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Synergistic Stimulation of Antigen Presenting Cells via TLR by Combining CpG ODN and Poly(γ-glutamic acid)-Based Nanoparticles as Vaccine Adjuvants

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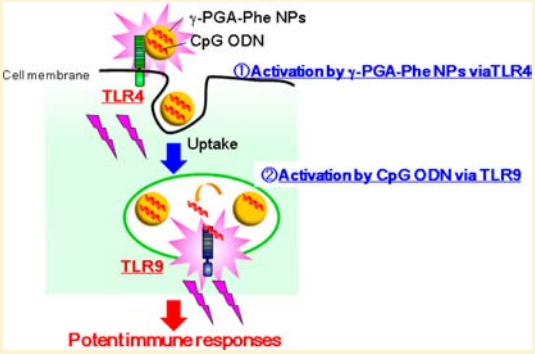
ABSTRACT: CpG oligodeoxynucleotide (ODN) encapsulated poly(γ-glutamic acid)-*graft*-L-phenylalanine ethyl ester (γ-PGA-Phe) nanoparticles (NPs) employing polycations were prepared to develop vaccine delivery and adjuvant systems. The CpG ODN was stably encapsulated into the NPs when protamine was used as the polycation. The CpG ODN-encapsulated γ-PGA-Phe NPs were taken up by macrophages and CpG ODN which was encapsulated into the NPs internalized into endo/lysosomes, where the toll-like receptor (TLR) 9, which recognizes CpG ODN, is expressed. The examination of release behavior *in vitro* revealed that the encapsulated CpG ODN into NPs was released when these NPs were immersed into the early endosomal environment. Interestingly, CpG ODN-encapsulated γ-PGA-Phe NPs synergistically activated macrophages. This may be due to the multiple stimulation of TLRs by γ-PGA-Phe NPs (TLR4 ligand) and CpG ODN (TLR9 ligand). We previously reported that γ-PGA-Phe NPs are excellent vaccine adjuvants for inducing potent innate and adaptive immune responses *via* TLR4. Moreover, coencapsulated CpG ODN and antigen in γ-PGA-Phe NPs induced potent antigen-specific cellular immunity at a higher level than the mixture of CpG ODN and antigen which is the conventional vaccine system. These findings suggest that the conjugation strategies of biologically derived adjuvant and polymeric NPs will aid the development of a novel approach for safe and effective vaccine delivery and adjuvant systems.



INTRODUCTION

Polymeric nanoparticles (NPs) formulated from biocompatible or biodegradable polymers have been studied extensively as carriers for the controlled delivery of different antigens including proteins, peptides, plasmid DNA (pDNA), and low molecular weight compounds. In particular, antigen-loaded NPs are being investigated as vaccine delivery carriers. These NPs are taken up by antigen presenting cells (APCs), particularly dendritic cells and macrophages, and then initiate immune responses.[1,2] The internalized antigens are degraded to peptides, and are presented on the surface of APCs by a major histocompatibility complex (MHC). Then naïve helper T cells bind the MHC through T cell receptors to eventually induce the differentiation of T cells into T helper 1 (Th1) and T helper 2 (Th2) cells, depending on what cytokines are induced.[3]

Recently, it has been revealed that the recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors called toll-like receptors (TLRs), which are distributed on/in the cells of the immune system, induces innate and adaptive immune responses.[4] To target and manipulate the immune responses, the vaccine adjuvants are coadministered with antigens and these adjuvants activate the APCs via TLRs. In particular, biologically derived oligodeoxynucleotides (ODNs) containing unmethylated CpG sequences (CpG ODNs) are known as an immune adjuvant that effectively induces Th1 responses.[5] The CpG ODN is recognized by TLR9 in endo/lysosome compartments.[6,7] The recognition of CpG ODN by TLR9 triggers the secretion of Th1-promoting cytokines, and finally induces Th1-biased cellular and humoral innate and adaptive immunity.[8] By taking advantage of this response, CpG ODN can be used to mediate protective immune responses to various infectious diseases, allergies, and cancers.[9] However, there are some drawbacks to CpG ODN therapy. CpG ODN is quickly eliminated by nuclease-mediated degradation under biological conditions.[10] A lack of specificity for target cells after systemic administration and poor cellular uptake also remain as challenges.[11] Therefore, it is important to deliver CpG ODN to the APCs while overcoming these undesirable interactions. On the other hand, some researchers have reported that the combination of multiple TLR ligands synergistically stimulated APCs and induced potent Th1 type immune responses.[12,13] To achieve both cellular uptake of CpG ODN by the target cells and the

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synergistic activation effect, a vaccine adjuvant system which possesses both antigen delivery and immune stimulating potentials is strongly required.

