ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 55 (2003) 11–18

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

The effect of co-administration of adjuvants with a nanoparticle-based genetic vaccine delivery system on the resulting immune responses

Zhengrong Cui, Russell J. Mumper*

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

Received 23 July 2002; accepted in revised form 24 September 2002

Abstract

Previously, we reported on a novel cationic nanoparticle-based DNA vaccine delivery system. In the present studies, the effects of co-administration of two well-known adjuvants, cholera toxin (CT) and lipid A (LA), with plasmid DNA (pDNA)-coated nanoparticles were investigated. Balb/C mice ($n = 6$) were immunized with either pDNA alone (cytomegalovirus-β-galactosidase, 5 μg) or pDNA-coated nanoparticles with either 0 or 50 μg of LA on days 0, 7, and 14 subcutaneously (s.c.), or topically on shaved skin with either pDNA (5 μg) alone or pDNA-coated nanoparticles with 0, 10, or 100 μg of CT on days 0, 6, 21, and 35. Mice were sacrificed on day 28 or day 45. Serum IgG titer, in vitro cytokine release and cell proliferation of the isolated splenocytes were determined. By the topical route, immunization of mice with 'naked' pDNA together with 10 and 100 μg of CT significantly enhanced the antigen-specific serum IgG titer by four- and 20-fold, respectively, compared to immunization with pDNA alone. Moreover, co-administration of 100 μg CT with the pDNA-nanoparticles enhanced the IgG titer by more than 300-fold over immunization with 'naked' pDNA alone with no CT. In vitro interferon-γ (IFN)-γ release from splenocytes isolated from mice immunized with pDNA-coated nanoparticles with CT (100 μg) was increased by three-fold over immunization with pDNA-nanoparticles without CT. Similarly, in vitro IFN-γ release from splenocytes isolated from mice immunized with 'naked' pDNA with CT (100 μg) was increased by two-fold over immunization with 'naked' pDNA without CT. Finally, pDNA-coated nanoparticles adjuvanted with 10 μg CT resulted in the strongest splenocyte proliferation. By the s.c. route, co-administration of LA (50 μg) with pDNA resulted in more than 16-fold enhancement in IgG titer over immunization with 'naked' pDNA alone. Immunization with pDNA-coated nanoparticles with LA (50 μg) led to 16-fold enhancement in specific serum IgG titer over immunization with pDNA-coated nanoparticles with no LA, and more than 250-fold enhancement over immunization with 'naked' pDNA alone with no LA. Moreover, in vitro IFN-γ release and proliferation by splenocytes isolated from LA co-immunized mice was also significantly enhanced. In conclusion, CT (topical) and LA (s.c.) are potential adjuvants to further enhance immune responses using a novel cationic nanoparticle-based DNA vaccine delivery system.

Keywords: DNA vaccine; Lipid A; Cholera toxin; Topical; Immunization

1. Introduction

Traditionally, vaccines have been comprised of live attenuated viruses or killed bacteria. However, plasmid DNA (pDNA)-based vaccines have attracted much attention recently [1]. DNA-based vaccines may be safer than traditional vaccines and are able to elicit both humoral and cellular immune responses [1,2]. In addition, DNA vaccines may be relatively stable, cost-effective for manufacture and storage, and may allow for potential simultaneous immunization against multiple antigens or pathogens [3]. Further, deoxy-cytidylate-phosphate-deoxy-guanylate (CpG) motifs on pDNA have been shown to have an adjuvant effect [2]. However, as compared to other vaccines, DNA vaccines are relatively poorly immunogenic in terms of eliciting antibody responses [2]. Also, since the first proof-of-concept immunization with 'naked' pDNA, DNA vaccines have mainly been administered by intramuscular injection. Intramuscular injection of 'naked' pDNA vaccines has proven to be very effective in several different small animal models. However, recent results from human and non-human primate studies have been disappointing due to sub-optimal immune responses even using multi-milligram doses of

* Corresponding author. Center for Pharmaceutical Science and Technology, College of Pharmacy, University of Kentucky, 907 Rose Street, Lexington, KY 40536-0082, USA. Tel.: +1-859-257-2300, ext. 258; fax: +1-859-323-5985.

