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Intranasal administration of plasmid DNA-coated nanoparticles results in enhanced immune responses

Zhengrong Cui and Russell J. Mumper

Abstract

Intranasal immunization offers potential for the elicitation of effective mucosal and systemic immune responses. In this study, a previously reported novel cationic nanoparticle engineered from a microemulsion precursor was further modified, optimized and applied intranasally to mice to explore its potential as a plasmid DNA (pDNA) vaccine delivery system. To this end, more uniform nanoparticles (around 100 nm) containing less cationic surfactant were developed. The pDNA-coated nanoparticles significantly enhanced the specific serum IgG and IgA titres to an expressed model antigen, β-galactosidase, by 18–28 and 25–30 fold, respectively, when compared with naked pDNA alone. An enhanced splenocyte proliferative response was also observed after immunization with the pDNA-coated nanoparticles. It was concluded that these plasmid DNA-coated nanoparticles may have potential for immunization via the nasal route.

Introduction

It is generally accepted that many new vaccines will require the induction of mucosal immunity since almost all viral, bacterial and parasitic pathogens causing common infectious diseases of the intestinal, respiratory and genital tract enter or infect through the body via mucosal surfaces (McGhee & Kiyono 1992; Mestecky et al 1997). Systemic administration of vaccines generally fails to induce mucosal immunity. However, with appropriate systems, it is possible to induce both mucosal and systemic immune responses by mucosal immunization (McGhee et al 1992, 1999). The nasal mucosa is an important arm of the mucosal system since it is often the first point of contact for inhaled antigens. As a consequence, intranasal immunization has proven to be an effective method for stimulating both upper and lower respiratory immunity (Partidos 2000; Davis 2001). Moreover, because of the properties of the common mucosal immune system, nasal immunization has the ability to induce both local and distal mucosal immunity in the nasal and upper respiratory mucosa and the draining lymph node, as well as the intestinal and vaginal mucosa (Mestecky & McGhee 1987; Klavinskis et al 1999). Thus, nasal immunization may have an important role in the prophylaxis of either (upper) respiratory infections or infections at distant mucosal sites. Additionally, intranasal immunization may require less vaccine presumably due to the minimization of vaccine loss or degradation expected after oral administration (Wu et al 2001). Intranasal immunization requires no needles and therefore may be amenable for widespread immunization of large populations. A nasal vaccine (Nasal Flu) is now commercially available, and the United States Food and Drug Administration is currently reviewing an application to market an additional influenza vaccine (Flumist) (Illum & Davis 2001).

Traditionally, vaccines have been comprised of (subunit) proteins, live attenuated viruses or killed bacteria. However, much attention has recently been focused on plasmid DNA (pDNA) vaccines. One important advantage of a pDNA vaccine is that it is able to induce in animals both humoral and cellular immune responses, including T helper type-1 (Th1) and cytotoxic lymphocyte (CTL) responses (Liu 1997). Moreover, the poor safety issues associated with the attenuated virus or bacterial vaccines may be

Division of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082, USA

Zhengrong Cui, Russell J. Mumper

Correspondence: R. J. Mumper, Assistant Professor of Pharmaceutical Sciences, Assistant Director, Center for Pharmaceutical Science and Technology, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082, USA. E-mail: rjmump2@uky.edu

avoided (Ulmer et al 1996). A pDNA vaccine may be relatively stable, and cost-effective for manufacture and storage. It is also possible to express multiple antigens on one plasmid. Finally, if properly controlled, the CpG motifs on pDNA can act as a potential immune adjuvant (Gurunathan et al 2000). However, until recently, intramuscular injection has been the primary route for administration of pDNA vaccines. Although pDNA immunization by the intramuscular route has proven to be very potent in small animal models, the potency in primates and humans has been disappointing (Mumper & Ledebur 2001). There exists a clear need to improve the potency of pDNA vaccines or to search for alternative route(s) to administer pDNA vaccines.

Intranasal immunization of mice with naked pDNA has been shown to induce antigen-specific IgA and cellular Th1 responses to the parasite *Cryptosporidium parvum* (Sagodira et al 1999) and to induce T-lymphocyte-dependent protection against *Chlamydia pneumoniae* (Svanholm et al 2000). However, to limit the possible enzymatic degradation of naked pDNA and to improve pDNA uptake at the nasal mucosal surface, a variety of formulations, including complexation of pDNA with liposomes and polymers, have been evaluated for mucosal delivery with some success (Klavinskis et al 1997, 1999; Sha et al 1999; Illum et al 2001). Over the last twenty years, the adjuvant effect achieved through the association of antigens with microparticles and nanoparticles has been repeatedly demonstrated (Vajdy & O'Hagan 2001). Most recently, Singh et al (2002) reported that intranasal mucosal immunization of mice with HIV-1 gag pDNA adsorbed on cationic poly-(lactide-co-glycolide) microparticles prolonged gene expression and enhanced local and systemic immunity.