In a previous study, we prepared NPs composed of poly(γ-glutamic acid) (γ-PGA) as the hydrophilic backbone and L-phenylalanine ethyl ester (Phe) as the hydrophobic segment (γ-PGA-Phe) for the development of safe and effective nanoparticle-based vaccines.[14] The antigen encapsulated into the γ-PGA-Phe NPs was efficiently taken up by the APCs, and an antigen specific immune response was effectively induced.[15,16] It also revealed that the γ-PGA-Phe NPs themselves possess adjuvant effects and can stimulate APCs via TLR4 expressed on the cell surface.[17] Moreover, the γ-PGA-Phe NPs could also encapsulate CpG ODN by employing cationic agents, which suggests that γ-PGA-Phe NPs can be used as an efficient carrier of oligonucleotide adjuvants.[18] These results suggest that the conjugation of adjuvant and γ-PGA-Phe NPs should be a promising strategy to induce potent immune responses. In this study, we determined the activation properties of CpG ODN-encapsulated γ-PGA-Phe NPs (CpG ODN-NPs). The APCs were treated with CpG ODN-NPs, and their activation was measured. We also investigated the cellular uptake behavior and intracellular distribution of CpG ODN-NPs. To determine their potential for vaccine delivery and adjuvant systems, CpG ODN- and protein-encapsulated γ-PGA-Phe NPs were prepared and the induction of antigen-specific immune response was evaluated. As γ-PGA-Phe NPs themselves possess potent adjuvant effects, our study revealed that the synergistic enhancement of adjuvant effects and immune responses were achieved by combining these NPs with CpG ODN and antigens, which will be a promising candidate for safe and effective vaccine delivery and immune stimulating systems.

EXPERIMENTAL SECTION

Preparation of CpG ODN Encapsulated γ-PGA-Phe Nanoparticles. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. γ-PGA (number-average molecular weight; M_n = 3.8 × 10^5, D/L = 6:4) was kindly provided by Meiji Seika Co. Ltd., Tokyo, Japan. γ-PGA was hydrophobically modified by L-phenylalanine ethylester (Phe) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Wako Pure Chemical Industries, Osaka Japan).[19] γ-PGA-*graft*-Phe (γ-PGA-Phe) with a 50% Phe grafting degree was used in the following experiments. In this study, CpG ODN 1826, which is the TLR9 ligand, was used. To prepare the CpG ODN encapsulated γ-PGA-Phe nanoparticles (CpG ODN-NPs), two biocompatible polycations were employed, poly(ε-lysine) (ε-PL) (M_w = 4.7 × 10^3), and protamine (M_w = 4.3 × 10^3). Each polycation solution (37−300 μg/mL) was mixed with a FITC-labeled CpG ODN (FITC-CpG ODN) (InvivoGen, San Diego, USA) solution (50 μg/mL in 0.1 M NaCl) to prepare a polyplex, and γ-PGA-Phe (10 mg/mL in dimethyl sulfoxide; DMSO) was added to the same volume of polyplex solution.[18] The CpG ODN solution and each polycation solution were mixed at a N/P molar ratio of 8. The charge ratio (N/P) was expressed as the ratio of the amino groups (N) on the polycations and the phosphate groups (P) on the CpG ODN. The resulting solution was centrifuged at 1.4 × 10^4 rpm for 10 min and repeatedly rinsed to remove unencapsulated polyplex, and then dispersed with phosphate-buffered saline (PBS). The final concentration of NPs was 10 mg/mL. The size of the CpG ODN-NPs was measured by dynamic light scattering (DLS) method using a Zetasizer Nano ZS (Malvern Instruments, UK), and the amount of CpG ODN encapsulated into the NPs was then measured by fluorescence spectrometry.

Evaluation of the Stability of CpG ODN Encapsulated γ-PGA-Phe Nanoparticles. FITC-CpG ODN was used to evaluate the stability of CpG ODN-encapsulated nanoparticles. To determine the release behavior of CpG ODN from the γ-PGA-Phe NPs in culture medium, FITC-CpG ODN-encapsulated NPs (FITC-CpG ODN-NPs) were immersed into medium containing 10% fetal bovine serum (FBS). The amount of CpG ODN released into the medium was measured by the fluorescence intensity.

Cell Culture. Mouse leukemic monocytes, RAW264 cells (Riken BRC Cell Bank, Japan), were used in the present study. The RAW264 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries, Japan) containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The cells were maintained in 5% CO_2 at 37 °C.

Uptake of CpG ODN-Encapsulated Nanoparticles by RAW264 Cells. FITC-CpG ODN was used to evaluate the uptake of CpG ODN-encapsulated nanoparticles by RAW264 cells. To determine the uptake of CpG ODN-NPs, the cells were incubated with FITC-CpG ODN-NPs for 1 h at 37 °C. FITC-CpG ODN-NPs containing 1.5 μg, 300 ng, and 150 ng of CpG ODN per 1 mg of NPs were used. The concentration of NPs and CpG ODN in the medium was 100 μg/mL and 15−150 ng/mL, respectively. The cells were then washed thoroughly with PBS, and were fixed in 10% formalin for 30 min at room temperature. The uptake of FITC-CpG ODN was then measured by flow cytometry (Guava easyCyte 8HT; Millipore, Billerica, MA, USA).

Stimulation of RAW264 Cells by CpG ODN Encapsulated γ-PGA-Phe Nanoparticles. CpG ODN 1826 (InvivoGen, San Diego, USA) was used to evaluate the activation properties of CpG ODN-NPs on RAW264 cells. RAW264 cells were seeded into 96-well plates at 1 × 10^4 cells/well, and then incubated for 24 h. The culture medium was then replaced by a medium containing the CpG ODN alone, the polyplex, or CpG ODN-NPs, and incubated for 24 h at 37 °C. CpG ODN-NPs containing 1.5 μg, 300 ng, and 150 ng of CpG ODN per 1 mg of NPs were used. The concentration of NPs was 100 μg/mL. To analyze the cytokine production, the supernatants were collected and the amount of secreted tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12 were measured by enzyme-linked immunosorbent assay (ELISA).

