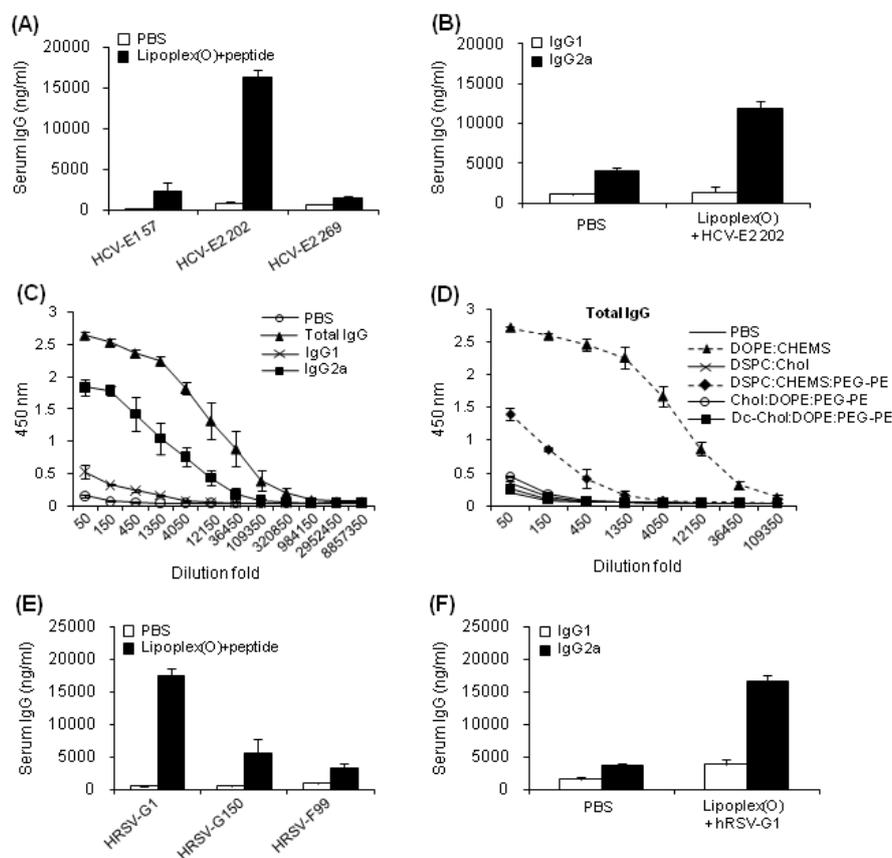
Fig. 4. Production of IgG by immunization with a complex of HCV-E2 (or RSV-G1) peptide and Lipoplex(O). (A) Selection of B cell epitopes from HCV- E2 protein by immunization with a complex of each predicted HCV-E2 peptide and Lipoplex(O). (B) Amounts of anti- HCV-E2 202 peptide-specific IgG isotypes. (C) The titers of anti-HCV-E2 202 peptide-specific IgG isotypes. (D) Production of DOPE : CHEMS encapsulation- dependent epitope-specific IgG. BALB/c mice (n = 5/group) were immunized with HCV-E2 202 (50 μg/mouse) and MB-ODN 4531(O) (50 μg/mouse) coencapsulated in one of the following complexes: DOPE : CHEMS (1 : 1 ratio), 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). The sera were collected, and titers of the anti-HCV-E2 202 peptide-specific total IgG were assayed with an ELISA kit. (E) Selection of B cell epitopes from HRSV F and G protein by immunization with a complex of each predicted HRSV peptide and Lipoplex (O). The sera were collected, and amounts of anti-HRSV-G1 peptide-specific IgG were assayed with an ELISA kit. (F) Amounts of anti-HRSV-G1 peptide- specific IgG isotypes.

TLR9 binding and MyD88- and NF-κB-mediated signal transduction. It also obviates the need for other adjuvants and carriers in production of antibody against peptide antigen. Our novel strategy using Lipoplex(O) might be extremely helpful for future applications in viral infectious diseases or cancers.

MATERIALS AND METHODS

ODNs and reagents
ODNs were synthesized by ST Pharm Co., Ltd (Seoul, Korea). The CpG-DNA sequences used in this study were either phosphodiester (O) or phosphorothioate-modified (S) versions of MB-ODN 4531 (14). The expression vectors for mutant IκBα protein (IκBαSR) and the mutant of MyD88 (△MyD88) were kindly provided by Dr. Harikrishna Nakshatri (Indiana University, USA) and Dr. Jurg Tschopp (University of Lausanne, Switzerland), respectively. When necessary, the cells were preincubated with BMS-345541 (50 μM) or pyrrolidine dithiocarbamate (PDTC, 200 μM) for 1 h before stimulation with CpG-DNA or liposome-encapsulated CpG-DNA.