Previously, we described a novel nanoparticle-based pDNA delivery system engineered from oil-in-water (O/W) microemulsion precursors. The microemulsions, formed at increased temperature (50–55°C), were comprised of emulsifying wax as the oil phase and a cationic surfactant, CTAB (hexadecyltrimethyl ammonium bromide) (Cui & Mumper 2001, 2002a, b). Upon simple cooling to room temperature, small cationic nanoparticles (< 100 nm) were formed. Plasmid DNA was then coated on the surface of the cationic nanoparticles to generate pDNA-nanoparticles. Both endosomolytic lipid, dioleoyl phosphatidylethanolamine (DOPE) and a dendritic-cell-targeting ligand, mannan, were successfully incorporated in, or attached on the surface of, the nanoparticles. Initial immunization with the pDNA-coated nanoparticles subcutaneously in mice led to enhanced Th-1 type and humoral immune responses to a model expressed antigen, β-galactosidase (Cui & Mumper 2002a). In addition, after topical application to shaved mouse skin, the pDNA-coated nanoparticles resulted in enhanced humoral and splenocyte proliferative immune responses (Cui & Mumper 2002b). Consequently, one of the objectives of this study was to further explore the potential of this pDNA-coated nanoparticle delivery system to elicit immune responses after intranasal administration to mice.

In the previous nanoparticle-based pDNA delivery system, 15 mM of CTAB was used as the surfactant to prepare the nanoparticles. Thus, the final amount of CTAB administered per mouse (per 100 μL injection) was estimated to be about 20 μg. Although no gross inflammatory, allergic or toxic effects were observed in mice after subcutaneous, intramuscular and topical routes, we sought to reduce the overall amount of CTAB used in the microemulsions by the co-incorporation of a non-ionic surfactant.

Materials and Methods

Materials

Plasmids containing a CMV promoter β-galactosidase reporter gene (CMV-β-gal) or CMV promoter luciferase gene (CMV-luc) were gifts from Valentis Inc. (Woodlands, TX). Both plasmids supplied by Valentis had endotoxin levels of < 0.1 EU mg^{-1}. Emulsifying wax (N.F. grade) was purchased from Spectrum Quality Products Inc. (New Brunswick, NJ). Hexadecyltrimethyl-ammonium bromide (CTAB), β-galactosidase antigen, normal goat serum (NGS), bovine serum albumin (BSA) and Sephadex G-75 were from Sigma Chemical Co. (St. Louis, MO). Brij 78 was a free sample from Uniqema (Wilmington, DE). PBS/Tween 20 buffer (20×) was from Scyteck Laboratories (Logan, UT). Anti-mouse IgG peroxidase-linked species specific F(ab′)$_2$ fragment (from sheep) was purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Goat anti-mouse IgA, peroxidase-linked, was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Tetramethylbenzidine (TMB) soluble reagent was from Pierce (Rockford, IL). Dioleoyl phosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL). CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit was purchased from Promega (Madison, WI).

Engineering of Brij-78-containing nanoparticles from microemulsion precursors

The engineering of cationic nanoparticles from microemulsion precursors was described previously (Cui & Mumper 2002a; Oyewumi & Mumper 2002). Briefly, exactly 2 mg of emulsifying wax was weighed and placed into twelve 7-mL glass scintillation vials. After melting at 50–55°C, a required volume of de-ionized and filtered (0.22 μm) water was added to the vials and vigorously stirred at 50–55°C until a homogenous milky slurry formed. Then, a required volume of Brij 78 (100 mM in water) was added while stirring to obtain a final Brij 78 concentration of 0–16 mM. Two minutes after the addition of Brij 78, a required volume of CTAB solution (50 mM in water) was added to obtain a final CTAB concentration of 0.8 mM. Stirring was maintained until microemulsions were formed (usually within about 3 min). The droplet sizes of the microemulsions were measured at 55°C by photon correlation spectroscopy (PCS) using a Coulter N4 Plus submicron particle sizer (Coulter Corporation, Miami, FL) at

90° angle for 90 s. The microemulsions were then cooled to room temperature while stirring to form solid cationic nanoparticles.

Stability of the nanoparticles in aqueous suspension

To study the short-term stability of these Brij-78-containing nanoparticles cured from microemulsions, the nanoparticle suspension prepared above was diluted 10 fold with water. The diluted nanoparticle suspensions were sealed in plastic cuvettes (Coulter) and kept at room temperature for 6 days. The particle size of the nanoparticles was measured daily as described above.

Effect of the final concentration of CTAB on the nanoparticles and their stability

The effect of CTAB concentration on the nanoparticles and their stability in suspension was studied by varying the final concentration of the CTAB in the microemulsion precursors from 0.2 to 0.8 mM, while keeping the Brij 78 concentration constant at 6 mM. The particle size of the nanoparticle suspensions in water at room temperature was measured daily for 7 days. Zeta potentials of the nanoparticle suspensions were measured using a Zeta Sizer 2000 (Malvern Instruments, Inc., Southborough, MA).

Purification of nanoparticles and its effect on the stability of the nanoparticles

The free Brij 78 or CTAB was removed using gel permeation chromatography (GPC) by passing the nanoparticles through a 6 × 210 mm Sephadex G-75 column. The column proved to be effective in separating the nanoparticles from the free surfactants. Previous studies showed that this purification process diluted the nanoparticles by roughly 10 fold. The particle sizes of these purified nanoparticle suspensions at room temperature were then measured daily for 7 days.