Intracellular Distribution of CpG ODN-Encapsulated Nanoparticles. FITC-CpG ODN was employed to determine the intracellular localization of the CpG ODN-encapsulated nanoparticles. RAW264 cells (1 × 10^5 cells/well) were incubated with 200 nm FITC-CpG ODN-NPs (FITC-CpG ODN 150 ng/mL, NPs 100 μg/mL) for 1 h at 37 °C. The cells were washed with PBS, and then stained with 100 nM Lyso Tracker Red (Molecular Probes, Eugene, OR) for 1 h at 37 °C to label the endosomes and lysosomes. Subsequently, the cells were washed and fixed in 10% formalin for 30 min at room temperature, and then the cells were washed with PBS. The nuclei were then stained with diamidino-2-phenylindole (DAPI). The intracellular distribution of FITC-CpG ODN was then observed using a confocal fluorescence microscope (CFM) (DSU-IX81-SET fluorescence microscope, Olympus, Japan).

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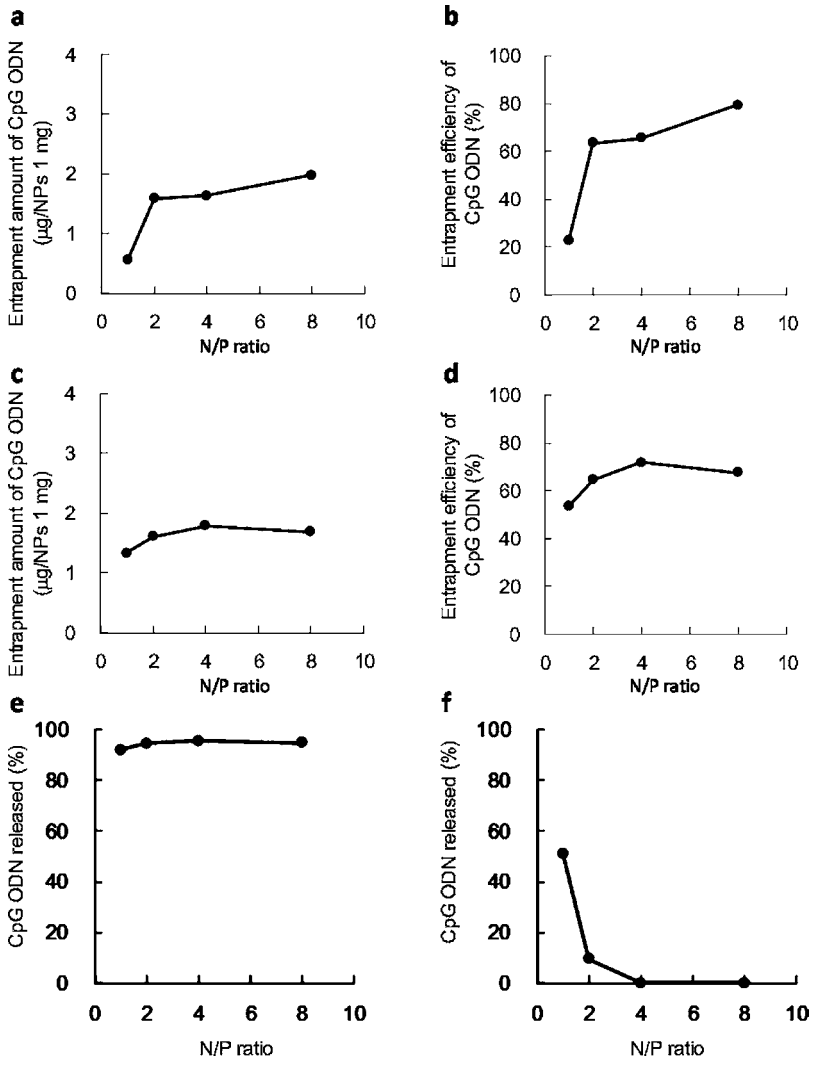

Figure 1. Effect of the N/P ratio on the entrapment behavior and stability of CpG ODN encapsulated into γ-PGA-Phe NPs in the presence of polycations. (a,c) The entrapment amount and (b,d) the efficiency of CpG ODN loaded into γ-PGA-Phe NPs in the presence of (a,b) ε-PL or (c,d) protamine were measured by fluorescence intensity. The release behavior of CpG ODN encapsulated into γ-PGA-Phe NPs in the presence of (e) ε-PL or (f) protamine was measured by fluorescence intensity.

Preparation of CpG ODN- and Protein-Encapsulated γ-PGA-Phe Nanoparticles. For the preparation of CpG ODN- and protein-encapsulated γ-PGA-Phe NPs, ovalbumin (OVA) was employed. A solution containing protamine (300 μg/mL) or FITC-CpG ODN (50 ng/mL) was mixed at the same volume to form a polyplex, followed by the addition of OVA solution (875 μg/mL). γ-PGA-Phe (10 mg/mL in DMSO) was added to the same volume of the solution containing OVA and polyplex to form CpG ODN- and OVA-encapsulated γ-PGA-Phe NPs (CpG-OVA-NPs). The N/P molar ratio was fixed at 8. The resulting solution was centrifuged at 1.4×10^4 rpm for 10 min and repeatedly rinsed to remove unencapsulated polyplex, and then dispersed with PBS. The final concentration of NPs was 10 mg/mL. The size of the CpG ODN-NPs was measured by the DLS method, and the amounts of CpG ODN and OVA encapsulated into the NPs were then measured by fluorescence spectrometry and the Lowry method, respectively.