Cell culture
The human B cell line RPMI 8226 and mouse macrophage cell line RAW 264.7 were maintained in RPMI 1640 medium containing 10% FBS (Hyclone, Logan, UT, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Viability was assayed by trypan blue dye exclusion.

Preparation of CpG-DNA co-encapsulated in liposome complexes
Liposome complexes consisting of CpG-DNA co-encapsulated with several liposome formulations were prepared as previously reported (15). Peptide and CpG-DNA co-encapsulated in liposome complexes were also prepared as previously described (15).

Transfection and luciferase assays
Transfection and luciferase assays were performed as previously described (15). After culturing RPMI 8226 cells and RAW 264.7 cells for 24 h in 12-well plates at a concentration of 2×10^5 cells per well, FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN, USA) was used to transfect the IL-8 promoter-reporter construct (pIL-8-*Luc*). The transfected cells were treated with CpG-DNA (5 μg/ml) or CpG-DNA encapsulated in liposomes for 12 h as indicated in the individual experiments.

Construction of recombinant human TLR9 expression vector and establishment of stable cell lines
hTLR9 cDNA was amplified from RPMI8226 cells by a reverse-transcription polymerase chain reaction (RT-PCR) using the following primer sets: 5' primer, 5'-CTAGCTAGCATGGG-TTTCTGCCGCAG-3'; 3' primer, 5'-GGATCCCTATTCGGCCG-TGGGTCCC-3'. The amplified PCR products were cloned into

pGEM-T easy vector (Promega, Madison, WI, USA) and then subcloned into the mammalian expression vector pcDNA-3.1/Myc-His(-)B (Invitrogen, Carlsbad, CA, USA) using the restriction enzyme sites *Nhe*I and *Bam*HI. For generation of hTLR9 stable cell lines, HEK293 cells were transfected with FuGENE 6 Transfection Reagent (Roche), after which the cells were selected in the presence of 500 μg/ml of G418 for 14 days. G418-resistant clones were selected and assayed for hTLR9 expression by immunoblotting with monoclonal antibody against hTLR9 (Cell Signaling Technology, Beverly, MA, USA).

Immunoprecipitation and immunoblotting
HEK 293 cells stably expressing hTLR9 were treated with 5 μg/ml of biotin-labeled CpG-DNA or biotin-labeled CpG-DNA encapsulated in DOPE : CHEMS (1 : 1 ratio) complex. After 30 min, the cells were harvested and cell lysates were incubated for 2 h at 4°C in a streptavidin-conjugated Protein A-Sepharose CL-4B (10% (v/v)) slurry (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Immunocomplexes collected by centrifugation were subjected to SDS-PAGE and Western blot analysis using anti-TLR9 antibody.

Selection and synthesis of B cell epitope peptides
The candidate epitope peptides of the HCV E protein (HCV-E1 57, [57]TRDGKLPTTQLRR[69]; HCV-E2 202, [202]CFRKHPEATYSR[213]; HCV-E2 269, [269]CDLEDRDRSELSP[281]), RSV G protein and F protein (HRSV-G1, [1]MSKHKNQRTARTLEKTWD[18]; HRSV-G150, [150]PRLKNPPKKPKDDY[163]; HRSV-F99, [99]NTPAANNRARREAPQYM[115]) were selected as previously described (15). The peptides were synthesized using an automated peptide synthesizer (Peptron III-R24, Peptron, Daejeon, Korea).

Mice and immunization
Mice were maintained under specific-pathogen-free conditions. We purchased 4-week-old male BALB/c (H-2[b]) mice from Central Lab. Animal, Inc. (Seoul, Korea). Our animal studies were approved by the Institutional Animal Care and Use Committee of Hallym University (Hallym 2009-47). On three occasions at 10 day intervals, the mice were injected intraperitoneally (i.p.) with 50 μg of peptides (50 μg) supplemented with either 50 μg of CpG-DNA or CpG-DNA encapsulated in liposomes.

Antigen-specific Ig ELISA assay
Mouse sera were obtained by orbital bleeding before each injection as well as by sacrifice 10 days after final injection. To measure the amounts and titers of total IgG, IgG1, and IgG2a, 96-well immunoplates were coated with 5 μg/ml of each peptide and then blocked with 0.05% of Tween-20 in PBS (PBST) containing 1% BSA. Total IgG, IgG1, and IgG2a levels were measured as previously described (15).

REFERENCES

1. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**, 740-745.

2. Krieg, A. M., Yi, A. K., Matson, S., Waldschmidt, T. J., Bishop, G. A., Teasdale, R., Koretzky, G. A. and Klinman, D. M. (1995) CpG motifs in bacterial DNA trigger direct B cell activation. *Nature* **374**, 546-549.