E-mail address: rjmump2@uky.edu (R.J. Mumper).

0939-6411/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0939-6411(02)00129-7

'naked' pDNA [3]. Therefore, there is a clear need to improve the potency of DNA vaccines.

Novel delivery systems and/or alternative routes for administration may be keys to address this need. Over the last 20 years, it has been established that the development of vaccines, including DNA vaccines, as particulates in the scale of micrometer or nanometer can help to improve the potency of the vaccines [4–6]. Previously, we reported on the development of a novel nanoparticle-based DNA vaccine delivery system engineered from oil-in-water (O/W) microemulsion precursors [7]. The microemulsions, formed at increased temperature (50–55 °C), were comprised of emulsifying wax as the oil phase and a cationic surfactant, cetyltrimethylammonium bromide (CTAB). Upon simple cooling of these microemulsion precursors to room temperature in the same container, cationic nanoparticles ($\leq$100 nm) were readily formed. pDNA was then coated on the surface of these pre-formed nanoparticles to form pDNA-coated nanoparticles. Both an endosomolytic lipid, dioleoyl phosphatidyl ethanolamine (DOPE), and a potential dendritic cell (DC)-targeting ligand, mannan, were successfully incorporated in, or deposited on, the surface of the nanoparticles to modify and/or improve the performance of the pDNA-coated nanoparticles both in vitro and in vivo. Immunization of mice with these pDNA-coated nanoparticles by subcutaneous (s.c.) injection, intradermal injection via a needle-free injection device, topical application on the skin, or intranasal application led to enhanced immune responses to a model expressed antigen, β-galactosidase [7–10]. For example, the antigen-specific total IgG titer in the sera of mice immunized with the pDNA-coated nanoparticles was enhanced by 16–200-fold over immunization with 'naked' pDNA alone by these routes of administration [7–10].

By definition, any material that aids the humoral and/or cellular immune responses to an antigen, but is itself immunologically inert, is referred to as an adjuvant [11]. Adjuvants have been used to enhance the immune responses to antigens for about 70 years [11]. During the last 70 years, many adjuvants have been developed, but few of them have been evaluated in clinical trials [12]. One of the most studied and best-defined immunomodulating molecules, cholera toxin (CT), has proven to be an extremely potent adjuvant [13]. CT has mainly been used as an adjuvant for mucosal immunization by the intranasal or oral routes [13]. Recently, Glenn et al. reported that CT, by co-administering with bovine serum albumin (BSA), can perform as an adjuvant to induce potent immune responses to BSA, when topically applied on shaved mouse skin [14]. This so-called 'transcutaneous immunization' has now proven to be a viable immunization modality in mice, sheep, cats, dogs, and even humans [15,16]. Topical immunization with DNA vaccines on skin has also proven to be feasible [17]. However, the potency of topical DNA immunization was found to be rather low. Therefore, one of the aims of the present studies was to test whether CT can be used as an adjuvant to improve immune responses after non-invasive topical pDNA administration. Specifically, it was hypothesized that co-administration of CT with our previously developed pDNA-coated nanoparticles would result in a further enhancement in immune responses.

The adjuvant effect of lipopolysaccharide (LPS) was first described as early as 1956 [18]. The lipid A (LA) region of the LPS was found to be responsible for the adjuvanticity. LA, which generally aids a T cell type 1 (Th1)-type response, enhances immune responses primarily through its ability to activate antigen-presenting cells (APCs) and to induce cytokine release [18]. The first evidence that LA, an adjuvant conventionally used for protein (subunit)-based vaccines and other traditional vaccines, had an adjuvant effect with a DNA-based vaccine was reported by Sasaki et al. in 1997 [19]. Following this initial report, there were several other attempts to use LA as DNA vaccine adjuvant by different routes [20,21]. Another interesting property of LA is that it can also be used to enhance or complement the activity of antigen delivery vehicles such as 'Alum', liposomes [22], and microparticles [23]. Recently, Wang et al. incorporated both pDNA and LA into poly(*d*,*l*-lactic-co-glycolic acid) (PLGA) microspheres for potential DNA vaccine delivery, although no in vivo results were reported [24]. Therefore, in the present studies, it was also hypothesized that co-administration of LA with our previously developed pDNA-coated nanoparticles would result in a further enhancement in immune responses.

