

Potent Antigen-specific Immune Responses Stimulated by Codelivery of CpG ODN and Antigens in Degradable Microparticles

Xue-Qing Zhang,* Christopher E. Dahle,† Nicki K. Baman,‡ Nathan Rich,† George J. Weiner,† and Aliasger K. Salem*†‡

Summary: CpG ODN stimulates a T_H1 response through its receptor Toll-like receptor 9 (TLR9). TLR9 is a receptor that is found intracellularly. Microparticles are efficiently internalized by dendritic cells (DCs) and macrophages and would thus be an ideal delivery vehicle for CpG ODN to reach its target site thereby enhancing the T_H1 response to an antigen also encapsulated in the microparticle. Here, we show that careful control over fabrication parameters can produce biodegradable microparticles with predictable size distributions, surface morphology, and shape. Entrapment efficiencies of the model antigen OVA ranged from 19% to 23% with an average loading of 10 µg/mg of microparticles. For CpG ODN, these values were 33% to 35%, which corresponded to an average loading of 8.5 µg/mg of microparticles. The microparticles release CpG ODN and OVA in a burst followed by sustained release profile. At the highest concentration of microparticles incubated with a pure DC cell line, 92% of DCs had internalized microparticles by 16 hours, confirming that DCs efficiently take up the microparticles. Microparticles are capable of inducing DC maturation as determined by up-regulation of CD80 and CD86 markers. Although the presence of CpG ODN in the microparticles did not impact on the phenotype of the DCs, it was necessary for DCs to induce activation of antigen-specific T cells as indicated by interferon-γ production. Microparticles entrapping both antigen and CpG ODN induced significantly higher amounts of anti-OVA antibody production than other preparations such as the soluble OVA and CpG ODN ($P < 0.01$) and stimulated stronger IgG2a production than delivery of microparticles entrapping antigen alone. We conclude that coencapsulating immunostimulatory CpG ODN and antigen in degradable microparticles is an effective approach to enhancing development of a T_H1 immune response.

Key Words: biodegradable microparticles, CpG oligonucleotides, immunotherapy

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Immunization with antigens alone produces poor immunity. By coadministering the antigen with an adjuvant, improved immune responses are generated.^{1–3} However, the severe inflammation induced by many adjuvants, such as killed bacteria, negates their potential for human application.⁴ Those adjuvants that are currently approved by the Food and Drug Administration (FDA), such as alum, are not very efficient. Although the mechanism of the adjuvant-enhanced immune response is not fully understood, it is believed that they promote specific types of immune responses, such as antigen-specific CD8⁺ cytotoxic T lymphocytes.⁵ In recent years, the Toll-like receptors (TLRs) have been identified as a key set of pattern-recognition receptors that trigger innate immune responses providing both immediate protection against various pathogens and instructing the adaptive immune system by the induction of dendritic cell (DC) recruitment and maturation. These TLRs represent a new and specific avenue for triggering strong immune responses against antigens.

We and others have previously observed that the use of CpG motifs as an adjuvant to antigens results in much stronger CD8⁺ responses than antigen delivery alone.⁶ Bacterial DNA and certain oligonucleotides containing unmethylated CpG dinucleotides can stimulate select murine and human mononuclear cells, whereas eukaryote DNA and methylated oligonucleotides cannot. CpG motifs are more common in bacterial DNA than in vertebrate DNA and, when present, are more likely to be methylated in vertebrates. CpG ODN stimulates B cells and DCs to secrete cytokines, especially T_H1-like cytokines such as interleukin 12 (IL-12) and IL-18, express costimulatory molecules and show increased antigen-presentation.^{2,3,7,8} TLR9-deficient mice are completely defective in their response to CpG ODN, including cytokine production by macrophages, B-cell proliferation, and maturation of DCs.⁹ Thus, TLR9 plays an essential role in the cellular response to CpG ODN.¹⁰ Endosomal acidification and maturation are prerequisites

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From the *Division of Pharmaceutics, College of Pharmacy; †Department of Internal Medicine, Holden Comprehensive Cancer Center; and ‡Department of Biomedical Engineering, College of Engineering, University of Iowa, Iowa City, IA.

Xue-Qing Zhang and Christopher E. Dahle have contributed equally.

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Reprints: Aliasger K. Salem, University of Iowa, 115 S. Grand Avenue, S228 Pharmacy Building, Iowa City, IA (e-mail: aliasger-salem@uiowa.edu).

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for the activation of the signaling pathways mediated by CpG ODN. In addition, TLR9 is found within endosomes.

The foregoing observations highlight the potential value of a delivery vehicle that can ensure CpG ODN and antigen is internalized by targeted cells. Degradable particles between 1 and 10 μm in size are reported to be preferentially internalized by antigen presenting cells and would therefore be an ideal delivery vehicle for CpG ODN.¹¹ Equally important is ensuring that *both* antigen and adjuvant are delivered to the same cell for enhanced antigen-specific immune responses. For example, when CpG motifs and antigens were bound to individual multicomponent nanorods that were delivered to cells by particle bombardment, significantly stronger CD8⁺ responses were generated than delivery of antigens alone.¹ Mutwiri et al¹² have shown in pigs that administration of CpG ODN and HBsAg vaccine in separate sites of the same muscle did not show an enhanced antibody response compared with administration of the HBsAg vaccine alone, whereas administration of CpG ODN at the same site as HBsAg vaccine significantly enhanced antibody responses. Coentrapping antigens and CpG ODN in microparticles prepared from biodegradable polymers would produce a delivery vehicle that is efficiently internalized by antigen presenting cells, and codelivers antigens and CpG to the same cell.