Mice. Female C57BL/6J (H-2K^b, 6−8 weeks of age) mice were purchased from Charles River (Yokohama, Japan). Experiments were approved by Osaka University and carried out in accordance with its guidelines for animal experimentation.

Immunization of Mice. The mice were anesthetized by an intraperitoneal injection of sodium pentobarbital and immunized with CpG-OVA-NPs. The mice (3 mice in each group) were immunized subcutaneously once with PBS, OVA alone, OVA-encapsulated γ-PGA-Phe NPs (OVA-NPs), CpG-OVA-NPs, a mixture of OVA with CpG ODN (OVA + CpG), or a mixture of OVA with CpG ODN and γ-PGA-Phe NPs (OVA + CpG + NPs). The injected amounts of OVA, CpG ODN, and γ-PGA-Phe NPs were fixed to 10 μg, 50 ng, and 500 μg per shot, respectively. On day 10 after immunization, spleen cells were collected. The spleen cells collected from each group of mice were pooled and the immune response was evaluated.

Enzyme-Linked Immunospot (ELISPOT) Assay. Interferon (IFN)-γ-producing cells were determined using an ELISPOT kit for mouse IFN-γ (BD Biosciences). The lymphocyte cells were isolated by density-gradient centrifugation. These cells (2×10^5 cells/well) were then stimulated with the $OVA_{257-264}$ peptide (10 μg/mL) in a 96-well ELISPOT plate. The plate was incubated for 24 h at 37 °C, washed, and

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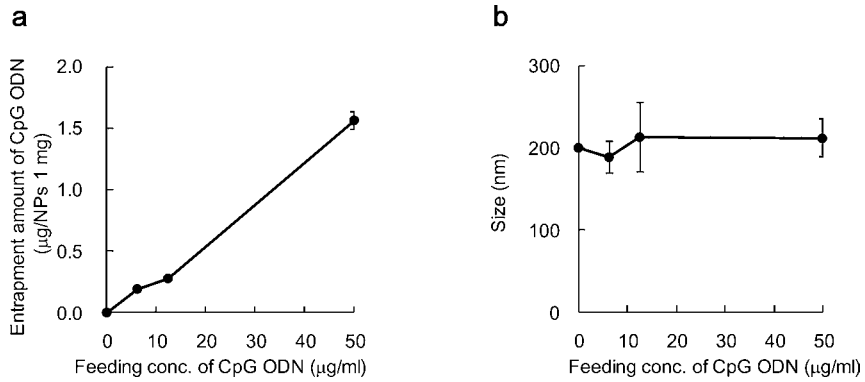

Figure 2. (a) Effect of the CpG ODN/protamine concentration on the amount of CpG ODN encapsulated into the NPs. (b) The sizes of CpG ODN-NPs prepared at various feeding concentrations of CpG ODN. The N/P ratio was 8.

further incubated with a biotin-conjugated detection antibody for 2 h at room temperature. After washing, the plate was incubated with streptavidin-conjugated horseradish peroxidase and incubated for 1 h. The plate was washed again and incubated with the substrate for 15 min. The colorimetric reaction was terminated by washing with water. After drying the plate, the number of spots was counted microscopically.

RESULTS AND DISCUSSION

Characterization of CpG ODN Encapsulated γ-PGA-Phe Nanoparticles. To prepare the CpG ODN-NPs, two biocompatible polycations were employed, ε-PL and protamine. Since these polycations are safe, biocompatible, and biodegradable, they are employed in regular clinical use, as food additives, and so on.[20,21] The amount of encapsulated CpG ODN in the NPs and the efficiency were increased according to the increase in the N/P ratios (Figure 1a–d). These findings revealed that γ-PGA-Phe could efficiently encapsulate CpG ODN by controlling the N/P ratios. When the CpG ODN-NPs were immersed into culture medium and incubated for 10 min, the encapsulated CpG ODN was immediately released at lower N/P ratios. In particular, when ε-PL was used to encapsulate CpG ODN, the encapsulated CpG ODN was almost completely released from the NPs. On the other hand, the amount released was drastically reduced when protamine was employed as a polycation (Figure 1e,f). It is known that protamine binds to DNA and provides a highly compact configuration of chromatin in the nucleus of sperm.[21] Therefore, this suggests that protamine formed a stable polyplex with CpG ODN compared with ε-PL, and therefore the leakage of CpG ODN from the NPs was suppressed, even when they were incubated in culture medium. According to these results, we employed protamine to encapsulate the CpG ODN in the NPs, and the N/P ratio was set at 8. Under these conditions, the amount of encapsulated CpG ODN was increased as the feeding amount of CpG ODN was increased (Figure 2a). In contrast, the size of the CpG ODN-NPs was about 200 nm under all conditions (Figure 2b). Each condition of the CpG ODN-NPs showed a negative zeta potential (about −20 mV) due to the ionization of the carboxyl groups of the γ-PGA located near the surface (data not shown). The size of the NPs which encapsulated the CpG ODN was the same as the γ-PGA-Phe NPs, which suggested that the encapsulation of the CpG ODN did not change the size of the NPs.