2. Materials and methods

2.1. Materials

Plasmid containing a cytomegalovirus (CMV) promoter with a β-galactosidase reporter gene (CMV-β-gal) was a gift from Valentis, Inc. (The Woodlands, TX). The plasmid had endotoxin levels of $<$0.1 EU/mg. Emulsifying wax (N.F. grade) was purchased from Spectrum Quality Products, Inc. (New Brunswick, NJ). CTAB, β-galactosidase, normal goat serum (NGS), BSA, triethanolamine (TEA), and Sephadex G-75 were from Sigma (St. Louis, MO). 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). Tetramethylbenzidine (TMB) soluble reagent was from Pierce (Rockford, IL). DOPE was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). {*N*-[2-(Chloesterylcarboxyamino)ethyl]carbamoylmethyl}mannan (chol-mannan) was purchased from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). LA from *Salmonella Minnesota* R595 (Re) LPS and CT from *Vibrio cholera* Inaba 569B were purchased from List Biological Laboratories, Inc. (Campbell, CA). Mouse interleukin-4 (IL-4) and interferon-γ (IFN-γ) ELISA Kits were from

Pierce-Endogen, Inc. (Woburn, MA). A CellTiter 96® Aqueous non-radioactive cell proliferation assay kit was purchased from Promega (Madison, WI).

2.2. Engineering of pDNA-coated nanoparticles

pDNA-coated nanoparticles were prepared by coating CMV-β-gal (pDNA) on pre-formed cationic nanoparticles as previously described [7,9]. Briefly, emulsifying wax (2 mg/ml) was melted at 55 °C. Seven hundred (700) μl of water was added into the melted wax and stirred until a homogenous milky suspension was obtained. Then, 0.3 ml of CTAB solution (50 mM) was added into the homogenate while stirring to obtain a clear microemulsion. Nanoparticles were engineered by simple and direct cooling of this warm microemulsion to room temperature in the same container. For the incorporation of endosomolytic agent, 100 μg of DOPE (final 5% w/w) was mixed with the emulsifying wax (2 mg/ml) prior to microemulsion preparation. Chol-mannan, dissolved in hot water (5 mg/ml), was deposited on the surface of the nanoparticles by mixing 1 ml of the pre-formed nanoparticle suspension (2 mg/ml) with 250 μg of chol-mannan and stirred at room temperature overnight. Free CTAB and chol-mannan were removed by passing the nanoparticle suspension through a Sephadex G-75 column (14 × 65 mm) using 10% lactose as the mobile phase [7]. pDNA (CMV-β-gal) was coated on the surface of these pre-formed cationic nanoparticles by gently mixing 1 ml of the purified and filtered nanoparticles in suspension with pDNA to obtain a final pDNA concentration of 50 μg/ml. This system was allowed to remain for at least 30 min at room temperature for complete adsorption of pDNA on the surface of the nanoparticles before further use. The particle sizes and zeta potentials of the engineered nanoparticles, before and after pDNA coating, were measured using N4 Plus Sub-Micron Particle Sizer (Coulter Corporation, Miami, FL) and Zeta Sizer 2000 (Malvern Instruments, Inc., Southborough, MA), respectively.

2.3. Immunization of mice

Ten- to 12-week-old female mice (Balb/C) from Harlan Sprague–Dawley Laboratories were used for all animal studies. NIH guidelines for the care and use of laboratory animals were observed. Two independent mouse studies were completed. Mice were immunized either by s.c. injection or by non-invasive topical application on the skin. Subcutaneous immunization was performed as previously described with modification [7]. Briefly, on day 0, day 7, and day 14, mice ($n = 6$/group) were immunized with either 'naked' pDNA alone (CMV-β-gal, 5 μg) or pDNA (5 μg)-coated nanoparticles, mixed with 0 or 50 μg of LA prepared as an aqueous solution in 0.5% (v/v) TEA in water [25]. Mice were anesthetized using pentobarbital (i.p.) prior to each immunization. A volume of 150 μl of each formulation (in 10% lactose) was injected using an Insulin Syringe with MICRO-FINE® IV Needle from Becton Dickinson and Company (Franklin Lakes, NJ) on one site on the back. Naive mice ($n = 6$) were not treated. On day 28, the mice were anesthetized and bled by cardiac puncture. Sera were separated and stored as previously described [7]. Spleens from every mouse were also collected on day 28.