The use of microparticles prepared from biodegradable polymers has increasingly been viewed as an attractive approach in vaccine development for controlled release of antigens.^{13,14} Microparticles can be formed using water in oil in water double emulsion solvent evaporation techniques. They can be delivered orally and to open areas susceptible to infections (eg, vaginal or nasal), can protect DNAs and other biomolecules from enzymatic degradation by nucleases, and can act as an adjuvant to antigens for promoting immune responses.^{4,15,16} Biodegradable polymers based on the poly (α hydroxy acids), such as the FDA-approved polylactic-co-glycolic acid (PLGA) are ideal for use in these applications.¹⁷⁻²¹

Using microparticle vaccines to stimulate strong antigen-specific immune responses is a therapeutic approach that can be applied to the prevention or treatment of a variety of diseases including tetanus,¹⁶ influenza,^{22,23} hepatitis,^{23,24} malaria,²⁵ and cancer.^{2,3} Herein, we report that careful control over the parameters for preparing microparticles loaded with CpG ODN and a model antigen OVA can produce microparticles with predictable size, morphology, and release rates. We show that the microparticles up-regulate markers for activation of DCs, are efficiently taken up by DCs, and stimulate strong IgG2a responses in vivo in a murine model.

MATERIALS AND METHODS

Materials

CpG ODN 1826: 5'TCCATGACGTTTCCTGAC GTT 3 was obtained from Coley Pharmaceutical Group

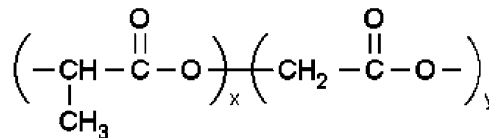


FIGURE 1. Structure of PLGA.

(Wellesley, MA) (CpG motif is in **bold**). Ovalbumin (OVA, chicken egg albumin, grade VI) was purchased from Sigma (St Louis, MO). PLGA (Fig. 1) with a lactide:glycolide ratio of 75:25 was purchased from Absorbable Polymers, Pelham, AL. The PLGA had an inherent viscosity of 0.47 dL/g in CHCl_3 at 30°C and MW of 56500.

Mice and Cell Lines

C57BL/6 female mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were housed at the animal care unit of the University of Iowa and used at 8 to 10 weeks of age. Naive C57BL/6 OT-1 mice that are transgenic for the T-cell receptor (TCR), which recognizes major histocompatibility complex (MHC) class I H-2Kb-restricted OVA257-264 (SIINFEKL) in H-2Kb were purchased from Jackson Labs (Bar Harbor, ME). The DC2.4 cell line (K. Rock, University of Massachusetts, Worcester, MA), which is derived from bone marrow progenitors from C57BL/6 mice was cultured using Dulbecco modified Eagle medium high glucose supplemented with 10% fetal calf serum, L-glutamine, and antibiotics.

Preparation of Microparticles for Immunization Studies

PLGA microparticles were prepared using a double emulsion solvent evaporation methodology. Briefly, 200 mg of 75:25 PLGA (MW = 56,500) was dissolved in 2-mL dichloromethane (DCM). A solution with OVA at a concentration of 100 $\mu\text{g}/\mu\text{L}$ and CpG ODN at 50 $\mu\text{g}/\mu\text{L}$ was prepared in 1% (wt/vol) PVA (87% to 89% hydrolyzed, Average MW = 13,000 to 23,000; Sigma) in deionized water. Using a microtip probe sonicator set at level 2 (Sonic Dismembrator Model 100, Fisher Scientific, Pittsburgh, PA), 100 μL of the protein/oligonucleotide solution containing 10 mg OVA and 5 mg CpG was mixed with the PLGA/DCM solution for 20 seconds (10-second pulses) to form the first emulsion. This emulsion was then rapidly added to 100 mL of 1% (wt/vol) PVA in deionized water with stirring at 6400 rpm. The mixture was stirred overnight during which time the DCM solvent evaporated. The microparticles were then washed 6 times with 50 mL of sterile filtered distilled/deionized water, collecting between washes by centrifugation and resuspension in deionized water. Microparticles were then lyophilized (Labconco freezezone 4.5, Kansas City, MO). After resuspending the microparticles in sterile filtered deionized water, they were placed under ultraviolet light in a laminar flow hood to sterilize for 1 hour before injection. For particle uptake studies in DCs, PLGA microparticles loaded with Rhodamine 123 were prepared using a single

emulsion evaporation methodology. Briefly, 200 mg of 75:25 PLGA (MW = 56,500) and 10 mg Rhodamine 123 (Sigma) was dissolved in 2 mL DCM. This was then rapidly added to 100 mL of 1% (wt/vol) PVA in deionized water with stirring at 6400 rpm. The rhodamine labeled particles were washed and collected as described above.

Characterization of PLGA Microparticles

Microparticle Size and Surface Morphology Analysis

Microparticle size analysis was conducted using the Zetasizer Nano ZS (Malvern, Southborough, MA). The cumulants analysis provides 2 values, a mean value for the size and a width parameter, known as polydispersity that reflects the broadness of the microparticle size distribution. The cumulants analysis is a fit of the polynomial to the log of the *GI* correlation function, where $\text{Ln}[GI] = a + bt + ct^2$. The coefficient of the squared term, *c*, when scaled as $2c/b^2$ is the polydispersity. Decreasing polydispersity values represent narrower particle size distributions. Microparticle morphology was assessed by scanning electron microscopy (SEM, Hitachi S-4000). Briefly, air-dried microparticles were placed on adhesive carbon tabs mounted on SEM specimen stubs. The specimen stubs were coated with approximately 5 nm of gold by ion beam evaporation before examination in the SEM operated at 5 kV accelerating voltage.