Uptake of CpG ODN-Encapsulated γ-PGA-Phe Nanoparticles by RAW264 Cells. To evaluate the uptake behavior of CpG ODN-NPs by macrophages, RAW264 cells were incubated with FITC-CpG ODN-NPs, polyplex, or FITC-CpG ODN alone for 1 h at 37 °C. The fluorescence intensity obtained by flow cytometry demonstrated that each sample was taken up into the cells (Figure 3). The amount of FITC-CpG

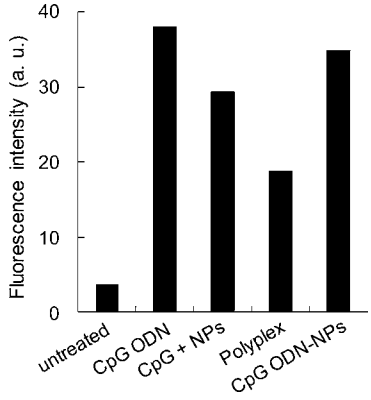

Figure 3. Cellular uptake of CpG ODN-NPs by RAW264 cells. The cells were incubated with CpG ODN, a mixture of CpG ODN and γ-PGA-Phe NPs (CpG + NPs), polyplex, or CpG ODN-NPs. The uptake amount was measured by flow cytometry. The N/P ratio was 8.

ODN taken up by the RAW264 cells, which was encapsulated into the NPs, was almost the same as the FITC-CpG ODN alone. Furthermore, the cellular uptake was measured at CpG ODN concentrations of 15 ng/mL and 30 ng/mL, and similar results were obtained at all conditions (data not shown). These findings indicated that the CpG ODN encapsulated into the γ-PGA-Phe NPs was taken up by the cells without any inhibition, and that γ-PGA-Phe NPs can be a promising carrier of CpG ODN adjuvant. Although the amount of CpG ODN, which was mixed with γ-PGA-Phe NPs, taken up by the RAW264 cells was slightly low as compared with CpG ODN alone, this may be due to the competition of uptake of CpG ODN and γ-PGA-Phe NPs. As it is known that particulate compounds are easily taken up by the APCs compared with soluble ones, γ-PGA-Phe NPs were more efficiently taken up by the cells than CpG ODN and the amount of uptake of CpG ODN decreased. On the other hand, the amount of polyplex taken up by the RAW264 cells was low as compared with CpG ODN-NPs and CpG ODN alone; this may be due to the stability of the polyplex in the medium. When the size of the polyplex in the medium was measured by the DLS method, it revealed relatively high aggregation. This suggested that the polyplex (N/P ratio 8) was

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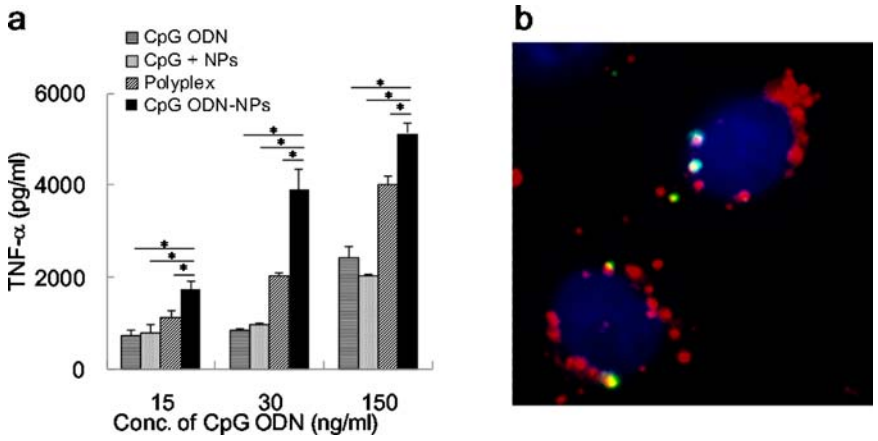

Figure 4. (a) Secretion of TNF-α by RAW264 cells. The cells were stimulated with CpG ODN, CpG + NPs, polyplex, or CpG ODN-NPs. The N/P ratio was 8. *$P < 0.01$. (b) Intracellular distribution of FITC-CpG ODN-NPs in RAW264 cells. The cells were incubated with FITC-CpG ODN-NPs for 1 h at 37 °C. The endo/lysosomes and nuclei were stained with Lyso Tracker Red (red) and DAPI (blue), respectively. The intracellular distribution of FITC-CpG ODN was observed using a CFM. The colocalization of FITC-CpG ODN (green) and the endo/lysosomes (red) appeared as yellow.

unstable in the medium because of the low electric interactions between the protamine and CpG ODN, and therefore the amount of polyplex taken up by the cells decreased.