Topical immunization on mouse skin was completed as previously described with modification [9]. Mice ($n = 6$/group) were immunized with either 'naked' pDNA or pDNA-coated nanoparticles, mixed with 0, 10, or 100 μg of CT, on days 0, 6, 21, and 35 with a pDNA dose of 5 μg. Again, mice were anesthetized using pentobarbital (i.p.) prior to each immunization. The hair covering the back of the mouse was shaved with an A5® Single-Speed Clipper (Oster Professional Products, McMinnville, TN). The skin was wiped with an alcohol swab, allowed to air dry for 5 min, and 120 μl of each formulation was dripped and subsequently spread with a pipette tip onto the skin covering an area of about 2 cm^2. Care was taken to disperse the solution over the skin without applying pressure to the skin. On day 45, the mice were anesthetized, and the blood and spleens were collected and treated as described above. One group of naive mice was not treated and was used as a negative control.

2.4. Determination of antibody titer

β-Galactosidase-specific serum IgG titer was quantified using ELISA [7]. Briefly, Costar® high binding 96-well assay plates were coated with 50 μl of β-galactosidase protein (8 μg/ml) overnight at 4 °C. The plates were then blocked for 1 h at 37 °C with 4% BSA/4% NGS (Sigma) solution (100 μl/well) made in 1 × PBS/Tween 20 (Scytek). Mouse serum (50 μl/well, serial diluted and starting at 1:10 (for topical) or 1:64 (for s.c.) in 4% BSA/4% NGS/PBS/Tween 20) was incubated for 2 h at 37 °C. After washing three times with PBS/Tween 20 buffer, anti-mouse IgG HRP $F(ab')_2$ fragment from sheep (diluted 1:3000 in 1% BSA) was added (50 μl/well) and incubated for 1 h at 37 °C. Plates were washed three times with PBS/Tween 20 buffer. Finally, the samples were developed with 100 μl TMB substrate for 30 min at room temperature and then stopped with 50 μl of 0.2 M H_2SO_4. The OD of each well was measured using a Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VM) at 450 nm.

2.5. In vitro cytokine release and splenocyte proliferation

Splenocyte preparation, cytokine release and splenocyte proliferation assays were performed as previously described [9]. Spleens from two mice in the same group were pooled together (i.e. $n = 3$ per treatment) and placed into 5 ml of HBSS (Hank's balanced salt solution) (1 ×) in a Stomacher Bag 400 from Fisher Scientific (Pittsburgh, PA). The spleens were homogenized at high speed for 60 s using a Stomacker Homogenizer. Cell suspensions were then transferred into a 15 ml Falcon tube and filled to 15 ml with 1 × ACK buffer (156 mM of NH_4Cl, 10 mM of $KHCO_3$, and 100

μM of EDTA) for red blood cell lysis. After 5–8 min at room temperature, the suspension was spun down at 1500 rev./min for 7 min at 4 °C. After pouring off the supernatant, the cell pellet was re-suspended in 15 ml 1× HBSS. The suspension was then spun down at 1500 rev./min for 7 min at 4 °C. After washing with 15 ml of RPMI-1640 (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO) and 0.05 mg/ml of gentamycin (Gibco BRL), the cells were re-suspended in RPMI-1640 media (2 ml total or 1 ml for each spleen in the pool).

For in vitro cytokine release, isolated splenocytes (5×10^6/well) were seeded into a 48-well plate (Costar), and stimulated with 0 or 3.3 μg/well of β-galactosidase (Spectrum) for 48 h at 37 °C. Cytokines (IL-4 and IFN-γ) in the supernatant were quantified using ELISA kits from Endogen.