CpG ODN and OVA Loading Estimation

OVA loading in PLGA microparticles was determined by dissolving 200 mg of lyophilized microparticles in 2 mL of acetonitrile. The suspension of dissolved polymer and precipitated OVA was centrifuged at $6000 \times g$ for 7 minutes and the polymer containing supernatant was then discarded. The pellet consisting of the precipitated OVA was redispersed in 0.1 N NaOH. The protein content of the neutralized alkaline solution was estimated using a microBCA protein assay kit (Pierce, Rockford, IL). Empty microparticles in acetonitrile were spiked with a known amount of OVA. After the procedure, recovery of the extracted OVA was found to be complete. Microparticles containing CpG ODN were also dissolved in acetonitrile and the CpG ODN was extracted in TE buffer (10 mM, pH 8.3). The amount of CpG ODN was determined based on absorbance at 260 nm. Empty microparticles in acetonitrile were spiked with a known amount of CpG ODN. After the procedure, recovery of the extracted CpG ODN was found to be complete.

In Vitro Release of CpG ODN and OVA

PLGA microparticles (200 mg) containing OVA was suspended in phosphate buffered saline (10 mL, 50 mM, pH 7.4) for 2 months. The suspension was gently shaken in a water bath at 37°C. At various time intervals, the supernatant was removed after centrifugation and replaced with fresh medium. OVA released into the supernatant was quantified using the micro-BCA protein

assay. CpG ODN containing microparticles were incubated in TE buffer and the released fraction estimated spectrophotometrically at 260 nm.

Flow Cytometry of Particle Uptake

0.01, 0.1, and 1 μL of 1% microparticles (wt/wt) with a rhodamine fluorescent marker (Sigma, St Louis, MO) were incubated with the DC2.4 cells for 30 minutes or 16 hours. Uptake of microparticles was assessed using flow cytometry (Becton Dickinson). Dot plots were gated on FSC/SSC properties of DCs to exclude free fluorescent-labeled microparticles. Data were analyzed using CellQuest^{Pro} software. Quadrant markers were set accordingly using controls. To confirm that rhodamine labeled particles had been internalized by the DCs at the 16 hours time-point, cells were viewed under a Confocal Microscope (Bio-Rad Radiance 2100MP). Orthogonal sections from stacked images confirmed that the particles were predominantly located inside the cell and not on the surface (data not shown).

In Vitro Generation of DCs and Assessment of Activation of DCs

DCs were generated from the bone marrow of wild type C57BL/6 mice by flushing the pelvis, femurs, and tibias and culturing the resulting cells in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 50 μM 2-ME, 10 U/mL penicillin, 10 $\mu\text{g}/\text{mL}$ glutamine supplemented with 1000 U/mL murine GM-CSF (R&D Systems, Minneapolis, MN), and 1000 U/mL murine IL-4 (Peprotech, Rocky Hill, NJ). Media was changed at 4 or 5 days, and DCs were harvested and used on day 7. DC phenotype and morphology was confirmed by flow cytometry and SEM. The cells were pulsed for 24 hours with microparticles containing CpG and OVA or either component individually. DCs were then washed twice and stained with FITC-labeled anti-CD80 and anti-CD86 as markers of DC activation, with subsequent analysis on a FACScan flow cytometer (Becton Dickinson). Splenocytes were harvested from naive C57BL/6 OT-1 mice transgenic for the TCR, which recognizes MHC class I H-2Kb-restricted OVA 257-264 (SIIN-FEKL) in H-2Kb. Red blood cells were lysed using 0.83% ammonium chloride solution. Purified CD8⁺ cells were positively selected by magnetic bead separation of T cells according to the manufacturers specifications (MiniMACS; Miltenyi Biotec, Auburn, CA). CD8⁺ OT-1 T cells were then added to DCs for 24 hours at a T cell: DC ratio of 100:1.

Supernatant from OT-1 T cell cultures with DCs were analyzed for the presence of interferon- γ (IFN- γ) by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (eBioscience, San Diego, CA). All samples were tested in triplicate.

Immunization Experiments

C57BL/6 mice of 8 to 10 weeks of age were used for immunization. Mice were immunized intraperitoneally on day 1 (week 0) and day 14 (week 2) with OVA and

CpG ODN in the following combinations: (group I) microparticles containing nothing, (group II) microparticles containing OVA (68 μ g), (group III) microparticles containing CpG ODN (76 μ g), (group IV) microparticles containing OVA (68 μ g) + CpG ODN (76 μ g), (group V) CpG ODN in solution (100 μ g) + microparticles containing OVA (68 μ g), (group VI) OVA (100 μ g) alone, and (group VII) OVA (100 μ g) + CpG ODN (100 μ g).

ELISA Determination of Anti-OVA Levels

Serum obtained from mice at predetermined time-points following immunization was obtained by retro-orbital puncture from mice. Microtiter plates were coated with 10 μ g/mL OVA antigen and incubated overnight at 4°C. The wells were then blocked with 5% milk and serial dilutions of serum were added. Plates were washed, and heavy chain-specific goat antimouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates, Birmingham, AL) added followed by the colorimetric substrate *p*-nitrophenylphosphate. Serum from naive mice to which a known concentration of monoclonal anti-OVA (4A9) antibody was added served as a standard. The negative control was pretreatment mouse serum. Plates were evaluated using a microplate reader and curves established for each sample. Test curves were compared with standard curves to determine the concentration of anti-OVA. Values were considered valid only if the standard curves and the sample curves had the same shape. The area under the curve (AUC) was measured for each sample using the trapezoidal rule.

Statistical Analysis

Group data were reported as mean \pm SEM. Differences between groups were analyzed by 1-way analysis of variance with a Tukey posttest analysis. Levels of significance were accepted at the $P < 0.05$ level. Statistical analyses were performed using Prism 3.02 software (Graphpad Software, Inc, San Diego, CA). All results were representative of at least 2 separate experiments.