Transmission electron micrographs (TEM) of the nanoparticles

The size and morphology of nanoparticles were observed using TEM (Philips Tecnai 12 Transmission Electron Microscope) in the Electron Microscopy & Imaging Facility at the University of Kentucky Medical Center (Cui & Mumper 2002a). Briefly, a carbon coated 200-mesh copper specimen grid (Ted Pella Inc., Redding, CA) was glow-discharged for 1.5 min. One-drop (5 μL) of the nanoparticle suspension was deposited on the grid and left to stand for 1.5 min and any excess fluid was removed with filter paper. The grid was stained with one drop of 1% uranyl acetate solution (0.2-μm filtered) for 30 s and any excess uranyl acetate was removed with filter paper. The grids were allowed to dry for an additional 30–60 min and then examined.

Coating of plasmid DNA on the surface of the cured cationic nanoparticles

For the following in-vitro cell transfection and in-vivo animal studies, selected nanoparticles were GPC-purified (with 10% w/w filtered lactose solution as the mobile phase), passed through a 0.22-μm filter unit (Gelman Sciences). Then, a required amount of pDNA was added to the cured nanoparticle suspension followed by brief vortexing for 4–5 s. The pDNA-coated nanoparticles were allowed to remain at room temperature for at least 30 min before measuring particle size and zeta potential.

In-vitro transfection of Hep G2 cells with the pDNA-coated nanoparticles

In-vitro cell transfection was completed as previously described (Cui & Mumper 2002a). Hep G2 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and were maintained in Eagle's Minimum Essential Medium (EMEM) (Gibco, BRL) containing 10% fetal bovine serum (Gibco, BRL) and 1% penicillin–streptomycin (Gibco, BRL). Transfections were performed on cells that were approximately 80% confluent. Cells were plated in 48-well plates at a density of 5×10^5 cells/well and incubated overnight. The cells were co-incubated with the formulations to provide a final plasmid (CMV-luc) concentration of 2.5 μg/well. Cells were harvested after 48 h by removing the media, washing with 1 × PBS buffer, and then adding 200 μL 1 × Lysis buffer (Promega), leaving for 5–10 min and freeze–thawing 3 times. A 20-μL sample was assayed for luciferase activity by injecting 100 μL reconstituted luciferase assay solution (Promega) using a ML2250 Dynatech Luminometer (Dynatech Laboratories, Chantilly, CA). The protein content of a 20-μL sample of the supernatant was determined using the Coomassie Plus Protein Assay Reagent (Pierce). As recommended by Dynatech, luciferase expression data were reported as the ratio of the full integral of the samples to that of the negative control, divided by the total amount of protein in the 20 μL of samples assayed.

Intranasal immunization of mice

Female Balb/C mice, 10–12 weeks old, from Harlan Sprague-Dawley Laboratories were used for all animal studies. NIH guidelines for the care and use of laboratory animal were observed. Twenty-five microlitres of pDNA (CMV-β-gal, 50 μg mL^{-1})-coated nanoparticle suspensions in 10% lactose or pDNA alone (50 μg mL^{-1}) in 155 mM saline was administered intranasally into mice (n = 5 per group) on day 0, day 7 and day 14. Mice were held upright, and the formulations were carefully dripped on one nostril with a pipette tip for the mouse to inhale. On day 28, the

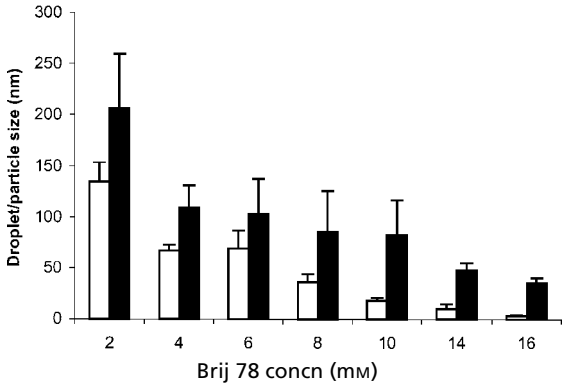

Figure 1 Engineering of cationic nanoparticles from microemulsion precursors. Nanoparticles (black bars) were cured from the microemulsion precursors (white bars), prepared with emulsifying wax (2 mg mL^{-1}), CTAB (0.8 mM), and increasing concentrations of Brij 78 (2–16 mM) at 55°C, by simply cooling to room temperature. Data were reported as mean ± s.d. (n = 3). A one-way analysis of variance showed both the microemulsion droplet size and the nanoparticle size were significantly affected ($P < 0.05$) by the final concentration of Brij 78.

mice were anaesthetized and then bled by cardiac puncture. The blood was transferred into a Vacutainer brand blood collection tube (Becton Dickinson and Company, Franklin Lakes, NJ). Serum was separated and isolated by centrifugation, and frozen at −20°C. After blood collection, the spleens of the mice were removed and placed into 5 mL of Hank's Balanced Salt Solution (HBSS) (1×) (Gibco BRL) for splenocyte preparation.

The two different pDNA-coated nanoparticle suspensions administered to the mice were, firstly, Brij-78-containing nanoparticles (Brij-NP/DNA) and, secondly, Brij-78-free nanoparticles (CTAB-NP/DNA). CTAB-NPs were engineered from microemulsions containing emulsifying wax (2 mg mL^{-1}) and CTAB (15 mM). The Brij-NPs were engineered from microemulsions containing emulsifying wax (2 mg mL^{-1}), CTAB (2.5 mM) and Brij 78 (6 mM). DOPE (5% w/w) was also incorporated into both nanoparticles.