A CellTiter 96® Aqueous non-radioactive cell proliferation assay kit was used to determine the isolated splenocyte proliferation [9]. Similarly, isolated splenocytes (5×10^6/well) were seeded into a 48-well plate (Costar), and stimulated with 0 or 3.3 μg/well of β-galactosidase (Spectrum). After incubation at 37 °C with 5% CO_2 for 94 h, 60 μl of the combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS) solution (Promega) was pipetted into each well (20 μl/100 μl of cells in medium). After an additional 1 h of incubation at 37 °C with 5% CO_2, the absorbance at 490 nm was measured using a Universal Microplate Reader. The cell proliferation was reported as the % increase of the OD_{490} of the stimulated cells (3.3 μg/well) over the OD_{490} of un-stimulated cells (0 μg/well) (i.e. $100 \times (OD_{490\ \text{stimulated}} - OD_{490\ \text{un-stimulated}})/OD_{490\ \text{un-stimulated}}$).

2.6. Statistical analyses

Except where mentioned, all statistical analyses were completed using a one-way analysis of variances (ANOVA) followed by pair-wise comparisons with Fisher's protected least significant difference procedure (PLSD). The type I error rate was set at 0.05 for all analyses. A P value of ≤ 0.05 was considered to be statistically significant.

3. Results and discussion

The discovery that pDNA vaccines can elicit both humoral and cellular immune responses has attracted much attention in the vaccine and immunology communities [1,2]. However, after over a decade of intensive investigations, researchers have concluded that the potency of 'naked' pDNA vaccines is sub-optimal, especially in humans and non-human primates [3]. Therefore, there exists a clear need to improve the effectiveness of DNA vaccines. To address this unmet need, we developed a novel cationic nanoparticle-based DNA vaccine delivery system [7,9]. Enhanced immune responses to an expressed model antigen, β-galactosidase, were observed when mice were immunized with these pDNA-coated nanoparticles by both s.c. injection and topical application, as compared to immunization with 'naked' pDNA alone [7,9]. The pDNA-coated nanoparticles were formed by coating pDNA on the surface of pre-formed cationic nanoparticles (≤100 nm), which were engineered from warm O/W microemulsion precursors by simple cooling at room temperature. In the present studies, the same pDNA-coated nanoparticles were used to immunize mice. The size and zeta potential of the nanoparticles before pDNA coating were 128 ± 18 nm and 29 ± 6 mV, respectively. The size and zeta potential of the pDNA-coated nanoparticles were 171 ± 29 nm and −24 ± 4 mV, respectively. The final pDNA concentration was 50 μg/ml. The particle size of the LA suspension (in 5%, v/v TEA solution) used to add to the final formulations was 258 ± 8 nm ($n = 3$) with a polydispersity index of 1.092. After mixing the pDNA-coated nanoparticles with 50 μg of LA, the resulting final particle size of the final formulation was 282 ± 103 nm. CT, which is soluble in water, did not affect the size of the pDNA-coated nanoparticles when they were mixed together.

3.1. Effect of co-administration of CT on non-invasive topical DNA immunization

Non-invasive topical immunization with DNA vaccines on the skin is attractive since the skin is readily accessible, and known to be one of the largest organs of the immune system [26]. The skin is rich in potent APCs such as Langerhan's cell (LCs) and DCs. It is also well equipped with other necessary immune cells and cytokines [26]. Topical immunization, due to its needleless nature, may be more cost-effective and have increased patient compliance, and therefore allow for widespread vaccination. Although the feasibility of non-invasive topical DNA immunization was established as early as 1997 [17], its very low potency has limited further applications. Therefore, methods to improve its potency are still needed. Through a series of experiments, Glenn and his co-workers established the feasibility of CT and other related enterotoxins as adjuvants for successful and efficient topical or 'transcutaneous immunization' with protein-based vaccines [14–16]. Although the mechanism(s) for the topical adjuvanticity of CT are still unknown, it is reasonable to hypothesize that CT may also be used as an adjuvant to enhance immune responses for DNA vaccines after non-invasive topical application.