RESULTS

Microparticle Properties can be Controlled by Fabrication Parameters

Initial studies were carried out to optimize microparticle fabrication processes. Lowering the PVA surfactant concentration from 9% to 0.1% wt/wt resulted in the average microparticle size increasing from 1.6 to 9.7 μ m at 3400-rpm stirring speed. Increasing the stirring speed from 3400 to 10000 rpm decreased the average microparticle size from 9.7 to 1.3 μ m at a fixed surfactant concentration of 0.1% wt/wt (Table 1). The preparation methodology selected for the immunization studies had a surfactant PVA concentration of 1% with a stirring speed of 6400 rpm producing microparticles with an average diameter of $2.1 \pm 0.35 \mu$ m. Entrapment efficiencies of OVA ranged from 19% to 23% with an average loading of 10 μ g/mg of microparticles. For CpG ODN, these values were 33% to 35%, which corresponded to an

TABLE 1. Influence of PVA Concentrations and Stirring Rate on Average Particle Size and Polydispersity (Averages Representative of 8 Measurements \pm SE)

PVA Concentration (% wt/vol)	Stirring Rate (Rev./min)	Average Size (μ m \pm SE)	Polydispersity (\pm SE)
0.1	3500	9.7 \pm 0.5	0.31 \pm 0.04
	6400	4.3 \pm 0.3	0.34 \pm 0.05
	10,000	1.3 \pm 0.2	0.39 \pm 0.07
1	3500	4.1 \pm 0.4	0.23 \pm 0.05
	6400	2.1 \pm 0.35	0.37 \pm 0.05
	10,000	1.5 \pm 0.42	0.43 \pm 0.07
9	3500	1.6 \pm 0.45	0.36 \pm 0.04
	6400	0.63 \pm 0.34	0.42 \pm 0.23
	10,000	0.19 \pm 0.16	0.57 \pm 0.42

average loading of 8.5 μ g/mg of microparticles. The spiking tests used to validate the methodology showed complete recovery for CpG ODN and OVA. Attempts to assess percentage CpG ODN entrapment with OVA were complicated by the strong absorption of OVA at 260 nm, thus necessitating measurements of CpG ODN entrapment in microparticles without the OVA. Rhodamine 123 is soluble in DCM. Therefore, PLGA microparticles loaded with Rhodamine 123 were prepared using a single emulsion technique that used identical stirring and PVA concentration conditions to the PLGA CpG ODN OVA microparticles. The single emulsion technique reduced the average microparticle size from 2.1 to 1.4 μ m and generated lower polydispersity. The microparticles were otherwise identical in morphology and shape to microparticles tested in the immunization studies.

PLGA CpG ODN OVA Microparticles are Smooth in Morphology and Produce an Initial Burst Release Profile Followed by More Sustained Release

SEM analysis (Fig. 2) showed that microparticles had a smooth morphology and spherical shape. Release profiles of CpG ODN and OVA in aqueous solution were

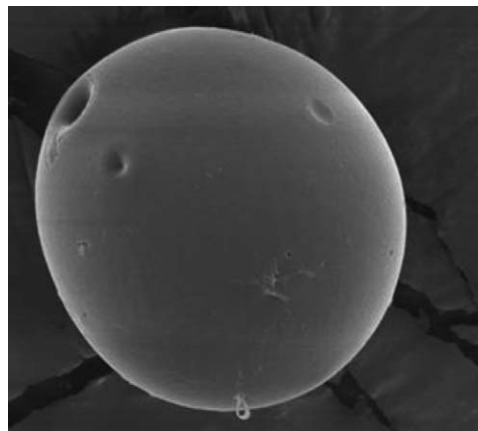


FIGURE 2. Left, SEM image shows that morphology of PLGA particles is smooth and spherical in appearance.

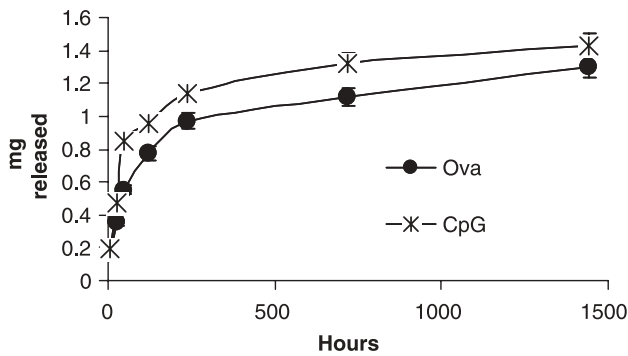


FIGURE 3. Right, Ovalbumin release profiles determined using the BCA assay and CpG release profiles determined using UV spectrophotometry at 260 nm show that ovalbumin and CpG released from 200 mg of PLGA particles demonstrate an initial burst release profile followed by a sustained release (averages representative of 8 measurements ± SE).

measured using UV spectrophotometry at 260 nm and the micro-BCA protein assay, respectively. Release of both CpG ODN and OVA (Fig. 3) followed a burst release profile with 0.164 mg OVA and 0.198 mg CpG ODN released by 4 hours. This was followed by a more sustained cumulative release of 1.3 mg OVA and 1.43 mg CpG ODN by 720 hours.

Efficient Cellular Uptake of Degradable Microparticles

Figure 4 shows flow cytometry data of rhodamine labeled microparticles incubated with the DC2.4 cells. At the highest concentration tested (1 μL of 1% solids), the percentage of DC2.4 cells that had taken up microparticles increased from 8.4% at 30 minutes to 92% of DC2.4 cells by 16 hours of incubation. Percentage uptake by DC2.4 cells was found to be concentration dependent.