Immunological assays

β-Gal-specific serum IgG and IgA titres were quantified by ELISA with slight modification (Cui & Mumper 2001). Specifically, for the IgA titre determination using a double antibody capture technique, anti-mouse IgA HRP-labelled was diluted 1:5000 in 1% BSA (instead of the 1:3000 as for IgG). Mouse splenocyte preparation and splenocyte proliferation assays were completed as previously described with minor modification (Cui & Mumper 2002b). Briefly, for the splenocyte proliferation assay, 5×10^6 cells/well of the isolated splenocytes (from individual mice) were co-incubated with 0 or 3.3 μg/well β-galactosidase for 4 days before assay using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay Kit (Rapaport & Robinson 1993). Proliferation results were reported as the percent increase in the OD490 after 3 more hours of co-incubation with 3-(4,5-dimethylthiazol- 2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium/phenazine methosulfate (MTS/PMS) solution (Promega).

Statistical analysis

Except where mentioned, all statistical analyses were completed using a one-way analysis of variance followed by pairwise comparisons with Fisher's protected least significant difference procedure (PLSD). A *P*-value of $\leqslant 0.05$ was considered to be statistically significant.

Results and Discussion

To prevent pathogen transmission, especially viral transmission, across the mucosal epithelium, and to prevent viral spreading to the local lymph nodes and organs, effective vaccines should ideally induce immune responses at the mucosal tissues and the associated lymph nodes (McGhee et al 1992). These responses include secretory IgA and IgG that were reported to mediate virus neutralization and to prevent virus adhesion (McGhee et al 1992; Almeida & Alpar 1996).

Previously, we reported on a novel nanoparticle-based pDNA delivery system prepared by adsorbing pDNA on pre-formed cationic nanoparticles engineered from microemulsion precursors (Cui & Mumper 2002a, b). The microemulsions, comprised of emulsifying wax (2 mg mL^{-1}) and CTAB, were prepared at increased temperature (50–55°C) and were simply cooled to room temperature to form nanoparticles (around 100 nm). It was found that to form microemulsions, a minimal final CTAB concentration of 5 mM was required (Cui & Mumper 2002a). The nanoparticles engineered from microemulsions containing 2 mg mL^{-1} of emulsifying wax and 15 mM of CTAB were used for further in-vivo mouse immunization studies since the nanoparticles engineered with this composition provided the highest stability in aqueous suspension and had relatively smaller particle sizes. An endosomolytic lipid, DOPE, was successfully incorporated into the nanoparticles. These pDNA-coated nanoparticles were administered to mice subcutaneously and topically, leading to significantly enhanced immune responses evidenced by the results of serum antigen-specific IgG level, antigen-specific in-vitro isolated splenocyte proliferation or in-vitro cytokine release from the isolated splenocytes, when compared with the naked pDNA alone (Cui & Mumper 2002a, b). One of the overall objectives of this study was to further explore the potential of this nanoparticle-based pDNA delivery system by assessing its ability to elicit immune responses in mice after intranasal administration.

In the previous studies, the final amount of CTAB administered per mouse was estimated to be about 20 μg (per 100 μL). At this dose of CTAB, no gross inflammatory, allergic or toxic effects were observed in mice by subcutaneous, intramuscular and topical routes. However, in this study, we sought to reduce the overall amount of

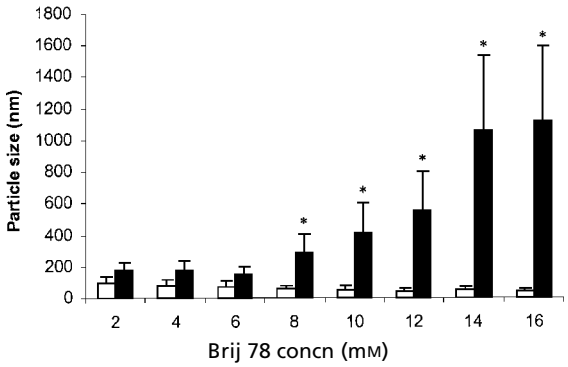

Figure 2 Short-term stability of the Brij-78-containing nanoparticles in aqueous suspension. Nanoparticles were engineered as described in Figure 1. After 10-fold dilution with water, the nanoparticle suspensions were kept at room temperature and the sizes were measured daily for 6 days. Reported (mean ± s.d., n = 3) are the sizes of the nanoparticles on day 0 (white bars) and day 6 (black bars). *Nanoparticles prepared with final Brij 78 concentration $\geqslant$ 8 mM; their sizes were significantly increased ($P < 0.05$) after 6 days of storage in aqueous suspension as determined by a two-sample *t*-test assuming equal variances. Moreover, a one-way analysis of variance showed that on day 6, the size of the nanoparticles was significantly affected ($P < 0.05$) by the final Brij 78 concentration.

CTAB used in the microemulsions by the co-incorporation of a non-ionic surfactant. This was approached by using Brij 78, in combination with decreasing amount of CTAB, to form O/W microemulsions with emulsifying wax as oil phase. Brij 78, a polyoxyethylene 20 stearyl ether, was previously used alone as surfactant to form neutral emulsifying wax nanoparticles (Oyewumi & Mumper 2002).