The results from these present studies support this hypothesis. As shown in Fig. 1, the co-administration of CT with 'naked' pDNA led to a significant enhancement in specific total IgG titer in sera to an expressed antigen, β-galactosidase, compared to immunization without CT. For example, the total serum IgG titer from mice immunized with the pDNA with CT (100 μg) and pDNA with CT (10 μg) was 20-fold ($P = 0.004$) and four-fold ($P = 0.02$) greater, respectively, than that from the mice immunized

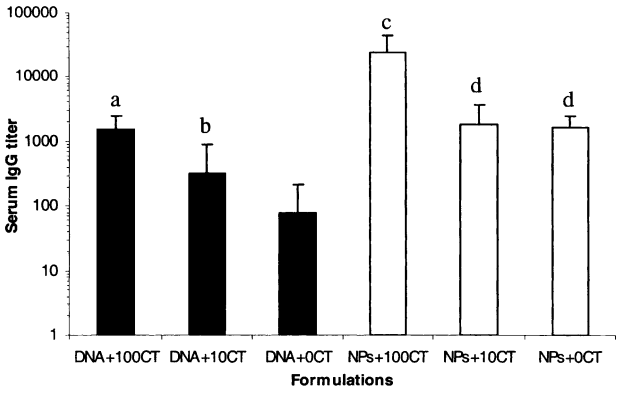

Fig. 1. Antigen-specific total IgG titer in sera to expressed β-galactosidase 45 days after non-invasive topical immunization on shaved mouse skin. Mice ($n = 6$/group) were immunized with 'naked' pDNA (CMV-β-gal, 5 μg) mixed with either 0 μg CT (DNA + 0CT), 10 μg CT (DNA + 10CT), or 100 μg CT (DNA + 100CT), or with pDNA-coated nanoparticles mixed with either 0 μg CT (nanoparticles (NPs) + 0CT), 10 μg CT (NPs + 10CT), or 100 μg CT (NPs + 100CT) on days 0, 6, 21, and 35. Data reported are the geometric mean ± standard deviation. A one-way ANOVA of the three mean serum IgG titers from mice immunized with 'naked' pDNA with or without CT resulted in a P value of 0.004, while similar analysis of mean serum IgG titer from mice immunized with DNA + 0CT, NPs + 0CT, NPs + 10CT, and NPs + 100CT resulted in a P value of 0.016. (a) indicates that the result for the DNA + 100CT was significantly greater than that of DNA + 10CT and DNA + 0CT. (b) indicates that the result for the DNA + 10CT was significantly greater than that of DNA + 0CT. (c) indicates that the result for NPs + 100CT was significantly greater than that of the others. (d) indicates that the results from NPs + 10CT and NPs + 0CT were significantly greater than that of the DNA + 0CT, although NPs + 10CT and NPs + 0CT are not significantly different ($P = 0.28$).

with 'naked' pDNA alone without CT. Moreover, the IFN-γ released from splenocytes isolated from mice immunized with pDNA with CT was significantly higher than that from mice immunized without CT (Table 1). These enhancements in IFN-γ release were also dependent on the CT dose. These results, in combination with the observation that the IL-4 release was not increased by the co-administration of the CT, demonstrated that CT may perform as an adjuvant for non-invasive topical DNA immunization, and that both enhanced antibody responses and more Th1-biased T cell responses were elicited.

Topical immunization with the pDNA-coated nanoparticles, compared to immunization with 'naked' pDNA alone, enhanced the specific total IgG titer in sera by 21-fold ($P = 0.002$), to a level that was comparable to immunization with 'naked' pDNA with CT (100 μg) (Fig. 1). This enhancement with pDNA-coated nanoparticles was similar to that observed in our previous studies [9]. Also, as shown in Fig. 1, the specific IgG titer in sera was enhanced by 14-fold ($P = 0.02$) when mice were immunized with the pDNA-coated nanoparticles with 100 μg CT, as compared to immunization with the pDNA-coated nanoparticles without CT. The specific total IgG titer from the mice topically immunized with pDNA-coated nanoparticles with 100 μg of CT was over 300-fold higher than that from mice immunized with 'naked' pDNA alone, strongly indicating a synergistic effect from the nanoparticles and CT in inducing antibody production. Shown in Table 1 and Fig. 2 are the results of in vitro cytokine release and proliferation by the isolated splenocytes. Again, co-administration of the pDNA-coated nanoparticles with CT helped to enhance both cytokine release and splenocyte proliferation, although the enhancement was not directly related to the dose of CT.