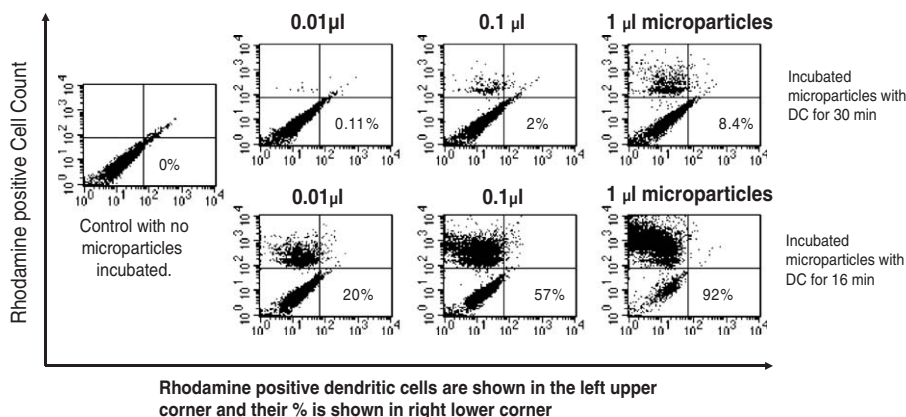


FIGURE 4. Flow cytometry data of degradable polymer microparticles (mean average size of $1.4 \pm 0.3 \mu\text{m}$, polydispersity 0.24 ± 0.01) that have been surface labeled with rhodamine and then incubated with DCs. At the highest concentration dosed (1 μL of 1% solids), the percentage of DCs that have taken up microparticles increased from 8.4% at 30 minutes incubation to 92% of DCs by 16 hours incubation. Percentage DCs with uptake of microparticles was also found to be concentration dependent with 20% of DCs having taken up microparticles with 0.01 μL of 1% solids to 57% of DCs for 0.1 μL of 1% solids to 92% of DCs that had taken up microparticles when incubated with 1 μL of 1% solids (at 16 h).

When the concentration of microparticles was increased from 0.01 μL of 1% solids to 0.1 μL of 1% solids, a corresponding increase in DC2.4 cells taking up microparticles from 20% to 57% was observed by 16 hours. A further increase in concentration to 1 μL of 1% solids resulted in 92% of DC2.4 cells taking up microparticles at the 16 hours time-point.

PLGA Microparticles Entrapping CpG ODN and OVA Trigger Activation and Maturation of Primary DCs

As seen in Figure 5, both CD80 and CD86 were up-regulated at 24 and 48 hours of incubation with biodegradable microparticles irrespective of whether the particles contained CpG ODN in comparison with untreated DCs. However, the presence of both CpG ODN and OVA significantly enhanced the ability of such DCs to activate antigen-specific T cells. Specifically, the microparticles entrapping CpG ODN and OVA generated 20-fold higher IFN-γ production (775 pg/mL) from OT-1 T cells cocultured with DCs pulsed with CpG ODN and OVA in solution (37 pg/mL) relative to all other conditions tested (Table 2). No T-cell activation was seen when the T cells were cultured with DCs pulsed with OVA-containing microparticles that lacked CpG ODN. DCs alone did not generate IFN-γ under any circumstances.

Microparticle Vaccines Stimulate Strong IgG Responses In Vivo

C57BL/6 mice were immunized on day 1 (week 0) and 14 (week 2) with either (1) microparticles containing nothing, (2) OVA 68 μg, (3) CpG ODN 76 μg, (4) OVA 68 μg + CpG ODN 76 μg, (5) microparticles entrapping OVA 68 μg + soluble CpG ODN 100 μg, (6) with soluble OVA (100 μg), or (7) OVA (100 μg) + CpG ODN (100 μg). Serum was sampled from selected groups every 2 weeks

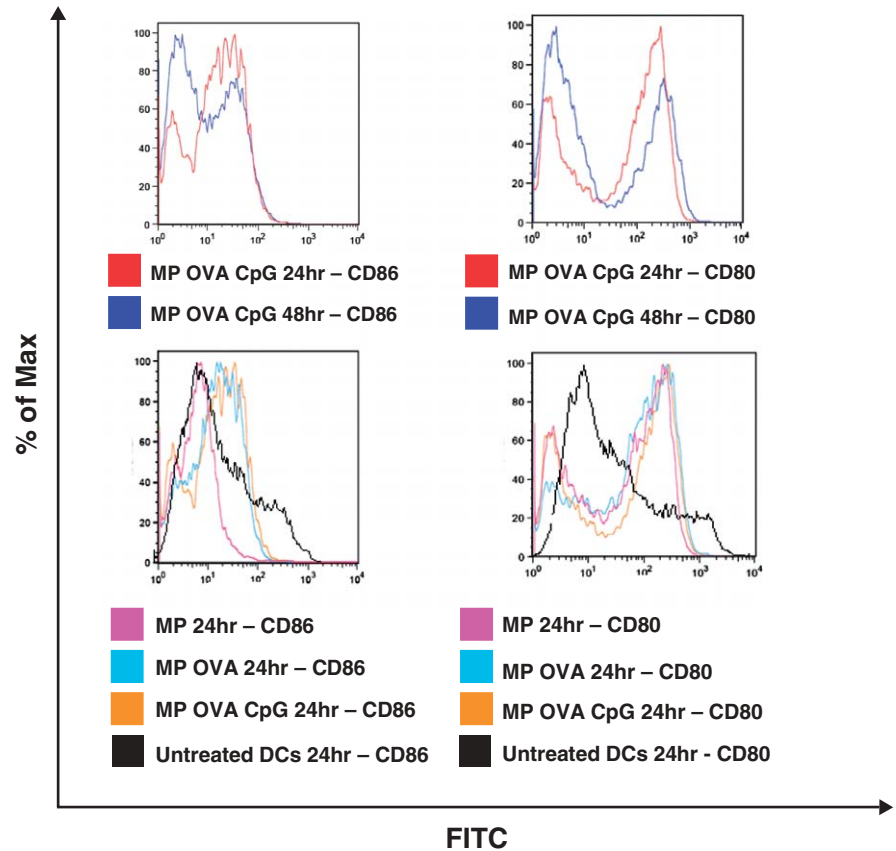


FIGURE 5. Flow cytometry data showing activation of CD80 and CD86 markers in DCs following incubation with PLGA microparticle formulations.