Engineering of cationic nanoparticles from microemulsion precursors

Nanoparticles were engineered by curing warm microemulsions, prepared with emulsifying wax (2 mg mL^{-1}), a fixed final CTAB concentration of 0.8 mM, and with a final Brij 78 concentration of 0–16 mM. Microemulsions were readily formed at Brij 78 concentration starting at 4 mM (Figure 1). As the final Brij 78 concentration increased from 4 to 16 mM, the microemulsion droplet sizes decreased from about 70 nm to 4 nm. In contrast, when the final Brij 78 concentration was $\leqslant$ 2 mM, microemulsions were not formed and the preparation remained very turbid. Simply cooling the warm microemulsions to room temperature produced solid nanoparticles having a size around 100 nm. Similar to observations in previously reported nanoparticle systems, the sizes of the cured nanoparticles were generally larger than their corresponding microemulsion droplets (Cui & Mumper 2002a). The reasons for these size differences between the droplets at 55°C and the nanoparticles at room temperature are currently under investigation. However, it was observed that the final concentration of the Brij 78 may be related to this phenomenon. For example, the size increase from the droplets to nanoparticles for the preparation with 16 mM Brij 78 was about

Table 1 The effect of final CTAB concentration on the size, zeta potential and stability of the engineered cationic nanoparticles.

Nanoparticles	CTAB concn (mM)	Size[a] on day 0 (nm)	Size[a] on day 7 (nm)	Size increase[b] (%)	Zeta potential[c] (mV)
Unpurified nanoparticles					
	0.2	62±23 (0.331)	107±37 (0.230)	74	25.1±5*
	0.3	76±30 (0.461)	97±36 (0.311)	28	41.1±4
	0.4	96±38 (0.453)	121±40 (0.201)	26	51.2±10
	0.5	87±34 (0.373)	114±41 (0.281)	30	42.7±3
	0.6	99±38 (0.362)	126±43 (0.220)	26	44.6±5
	0.7	116±38 (0.195)	133±47 (0.258)	15	44.5±6
	0.8	113±39 (0.233)	143±45 (0.171)	27	41.9±8
GPC-purified nanoparticles					
	0.2	65±24 (0.319)	84±25 (0.133)	29	N/D
	0.3	90±34 (0.326)	103±35 (0.221)	14	N/D
	0.4	91±31 (0.217)	108±38 (0.261)	18	N/D
	0.5	88±32 (0.275)	108±29 (0.108)	23	N/D
	0.6	102±35 (0.229)	119±26 (0.062)	17	N/D
	0.7	124±40 (0.190)	N/D	N/D	N/D
	0.8	116±42 (0.286)	132±35 (0.094)	14	N/D

Nanoparticles were prepared from microemulsion precursors comprised of emulsifying wax (2 mg $mL)^{-1}$, Brij 78 (6 mM) and increasing concentration of CTAB (0.2–0.8 mM). The nanoparticles were then either diluted 10 fold with water or were gel permeation chromatography (GPC)-purified and particle size was measured daily for 7 days. [a]Particle sizes were reported as size ± s.d. (polydispersity index). [b]A two-sample *F*-test for variances showed that the % size increases for the GPC-purified nanoparticles over 7 days were significant lower than that for the unpurified nanoparticles. [c]The zeta potentials of the unpurified nanoparticles on day 0 were reported. N/D, not determined. *$P < 0.05$, vs other zeta potentials.

1000%, while that for the preparation with 6 mM Brij 78 was only about 44%.

Figure 2 shows the short-term stability of diluted nanoparticles in aqueous suspension at room temperature. In general, the particle sizes increased over time. Overall, although Figure 2 points to the need for alternative means to store these nanoparticles, it also strongly demonstrated that high concentration of Brij 78 surfactant in the preparation resulted in increased particle size of the nanoparticles in aqueous suspension. The mechanism of Brij-78-induced instability is currently under investigation. However, the nanoparticles prepared with lower concentrations of Brij 78 were relatively stable. Therefore, final Brij 78 concentrations of 4 or 6 mM were used to prepare nanoparticles for further studies.

The system was further studied by generating nanoparticles from microemulsions prepared with final Brij 78 concentration fixed at 6 mM while varying the concentration of the CTAB from 0.2 to 0.8 mM. These nanoparticles were also successfully purified using gel permeation chromatography (GPC) with a Sephadex G75 column. Table 1 shows some physical properties of these GPC-purified and unpurified nanoparticles. As expected, the stability of these nanoparticles in aqueous suspension was improved at lower Brij 78 concentration (6 mM). Also, purification by GPC to separate free surfactants from the nanoparticles appeared to improve the stability of these nanoparticles, evidenced by the smaller percent size increase of the GPC-purified nanoparticles than that of the unpurified ones ($P = 0.01$; using a two-sample F-test for variances) (Table 1). Interestingly, although the zeta potential of the nanoparticles increased when the final concentration of CTAB increased from 0.2 to 0.3 mM, the zeta potential reached a plateau at a final CTAB concentration of 0.3 mM (Table 1).