Activation of RAW264 Cells by CpG ODN-Encapsulated γ-PGA-Phe Nanoparticles. It is known that when the APCs are stimulated by pathogens or TLR ligands like CpG ODN, they release many proinflammatory cytokines such as TNF-α and ILs and immune responses are induced.[22] To evaluate the activation effect of CpG ODN-NPs, RAW264 cells were incubated with the polyplex, CpG ODN-NPs, or CpG ODN alone, and the amount of TNF-α, IL-6, and IL-12 secreted was measured. The amount of IL-6 and IL-12 secreted by RAW264 cells was identical with control at these CpG ODN concentrations (data not shown). When CpG ODN-NPs were exposed to the cells, the amount of TNF-α secreted into the supernatant was the highest as compared with the mixture of CpG ODN and γ-PGA-Phe NPs, polyplex, or CpG ODN alone (Figure 4a). Moreover, the activation of these cells was dose-dependent with each sample. However, the amount of cellular uptake of CpG ODN was almost the same between CpG ODN alone and CpG ODN-NPs *in vitro* (Figure 3). Taken together, the highest activation effect of CpG ODN-NPs may be due to the synergistic stimulation of both TLR4 and TLR9. Some researchers have reported that the combination of multiple TLR ligands synergistically stimulated APCs and induced potent Th1 type immune responses.[12,13] In a previous study, we demonstrated that γ-PGA-Phe NPs can induce potent innate and adaptive immune responses through TLR4, which is expressed on the surface of the cell membrane.[17,23] When the RAW264 cells were stimulated with 100 μg/mL γ-PGA-Phe NPs, they secreted TNF-α (661 ± 146 pg/mL), and this activation behavior was dose-dependent, whereas unmodified γ-PGA did not show such behavior (data not shown). In this study, we encapsulated the CpG ODN (TLR9 ligand) into γ-PGA-Phe NPs (TLR4 ligand). Therefore, these NPs may synergistically activate the APCs by interacting with both TLR4 and TLR9, although the activation property of RAW264 cells by the mixture of CpG ODN and γ-PGA-Phe NPs was almost the same as CpG ODN alone. This may be due to the low efficiency of the cellular uptake of both CpG ODN and γ-PGA-Phe NPs by the same cells, and therefore the synergistic activation could not be induced. Furthermore, we investigated the cytotoxicity of CpG ODN-NPs and they did not show any appreciable cytotoxicity up to concentrations of NPs, 1 mg/mL; and CpG ODN, 1.5 μg/mL. This suggests that these NPs may be safe and effective adjuvants, and are superior to conventional adjuvant systems.

Activation Mechanism of RAW264 Cells by CpG ODN Encapsulated into γ-PGA-Phe Nanoparticles. To determine the activation mechanism of RAW264 cells by CpG ODN-NPs, the intracellular distribution of CpG ODN was investigated. FITC-CpG ODN-NPs were incubated with RAW264, cells and the localization of the FITC-CpG ODN was visualized by staining the RAW264 cells with Lyso Tracker Red, which accumulates in endo/lysosomes. As shown in Figure 4b, the FITC-CpG ODN loaded into the γ-PGA-Phe NPs was mostly detected along with the endo/lysosomes. Colocalization signals (yellow) of the FITC-CpG ODN (green) and endo/lysosomes (red) were observed inside the cells. It is known that TLR9 is expressed within endo/lysosomes, and detects viral nucleic acid and unmethylated CpG sequences.[7] Therefore, it is important to deliver the CpG ODN adjuvant to the endo/lysosomes. In this study, we demonstrated that the CpG ODN encapsulated in NPs was internalized into the endo/lysosomes. Moreover, Honda et al. suggested that the internalization of TLR9 ligands in early endosomes led to a more effective immune response than in late endo/lysosomes.[24] Therefore, to stimulate TLR9 with CpG ODN effectively, the encapsulated CpG ODN must be released from the NPs when it is internalized into the early endosomes. When the CpG ODN-NPs were immersed for 1 h into the PBS buffers which were mimicking the early endosomes (10 mM PBS, pH 6.9, and 20 mM NaCl),[25] 40% of the encapsulated CpG ODN was released from the NPs. This indicated that after the CpG ODN-NPs were taken up by RAW264 cells, the encapsulated CpG ODN was released into the early endosomes and stimulated TLR9, which then led to the secretion of cytokines. The detailed mechanism of the synergistic activation of the APCs by CpG ODN-NPs still remains to be elucidated. Further investigation is in progress. On the other hand, it has been demonstrated that the coencapsulation of the antigen and the adjuvant into NPs leads to efficient uptake and activation of the APCs as compared to APCs fed with a mixture of antigens and adjuvants.[26] In our previous study, we showed that γ-PGA-

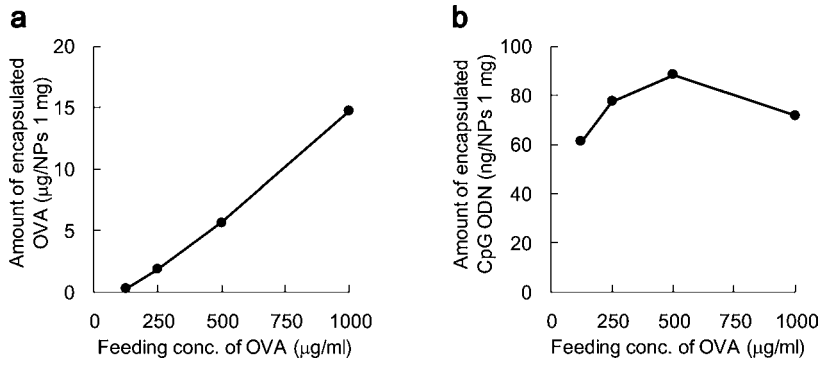

Figure 5. Effect of the OVA concentration on the amount of (a) OVA- and (b) CpG ODN-encapsulated into the NPs. The N/P ratio was 8.