3. Ballas, Z. K., Rasmussen, W. L. and Krieg, A. M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* **157**, 1840-1845.

4. Krieg, A. M. (2002) CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* **20**, 709-760.

5. Klinman, D. M., Yi, A. K., Beaucage, S. L., Conover, J. and Krieg, A. M. (1996) CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon-γ. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 2879-2883.

6. Dong, L., Mori, I., Hossain, M. J., Liu, B. and Kimura, Y. (2003) An immunostimulatory oligodeoxynucleotide containing a cytidine-guanosine motif protects senescence-accelerated mice from lethal influenza virus by augmenting the T helper type 1 response. *J. Gen. Virol.* **84**, 1623-1628.

7. Lee, K. W., Lee, Y., Kim, D. S. and Kwon, H. J. (2006) Direct role of NF-κB activation in Toll-like receptor-triggered HLA-DRA expression. *Eur. J. Immunol.* **36**, 1254-1266.

8. Ballas, Z. K., Krieg, A. M., Warren, T. L., Rasmussen, W. L., Davis, H. L., Waldschmidt, M. and Weiner, G. J. (2001) Divergent therapeutic and immunologic effects of oligodeoxynucleotides with distinct CpG motifs. *J. Immunol.* **167**, 4878-4886.

9. Krieg, A. M. (2006) Therapeutic potential of Toll-like receptor 9 activation. *Nat. Rev. Drug. Discov.* **5**, 471-484.

10. Heikenwalder, M., Polymenidou, M., Junt, T., Sigurdson, C., Wagner, H., Akira, S., Zinkernagel, R. and Aguzzi, A. (2004) Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. *Nat. Med.* **10**, 187-192.

11. Deng, G. M., Nilsson, I. M., Verdrengh, M., Collins, L. V. and Tarkowski, A. (1999) Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. *Nat. Med.* **5**, 702-705.

12. Kim, D., Rhee, J. W., Kwon, S., Sohn, W. J., Lee, Y., Kim, D. W., Kim, D. S. and Kwon, H. J. (2009) Immunostimulation and anti-DNA antibody production by backbone modified CpG-DNA. *Biochem. Biophys. Res. Commun.* **379**, 362-367.

13. Choi, Y. J., Lee, K. W., Kwon, H. J. and Kim, D. S. (2006) Identification of immunostimulatory oligodeoxynucleotide from Escherichia coli genomic DNA. *J. Biochem. Mol. Biol.* **39**, 788-793.

14. Lee, K. W., Jung, J., Lee, Y., Kim, T. Y., Choi, S. Y., Park, J., Kim, D. S. and Kwon, H. J. (2006) Immunostimulatory oligodeoxynucleotide isolated from genome wide screening of Mycobacterium bovis chromosomal DNA. *Mol. Immunol.* **43**, 2107-2118.

15. Kim, D., Kwon, S., Rhee, J. W., Kim, K. D., Kim, Y. E, Park C. S., Choi, M. J., Suh, J. G., Kim, D. S., Lee, Y. and Kwon, H. J. (2011) Production of antibodies with peptide-CpG-DNA-liposome complex without carriers. *BMC Immunol.* **12**, 29.

16. Zhao, Q., Matson, S., Herrera, C. J., Fisher, E., Yu, H. and Krieg, A. M. (1993) Comparison of cellular binding and uptake of antisense phosphodiester, phosphorothioate, and mixed phosphorothioate and methylphosphonate oligonucleotides. *Antisense Res. Dev.* **3**, 53-66.

17. Zhao, Q., Waldschmidt, T., Fisher, E., Herrera, C. J. and Krieg, A. M. (1994) Stage-specific oligonucleotide uptake in murine bone marrow B-cell precursors. *Blood* **84**, 3660-3666.

18. Yasuda, K., Yu, P., Kirschning, C. J., Schlatter, B., Schmitz, F., Heit, A., Bauer, S., Hochrein, H. and Wagner, H. (2005) Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. *J. Immunol.* **174**, 6129-6136.

19. Magnusson, M., Tobes, R., Sancho, J. and Pareja, E. (2007) Natural DNA repetitive extragenic sequences from Gram-negative pathogens strongly stimulate TLR9. *J. Immunol.* **179**, 31-35.

20. Kim, D., Rhee, J. W., Kwon, S., Kim, Y. E., Choi, S. Y., Park, J., Lee, Y., and Kwon, H. J. (2010) Enhancement of immunomodulatory activity by liposome-encapsulated natural phosphodiester bond CpG-DNA in a human B cell line. *BMB Rep.* **43**, 250-256.