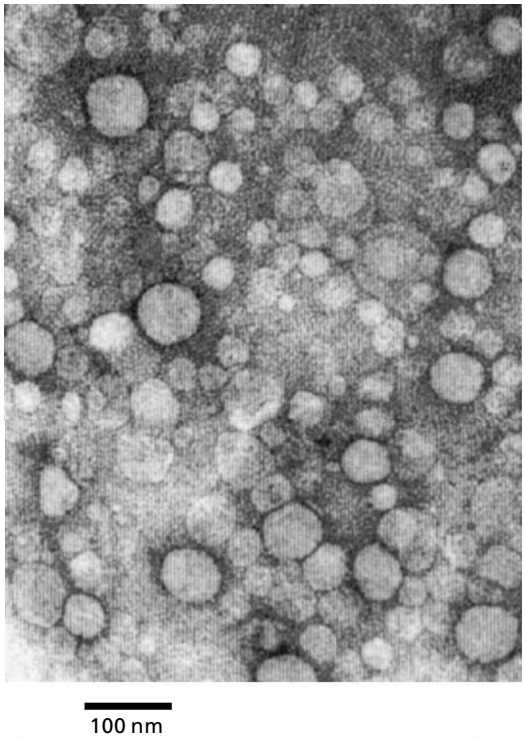

Figure 3 Transmission electron micrograph (TEM) of Brij-78-containing cationic nanoparticles.

Transmission electron micrograph (TEM) of the nanoparticles

Based on the aforementioned studies, nanoparticles were engineered from microemulsion precursors comprised of 2 mg mL^{-1} emulsifying wax, 6 mM Brij 78 and 0.8 mM of CTAB. The nanoparticles were spherical, relatively uniform in size and mostly under 100 nm (Figure 3).

In-vitro cell transfection

Coating of pDNA on the nanoparticles increased the transfection efficiency by more than 10 fold over the naked pDNA alone (Figure 4). The in-vitro cell transfection efficiency of these pDNA-coated nanoparticles was also found to be over 3 times that of the pDNA alone in other cell lines such as A549, a lung carcinoma cell, and C_2C_{12}, a myoblast (unpublished data). It was not surprising to find these improvements in transfection efficiency since it is well known that naked pDNA alone, a very negatively charged macromolecule, does not transfect cells well in-vitro. Importantly, these in-vitro transfection studies demonstrated that the pDNA adsorbed on the cationic nanoparticles retained its functionality.

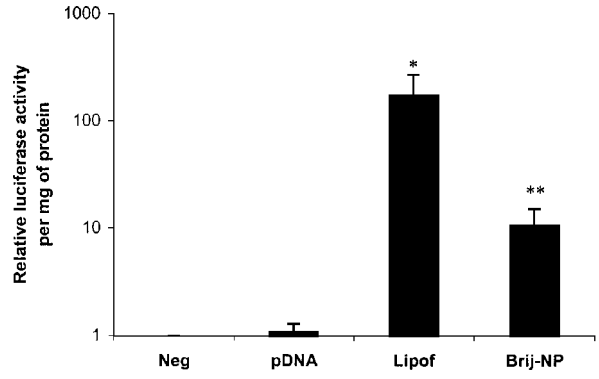

Figure 4 In-vitro transfection of liver Hep G2 cells (50000 cells) (n = 3) in the presence of 10% FBS after 48 h with pDNA alone, pDNA-complexed Lipofectin (Lipof) or pDNA-coated Brij-78-containing nanoparticles (Brij-NP). The pDNA dose was 2.5 μg for all samples. The pDNA-coated nanoparticles had particle size and zeta potential of 124 ± 30 nm and 36 ± 5 mV, respectively. As recommended by Dynatech, luciferase expression data were reported as the ratio of the full integral of the samples to that of the negative control (Neg), divided by the total amount of protein in the 20 μL of samples assayed. *The result of the Lipofectin was significantly different ($P < 0.05$) to that of the others. **The result of the Brij-NP was significantly different ($P < 0.05$) to that of the others.

Immune responses after intranasal immunization to mice

DOPE, a known endosomal disrupting lipid (Farhood et al 1995), was found to be necessary to significantly enhance the immune responses elicited by our previously reported pDNA-coated nanoparticles (Cui & Mumper 2002a, b). As mentioned in Materials and Methods, to incorporate 5% (w/w) of DOPE into these Brij-78-containing nanoparticles, a final CTAB concentration of 0.8 mM was insufficient. Consequently, for the following animal studies, the Brij-78-containing nanoparticles (Brij-NP/DNA) were generated from microemulsion precursors prepared with 2.5 mM of CTAB, while the concentration of the emulsifying wax and Brij 78 was kept constant at 2 mg mL^{-1} and 6 mM, respectively. The size and zeta potential of the Brij-NPs used in the mouse studies before and after pDNA coating (50 μg mL^{-1}) were 123±2 nm (P.I. 0.183), 45.7±1.4 mV and 302±19 nm (P.I. 0.380), 9.9±2.6 mV, respectively. CTAB-NPs containing no Brij 78 were the same nanoparticles used in previous subcutaneous and topical immunization studies. The size and zeta potential before and after pDNA coating (50 μg mL^{-1}) were 110±2 nm (P.I. 0.072), 29.6±6.6 mV, and 197±13 nm (P.I. 0.062), −20.7±2.6 mV, respectively. As expected, after pDNA coating, the nanoparticle size increased and the zeta potentials decreased, providing evidence for the successful coating of pDNA on the cationic nanoparticles. The phenomenon of particle size increase after plasmid DNA coating was observed previously (Cui & Mumper 2002a,b). It was believed that aggregation caused by the cross-link of several nanoparticles by one pDNA molecule, or vice versa, was responsible for the observed size increase. An improved procedure to mix pDNA with preformed nanoparticles in suspension may be required to allow pDNA to uniformly coat the surface of the nanoparticles and, at the same time, avoid the formation of multi-particle aggregates. The zeta potential difference between the Brij-NPs and CTAB-NPs, both before and after pDNA coating, may be attributed to the shift of shear plane of the nanoparticles due to the presence of the longer Brij 78 chain on the surface of the particles (Tobio et al 1998). Steric hindrance from the Brij 78 chain may also contribute to the observed zeta potential differences.