and from all groups at week 4 and stored at -20°C . The dose of CpG ODN and OVA entrapped in the PLGA microparticles was 24% and 32% lower than the doses of CpG ODN and OVA, respectively in the soluble preparations. PLGA particles containing OVA alone induced a 10-fold higher level of anti-OVA IgG than did soluble OVA ($P < 0.01$). The highest levels of anti-OVA IgG were seen in mice immunized with PLGA

particles containing OVA plus CpG ODN. This level was 3-4 fold greater than that seen in response to codelivery of soluble OVA and CpG ODN ($P < 0.01$) (Fig. 6A). The presence of increased amounts of anti-OVA IgG was confirmed using serial dilutions of serum obtained at week 4 (Fig. 6B). Mean AUC measurements determined using the trapezoidal rule suggested an increase from 2.88 for soluble OVA and CpG to 6.44 for microparticles entrapping CpG and OVA.

TABLE 2. IFN- γ Measurements

	IFN- γ (pg/mL)
DC+OT-1	
OVA	None detected
PLGA OVA	None detected
PLGA OVA CpG	775
OVA/CpG	37
SIINFEKL	1423
Untreated DC	None detected
Splenocytes only	None detected
DC alone	
OVA	None detected
PLGA OVA	None detected
PLGA OVA CpG	None detected
OVA/CpG	None detected
SIINFEKL	None detected
Untreated DC	None detected

Results are from DCs alone incubated with vaccination groups or DCs incubated with vaccination groups and cocultured with OT-1 T cells.

PLGA CpG ODN OVA Microparticle Vaccinations Stimulate IgG2a Responses

As outlined above, when the IgG response from week 4 serum from mice was measured using an ELISA assay, microparticles that codelivered CpG ODN and OVA generated the strongest responses. Murine IgG2a responses are known to be associated with a T_H1 response, whereas IgG1 is found in both T_H1 and T_H2 responses. We therefore evaluated levels of anti-OVA IgG1 and IgG2a. Microparticles with OVA but without CpG ODN (AUC 11.93) and microparticles with OVA and CpG ODN (AUC 9.43) produced a similar IgG1 response (Figs. 7A, B). In contrast, as illustrated in Figure 7C, microparticles that codelivered CpG ODN with OVA (AUC 8.68) stimulated stronger IgG2a production than delivery of microparticles entrapping OVA alone (AUC 1.45). In control experiments with microparticles entrapping CpG ODN but not OVA, low

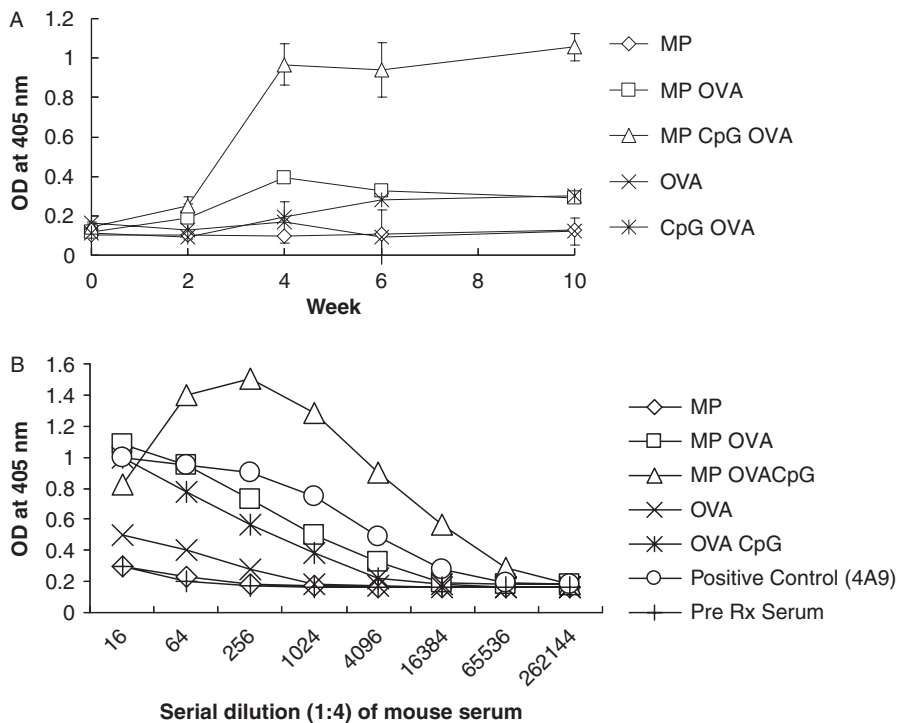


FIGURE 6. A, ELISA assay of anti-OVA IgG response in mouse serum. Mice (n=4) were inoculated twice on day 1 (week 0) and 14 (week 2) with either micro particles containing nothing (MP), OVA 68 μg (MP OVA), or OVA 68 μg+CpG 76 μg (MP CpG OVA), or with OVA (100 μg) or OVA (100 μg)+CpG (100 μg) alone. B, Serial dilution in phosphate buffered saline of week 4 serum from mice immunized at week 0 and week 2. Each group contains 4 mice. Each mouse's serum was run in triplicate. Each plate was normalized to the positive control. The positive control is a monoclonal anti-Ova antibody (4A9). The negative control is pretreatment mouse serum. The secondary antibody is goat-anti-mouse-Ig(H+L)-AP. MP=microparticle.