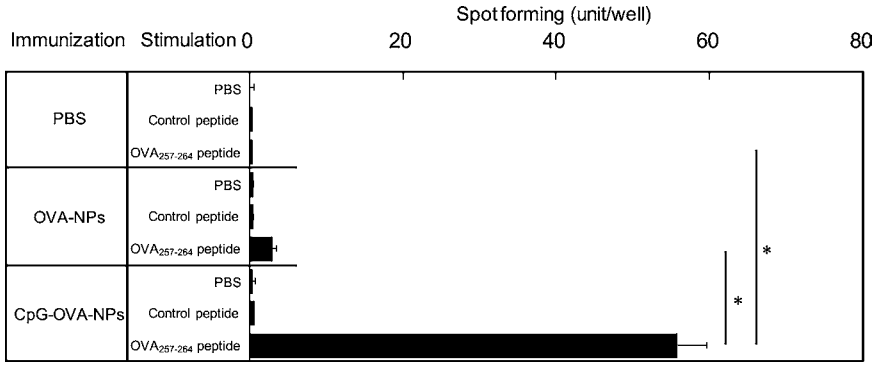

Figure 6. Antigen-specific IFN-γ producing T cells induced by OVA-NPs or CpG-OVA-NPs Spleen cells (2×10^5 cells/well) of mice immunized with the indicated samples were stimulated with no peptide (PBS), control peptide, or the $OVA_{257-264}$ peptide (10 μg/mL) and evaluated for their INF-γ production by ELISPOT. Data are expressed as the number of IFN-γ positive spots per well. The data represent means ± SD in each group (n = 3). *p < 0.01.

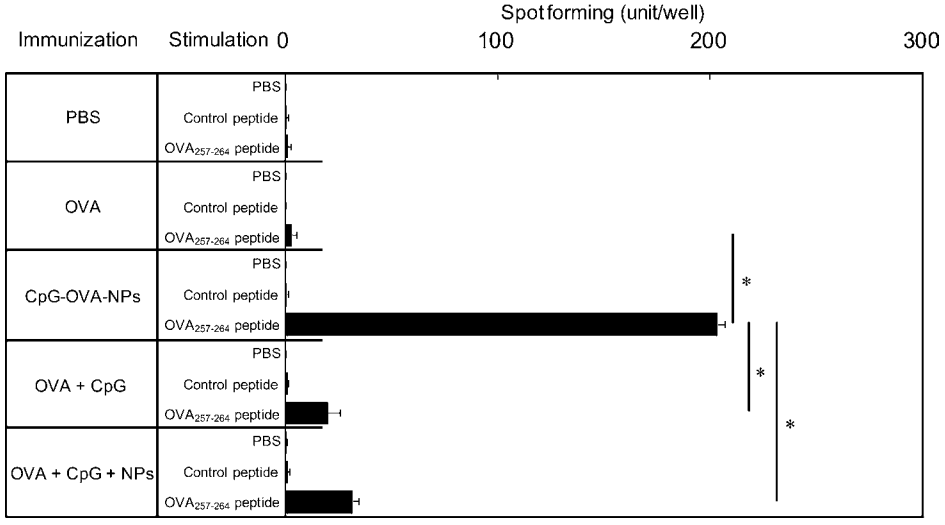

Figure 7. Antigen-specific IFN-γ producing T cells induced by CpG-OVA-NPs Spleen cells (2×10^5 cells/well) of mice immunized with the indicated samples were stimulated with no peptide (PBS), control peptide, or the $OVA_{257-264}$ peptide (10 μg/mL) and evaluated for their INF-γ production by ELISPOT. Data are expressed as the number of IFN-γ positive spots per well. The data represent means ± SD in each group (n = 3). *p < 0.01.

Phe NPs could efficiently encapsulate antigens.[15] Therefore, we hypothesized that the coencapsulation of the antigen and CpG ODN into the γ-PGA-Phe NPs may enable them to be efficiently taken up by APCs, and should lead to the effective activation of these cells.

Characterization of CpG ODN- and Protein-Encapsulated γ-PGA-Phe Nanoparticles. To clarify that this adjuvant system can lead potent antigen-specific immune responses, we evaluated whether γ-PGA-Phe NPs can encapsulate both CpG ODN and antigen. In this study, OVA was used for the model antigen. FITC-CpG ODN was mixed with protamine (N/P ratio 8) and a polyplex was formed. Then OVA was added, followed by the addition of γ-PGA-Phe to form CpG ODN- and OVA-encapsulated γ-PGA-Phe NPs (CpG-OVA-NPs).

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The amounts of encapsulated CpG ODN and OVA were evaluated by fluorescence intensity and the Lowry method, respectively. As shown in Figure 5, it was demonstrated that γ-PGA-Phe NPs could encapsulate both CpG ODN and OVA at the same time. The encapsulated amount of OVA increased (0.25−15 μg/NPs 1 mg) as the amount of OVA fed increased (125−1000 μg/mL). On the other hand, the amount of CpG ODN encapsulated into the γ-PGA-Phe NPs only changed slightly (61−88 ng/NPs 1 mg) with the amount of OVA fed. For the evaluation of the cellular uptake of CpG-OVA-NPs, RAW264 cells were incubated with CpG-OVA-NPs and uptake behavior was measured by flow cytometry. The cellular uptake of CpG ODN and OVA encapsulated into γ-PGA-Phe NPs showed almost the same behavior as CpG ODN-NPs and OVA-NPs, respectively (data not shown). These findings suggest that the encapsulation of neither CpG ODN nor OVA affected the interaction between CpG-OVA-NPs and cells.