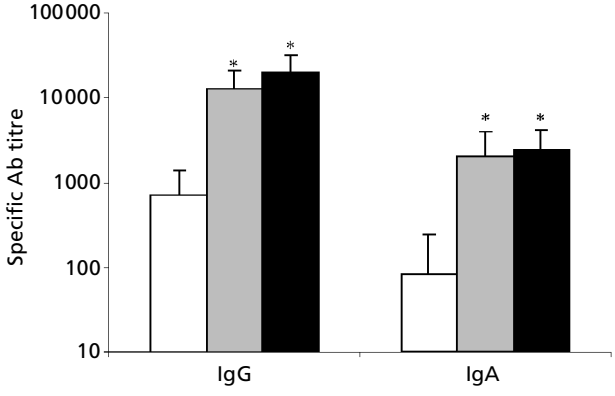

Figure 5 Specific total IgG titre and IgA titre in serum to expressed β-galactosidase antigen 28 days after intranasal administration. Twenty-five microlitres (25 μL = 1.25 μg) of pDNA was administered to Balb/C mice (n = 5) on days 0, 7 and 14. The data were reported as the mean±s.d. *The IgG and the IgA titres of the Brij-NP/DNA (grey bars) and CTAB-NP/DNA (black bars) immunized mice were significantly higher than those of the mice immunized with pDNA alone (white bars), while there was no significant difference ($P > 0.05$) between the two different nanoparticles in inducing IgG and IgA titres.

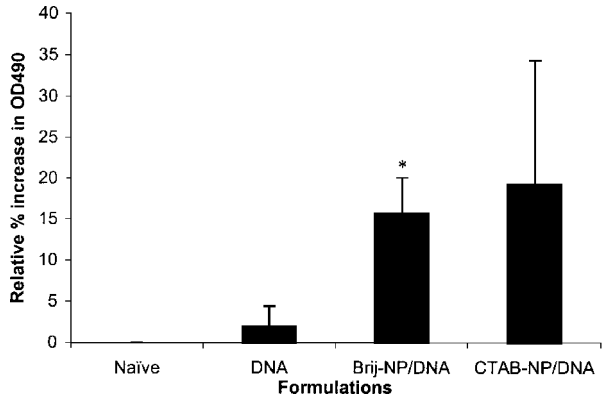

Figure 6 In-vitro proliferation of isolated splenocytes 28 days after intranasal immunization with pDNA alone or pDNA-coated nanoparticles (Brij-NP/DNA and CTAB-NP/DNA). Twenty-five microlitres (25 μL = 1.25 μg) of pDNA was administered to Balb/C mice (n = 5) on days 0, 7 and 14. At day 28, the mice were sacrificed and their spleens were removed. Isolated splenocytes (5×10^6 cell/well) were incubated with either 0 or 3.3 μg/well of β-galactosidase for 4 days. The cell proliferation was reported as the relative% increase of the OD490 of the stimulated cells (3.3 μg/well) over the corresponding un-stimulated splenocytes (0 μg/well). The data were reported as the mean±s.d. *The result of the Brij-NP/DNA immunized mice was significantly different ($P < 0.05$) from that of the pDNA-alone immunized mice.

After intranasal administration, both of the pDNA-coated nanoparticles led to significantly enhanced antigen-specific IgG level in serum, compared with the naked pDNA alone (i.e. 18-fold IgG titre using Brij-NP/DNA and 28-fold using CTAB-NP/DNA) (Figure 5). Moreover, the antigen-specific IgA titres in the pDNA-coated nanoparticles immunized mice were 25- to 30-fold higher than that in the naked pDNA immunized mice, strongly indicating an enhancement of mucosal immunity caused by these pDNA-coated nanoparticles. Although it would be more persuasive if the local specific IgA levels, such as in nasal, vaginal and genital fluids, were measured in these studies, such a marked enhancement of the antigen-specific IgA level in serum strongly suggested the local specific IgA level should also be enhanced.

Finally, based on the percent increase in OD490, immunization with the pDNA-coated Brij-NP led to significant enhancement in the in-vitro proliferation of isolated splenocytes after stimulation with the protein antigen (β-galactosidase), as compared with immunization with pDNA alone (Figure 6). OD490 increase (instead of the splenocyte number increase) was reported, since a linear

relationship between OD490 and splenocyte number could not be attained. Actually, the co-incubation of MTS/PMS solution with the splenocytes for 3 h was most likely too long since most of the samples were found to have OD 490 nm of more than 2.0. Thus, when the assay conditions are further optimized, the actual OD490 increase would be much greater than those reported in these studies.