anti-OVA IgG (AUC 0.95), IgG1 (AUC 0.83), and IgG2a (AUC 0.915) responses were generated. PLGA microparticles entrapping OVA that were coinjected with CpG ODN in solution generated IgG (AUC 12.1) and IgG1 (AUC 9.1) responses that were equivalent to microparticles entrapping OVA injected alone. In addition, no significant increase in IgG2a (AUC 2) was observed for this formulation in comparison with microparticles entrapping OVA alone.

DISCUSSION

CpG ODN enhances the immune response via binding to the TLR9 receptor. TLR9 is a receptor that is found within endosomes. In previous studies, we and others have observed that codelivery of an antigen with a CpG ODN significantly enhances the antigen-specific immune response. Microparticles are efficiently internalized by macrophages and DCs. Particles that contain both CpG ODN and antigen would be expected to deliver

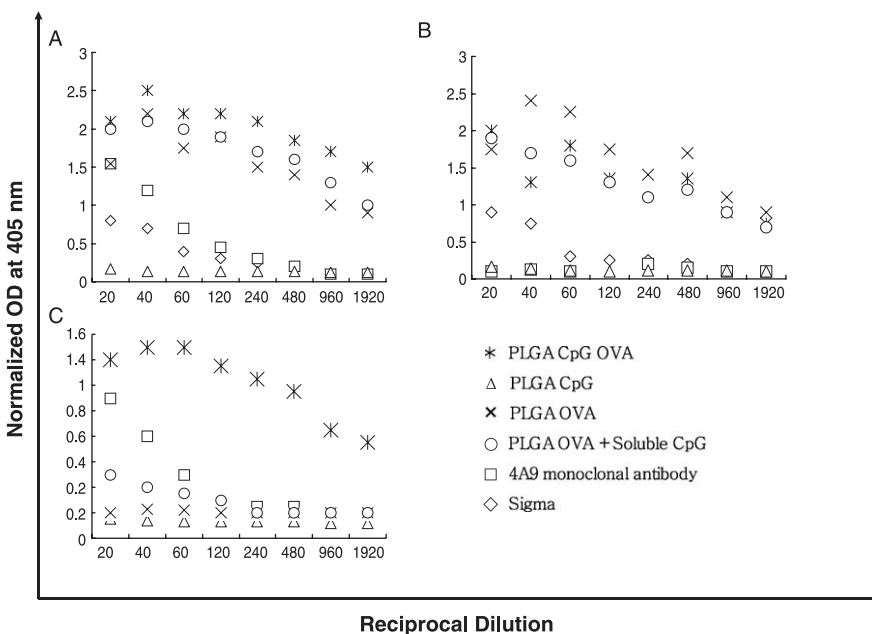


FIGURE 7. ELISA assay measuring IgG, IgG1, and IgG2a antibodies to ovalbumin in week 4 mouse serum. A, 10 μg/mL Ova coat and GAM IgG. B, 10 μg/mL Ova coat and GAM IgG1. C, 10 μg/mL Ova coat and GAM IgG2a. Sigma=anti-OVA antibody positive control purchased from Sigma. 4A9 is a monoclonal antibody positive control (University of Iowa Hybridoma Research Facility).

both antigen and CpG ODN to the same cell at the same time, and therefore could be expected to induce an enhanced immune response. In this study, we evaluated whether entrapping CpG ODN and OVA into biodegradable microparticles are effective in inducing a stronger immune response. Microparticles were prepared using PLGA, because the polymer is biocompatible and has a long track record of use in FDA approved biomedical applications.

As outlined in the Methods section, the CpG ODN and the model antigen OVA were entrapped in PLGA microparticles using the water in oil in water double emulsion solvent evaporation technique.²⁶ The method involves the use of 3 phases, each of which can be modified to control the composition and structure of the microparticles. These phases include: (1) an inner water phase containing the CpG ODN to be incorporated, (2) an intermediate organic phase consisting of a polymer/methylene chloride solution, and (3) an outer water phase containing an emulsifying agent. A commonly used emulsion stabilizer in the solvent evaporation method for PLGA microparticle preparation is partially hydrolyzed PVA, which is a copolymer of poly (vinyl acetate) and poly (vinyl alcohol). An 88% hydrolyzed PVA was chosen because a study by Murakami et al²⁷ found this to be the optimum degree of hydrolyzation of PVA for the manufacture of nano/microparticles. The ability to control the size of microparticles prepared from degradable polymers (Table 1), was governed by the stirring rate and the PVA concentration of the continuous phase. The general trend was increasing stirring speed and surfactant concentration decreased the average size of the microparticles. As the microparticle size distribution got smaller, the polydispersity increased. This is important, as it has been found that there is a direct relationship between the degradation rate and microparticle size. In smaller microparticles, degradation products formed within the microparticle can diffuse easily to the surface, whereas in larger microparticles degradation products have a longer path to the surface of the microparticle, during which autocatalytic degradation of the remaining polymer can occur.²⁸ The CpG ODN 1826 that was entrapped in our microparticles is highly stimulatory for mice but not humans or other animals. However, the procedures described here can easily be adapted to CpG ODN sequences such as CpG ODN 7909 (Coley Pharmaceuticals) that is being evaluated clinically.