Induction of Antigen-Specific Immune Response by CpG ODN- and Protein-Encapsulated γ-PGA-Phe NPs. The immune stimulatory potential of CpG-OVA-NPs was evaluated by INF-γ ELISPOT assay. The lymphocytes isolated from the spleens were stimulated with $OVA_{257-264}$ CTL epitope peptide and $OVA_{257-264}$-specific INF-γ-producing cells were determined. As shown in Figure 6, $OVA_{257-264}$-specific INF-γ-producing cells were not observed in the spleen cells, when mice were immunized with PBS. A number of $OVA_{257-264}$-specific IFN-γ-producing cells were identified in the mice immunized with OVA-NPs and CpG-OVA-NPs. As expected, the CpG-OVA-NPs induced about 18-fold more IFN-γ-producing cells compared with OVA-NPs.

To confirm that the effective OVA specific-immune response was due to the combination of CpG ODN, OVA, and γ-PGA-Phe NPs, the mice were immunized with OVA, OVA + CpG ODN, OVA + CpG ODN + γ-PGA-Phe NPs, and CpG-OVA-NPs. The lymphocytes were then isolated from the spleen, stimulated with $OVA_{257-264}$ CTL epitope peptide, and $OVA_{257-264}$-specific IFN-γ cells were evaluated. A number of IFN-γ-producing cells were identified in the mice immunized with OVA, OVA + CpG ODN, OVA + CpG ODN + γ-PGA-Phe NPs, and CpG-OVA-NPs. (Figure 7). CpG-OVA-NPs induced a higher number of IFN-γ-producing cells than the mixture of OVA and CpG ODN, or OVA, CpG ODN, and γ-PGA-Phe NPs. Surprisingly CpG-OVA-NPs induced about 10 times higher antigen-specific immune response than the mixture of OVA and CpG ODN, which is the conventional vaccine system. Therefore, it was shown that the antigen- and adjuvant-encapsulated γ-PGA-Phe NPs can serve as a novel nanoparticle-based vaccine adjuvant system. Although the detailed mechanism responsible for the effective induction of antigen-specific immune response by CpG-OVA-NPs still remains unclear, it may be due to the more efficient delivery by γ-PGA-Phe NPs of both CpG ODN and OVA to the APCs. In a previous study, we revealed that γ-PGA-Phe NPs delivered antigens to the APCs and induced potent antigen-specific immune responses.[16] On the other hand, naïve helper T cells differentiate into Th1 and Th2, when stimulated with cognate antigens by APCs. Th1 cells secrete IFN-γ and mainly promote cellular immunity.[3] CpG ODN has been known to induce Th1-promoting cytokines, and induces Th1-biased immune responses.[8] However, easy degradation of CpG ODN and nonspecificity for target cells still remain challenges. In this study, we demonstrated that γ-PGA-Phe NPs could encapsulate both CpG ODN and OVA. These CpG-OVA-NPs efficiently induced antigen-specific IFN-γ-producing cells. Taken together, these effects may be due to the protection of CpG ODN from degradation in biological conditions and specific delivery to APCs.

Recently some researchers have reported that the combination of multiple TLR ligands synergistically stimulated APCs and induced potent Th1 type immune responses.[12,13] Sokolova et al. reported that the combination of TLR ligands and antigen induced DC maturation and T cell activation.[26] It is known that CpG ODN is recognized by TLR9 in endo/lysosomes.[6,7] We also revealed that γ-PGA-Phe NPs stimulated DCs via TLR4.[17] According to these findings, it is suggested that CpG-OVA-NPs might stimulate APCs via TLR4 (γ-PGA-Phe NPs) and TLR9 (CpG ODN) in the same cell, resulting in the efficient induction of antigen-specific cellular immunity.

In conclusion, we prepared CpG ODN encapsulated γ-PGA-Phe NPs using polycations. The CpG ODN was stably loaded into the NPs when protamine was used as the polycation. The CpG ODN encapsulated into the NPs was taken up by macrophages, and localized to the endo/lysosomes. Moreover, CpG ODN- and OVA-encapsulated γ-PGA-Phe NPs induced potent OVA-specific immune responses as compared with the conventional vaccine system (mixture of antigen and CpG ODN). These results may be due to the synergistic stimulation of the APCs via TLR4 (γ-PGA-Phe NPs) and TLR9 (CpG ODN). The different TLR ligands of CpG ODN and NPs cooperated in activation of APCs and induction of immune responses. In the conventional vaccine adjuvant system, aluminum salts (Alum) and 3-*O*-desacyl-4′-monophosphoryl lipid A (MPLA) adsorbed with Alum have been licensed for clinical use in humans. Our findings clearly demonstrate that potent immune stimulation and immune responses were achieved by conjugating polymeric NPs (γ-PGA-Phe NPs) and biologically derived adjuvant (CpG ODN) due to the efficient delivery of both antigen and CpG ODN by γ-PGA-Phe NPs and the synergistic immune stimulating properties of γ-PGA-Phe NPs and CpG ODN. The strategies of synergistic stimulation of APCs via selected TLRs and codelivery of antigens and adjuvants to the same APCs by the conjugation of adjuvant and the NPs will aid in the development of novel approaches for safe and effective vaccine delivery and immune stimulating systems.

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Notes

The authors declare no competing financial interest.

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