As mentioned above, it is well established that mucosal immunization is an effective method to induce long-term immunity. Intranasal immunization offers a practical and effective means to elicit secretory IgA (sIgA) in both local and distant mucosa, such as the nasal, upper respiratory and vaginal mucosa, and the circulation (serum IgA) (Klaviskis et al 1999; Partidos 2000; Davis 2001). For example, by intranasal immunization of mice with 100 μg (for 4 times) HIV-1 gag encoding plasmid adsorbed on cationic PLGA nanoparticles, Singh et al (2002) reported a 5-fold enhancement in the antigen-specific serum IgA titre over naked pDNA alone. Similarly, a comparison of immunization with a single dose (50 μg) of firefly luciferase gene encoding plasmid as either naked pDNA or complexation of pDNA with liposome (DMRIE/DOPE) showed that the complexed pDNA immunization enhanced the specific IgA titres in mouse serum, vaginal and rectal fluid by 17-, over 6- and 6-fold, respectively (Klavinskis et al 1999). In this study, using a pDNA dose as low as 1.25 μg, intranasal administration of these novel nanoparticle-based pDNA delivery systems resulted in the enhancement of antigen-specific serum IgG and IgA titres by 18–28 fold and 25–30 fold, respectively, over the naked pDNA alone. These results strongly suggested the potential of these nanoparticles as a pDNA vaccine delivery system by intranasal route.

A comparison of the two pDNA-coated nanoparticle systems used in the present study, the pDNA-coated Brij-78-containing nanoparticles (Brij-NP) and the pDNA-coated nanoparticles containing no Brij 78 (CTAB-NP), did not reveal significant differences in their ability to induce immune responses (Figures 5 and 6). However, the cationic surfactant (CTAB) level in the Brij-NPs was lowered by at least 6 fold, resulting in the final administered CTAB amount of about 500–600 ng (in the 25-μL volume). CTAB, a quaternary ammonium surfactant, is generally used in cosmetics and pharmaceutical formulations as an antimicrobial preservative. In eye-drops, CTAB was used as a preservative at a concentration of 0.005% w/v (Wade & Weller 1994). Although at a dose of about 20 μg per mouse (in 100 μL), no gross inflammatory, allergic or toxic effects were observed after intramuscular, subcutaneous and topical application, it was one of our objectives to further reduce the amount of CTAB used. Brij 78, a polyoxyethylene alkyl ether, is widely used as emulsifying agent for W/O and O/W emulsions (Wade & Weller 1994). The final intranasal Brij dose per mouse in our studies was about 5 μmol. The nonionic emulsifying wax is comprised of cetyl alcohol and polysorbate 60 in a molar ratio of about 20:1. The wax is typically used in cosmetics and topical pharmaceutical formulations and is generally regarded as a non-toxic and non-irritant excipient. For example, cetyl alcohol is currently used as an excipient in the marketed product Exosurf Neonatal. Polysorbate 60 is used in many pharmaceutical products, including parenteral products. Moreover, we recently found that the emulsifying wax may be replaced by phosphatidyl choline (lecithin), a USP excipient used in a wide variety of pharmaceutical applications, as well as cosmetics and food products (unpublished data). If necessary, future formulations may use lecithin instead of emulsifying wax.

In these studies, no effort was made to elucidate the mechanism(s) of the enhancement in immune response after immunization with these pDNA-coated nanoparticles. The possible mechanisms include: adjuvant effect by the nanoparticles; enhanced antigen expression; enhanced pDNA stability by coating on the nanoparticles; and possible enhancement of uptake of the pDNA-coated nanoparticles into the nasal-associated lymphoid tissues (NALT) and their delivery to antigen-presenting cells (APC). Generally, soluble antigens are able to penetrate the nasal epithelium and interact with dendritic cells, macrophages and other lymphocytes. In contrast, antigen in the form of colloidal particles is mostly taken up by M-cells overlaying the follicle-associated epithelium of the NALT (Kuper et al 1992). It is possible that by coating pDNA on the surface of these nanoparticles, it will more likely be taken up by the M-cells and thus lead to enhanced immune responses. Possible mechanisms of enhancement of immune responses with these nanoparticle-based pDNA delivery systems are currently under investigation.

Conclusions

In this study, a previously reported novel cationic nanoparticle-based pDNA vaccine delivery system was further modified and optimized. A non-ionic surfactant, Brij 78, was co-incorporated into these nanoparticles to reduce the amount of cationic surfactant, CTAB, by 6 fold. When administered intranasally, both the modified and original pDNA-coated nanoparticles were able to enhance specific IgG and IgA titres in mouse serum to an expressed model antigen, β-galactosidase, by 18–28 fold and 25–30 fold, respectively, over the naked pDNA alone. Moreover, an enhanced splenocyte proliferative response was also observed after immunization with these pDNA-coated nanoparticles. These results strongly suggested the potential of these nanoparticles as a pDNA vaccine delivery system by intranasal route.

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