The morphology of the microparticles as observed by SEM was smooth and spherical in appearance. Release profiles of CpG ODN and OVA provided confirmation that both components were entrapped within the microparticle matrix and could provide an initial high dose followed by lower doses released over a sustained period of time. The release of CpG ODN was faster than OVA. This is likely due to the fact that CpG ODN has an MW of 6363 d, which is a much smaller molecule than OVA (44.3 kd). It is important to note that these release studies were performed in cell-free, protein-free conditions. The effects of enzymatic degradation, and other differences

between in vitro incubation and in vivo therapy, could result in modified release kinetics.

Next, we determined the uptake efficiency of microparticles incubated with a DC cell line developed by Shen et al²⁹ that is phagocytic, has high levels of MHC molecules and costimulatory molecules and has been shown to present exogenous OVA on both MHC class I and class II molecules. At the highest concentration of microparticles dosed, 92% of DCs had internalized microparticles by 16 hours. These results confirm that cells can take up the microparticles. Our ability to control the microparticle size, as seen in Table 1, was critical because a number of studies have shown that particle size and cellular uptake are correlated. The general trend is that smaller particles are more efficiently internalized than larger particles.³⁰ This is also consistent with the observations from Manzel and Macfarlane³¹ that showed an immune response was not generated when CpG ODN was bound to particles that were larger than the cells, presumably owing to the inability of the cells to internalize the particles.

To evaluate the effect of microparticles on primary DCs, DCs generated from the marrow of mice were incubated with microparticles for 24 or 48 hours and assessed for CD80 and CD86 markers by flow cytometry. CD80 and CD86 are both markers for activation and maturation of DCs. The CD80 phenotypic change was observed whether or not the microparticles encapsulated CpG ODN and OVA, that is, empty microparticles also stimulated up-regulation of CD80 markers. In contrast, up-regulation of CD86 was only significantly observed when microparticles entrapped OVA and CpG ODN or OVA alone. These studies demonstrated that microparticles are capable of inducing DC maturation. Supernatants from cocultures containing OT-1 T cells and DCs incubated with the microparticles were then analyzed for the presence of IFN- γ by ELISA. The presence of IFN- γ indicated a T_H1 type response. Although the presence of CpG ODN in the microparticles did not impact on the phenotype of the DCs, it had a major impact on the ability of the DCs to induce IFN- γ production by antigen-specific T cells (Table 2). OT-1 mice are transgenic for the TCR, which recognizes MHC class I H-2K^b restricted OVA_{257–264} (SIINFEKL). The control peptide SIINFEKL provided confirmation of the integrity of the assay generating high IFN- γ production consistent with previous observations. In those studies, we have also shown that CpG ODN could enhance the IFN- γ production by 8–10 fold when coincubated with the SIINFEKL peptide.³² Further studies are also warranted to investigate the MHC-class II restricted OVA presentation to OT-II T cells.

Next, we determined if microparticles entrapping CpG ODN and OVA significantly enhanced the immune response in comparison with immunization with soluble CpG ODN and OVA. IgG measurements made using ELISA were plotted over a 10-week time-course. From week 4 to week 10, significant 3–4 fold increases ($P < 0.01$) in the antigen-specific antibody response were observed

from mice inoculated with microparticles entrapping CpG ODN and OVA in comparison with codelivery of soluble OVA and CpG ODN (Fig. 6A). The increases in IgG response observed at week 4 were confirmed using serial dilution curves (Fig. 6B). These results highlight the substantial improvements that can be achieved when CpG ODN is delivered in microparticles in comparison with delivery in solution and are consistent with previous studies that showed particulate delivery of CpG ODN either adsorbed on the surface of cationic microparticles, on nanorods, as nanoparticles or in liposomal formulations enhanced the efficacy of CpG ODN as an adjuvant.^{6,16,33–35} In humans, CpG ODN activates plasmacytoid cells but not myeloid cells. Previous studies have shown that plasmacytoid DCs that internalize CpG ODN absorbed on particles generate stronger IFN- γ production than CpG ODN in solution. Thus, the results obtained from PLGA microparticles in these studies will have impact within a human clinical setting.³⁶

A breakdown of the IgG response at week 4 showed that codelivery of *both* CpG ODN and antigens in the microparticles is necessary for optimal IgG2a responses (Fig. 7). Higher IgG2a responses have been previously reported with CpG ODN, and are consistent with induction of a T_{H1} response. The strong immune responses stimulated by the microparticle vaccines developed in this study suggest significant potential in a wide variety of immunotherapeutic and prophylactic vaccine applications.

In summary, our data demonstrate that biodegradable microparticles that entrap CpG ODN and antigen ensure that both components are delivered to the same cell—thus, stimulating stronger T_{H1} type responses than CpG ODN and antigen in solution. CpG ODN triggers stronger T_{H1} immune responses by binding to TLR9, a receptor found within endosomes. It was also demonstrated that microparticles trigger activation and maturation of DCs and are efficiently internalized by DCs. It is likely that this efficient microparticle internalization facilitates delivery of the CpG ODN to its intracellular binding site. In future studies, it will be necessary to evaluate the impact of increasing the number and concentration of doses to enhance the response further. In addition, as microparticles are internalized efficiently and deliver CpG ODN to the intracellular TLR9, it follows that alternative TLR agonists such as Poly I:C (TLR3 ligand) and the imiquods (TLR7/8) that also bind to intracellular/endosomal TLRs would benefit from using this delivery system.^{37–39} Finally, the microparticle vaccines should prove to be highly effective as cancer vaccines given our previous observations that CpG ODN was an effective adjuvant in a tumor immunization strategy using the 38C13 B cell lymphoma model.⁴⁰

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