Table 1
In vitro cytokine release profiles from isolated splenocytes[a]

	IFN-γ (pg/ml)	IL-4 (pg/ml)
DNA + 100CT	722.6 ± 51.3*	45.5 ± 0.6
DNA + 10CT	422.3 ± 67.3*	33.4 ± 6.7
DNA + 0CT	216.9 ± 52.2	51.5 ± 14.8
NPs + 100CT	224.9 ± 77.8	33.6 ± 16.6
NPs + 10CT	640.6 ± 35.5**	51.8 ± 6.6***
NPs + 0CT	342.4 ± 133.5	24.8 ± 7.6
Naive	194.1 ± 2.5	32.3 ± 5.4

[a] Mice were immunized topically on shaved skin with either 'naked' pDNA mixed with 0 μg CT (DNA + 0CT), 10 μg CT (DNA + 10CT), or 100 μg CT (DNA + 100CT), or with pDNA-coated nanoparticles mixed with 0 μg CT (NPs + 0CT), 10 μg CT (NPs + 10CT), or 100 μg CT (NPs + 100CT). Naive mice were not treated. Splenocyte preparation and cytokine release studies were completed as mentioned in Section 2. Data reported are the mean ± standard deviation ($n = 3$). * indicates that, for IFN-γ, the results for DNA + 100CT and DNA + 10CT were significantly different from those for the DNA + 0CT and naive. ** indicates that, for IFN-γ, the result for NPs + 10CT was different from those for the NPs + 100CT, NPs + 0CT, and naive. *** indicates that, for IL-4, the result for NPs + 10CT was different from those for the NPs + 100CT, NPs + 0CT, and naive.

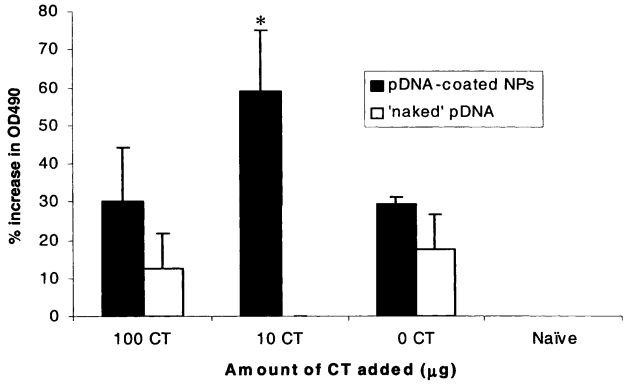

Fig. 2. In vitro proliferation of isolated splenocytes 45 days after topical immunization on shaved mouse skin. Mice ($n = 6$/group) were immunized with 'naked' pDNA (CMV-β-gal, 5 μg) mixed with either 0 μg CT (DNA + 0CT), 10 μg CT (DNA + 10CT), or 100 μg CT (DNA + 100CT), or with pDNA-coated nanoparticles mixed with either 0 μg CT (NPs + 0CT), 10 μg CT (NPs + 10CT), or 100 μg CT (NPs + 100CT) on days 0, 6, 21, and 35. The cell proliferation was reported as the % increase of the OD_{490} of the stimulated cells over their corresponding un-stimulated cells. Data reported are the mean ± standard deviation ($n = 3$). * indicates that the result from NPs + 10CT was significantly different from that of the NPs + 100CT, NPs + 0CT, and Naive. Splenocytes isolated from the naive mice showed no response.

In fact, for reasons that need to be further investigated, pDNA-coated nanoparticles with 10 μg of CT led to enhanced IFN-γ release, IL-4 release, and splenocyte proliferation, while immunization with 100 μg of CT did not show any apparent effect. Also, the splenocyte proliferation results from mice immunized with 'naked' pDNA, with or without CT, were uninformative. These results suggested that the amount of CT co-administered with pDNA-coated nanoparticles needs to be further optimized to obtain optimal immune responses. However, CT co-administrated with either 'naked' pDNA alone or with pDNA-coated nanoparticles boosted the production of specific antibody (IgG), increased the release of Th1-type cytokine (IFN-γ) from isolated splenocytes, and enhanced splenocyte proliferation.

The exact mechanism(s) behind the observed adjuvant effect are currently unknown. Using skin transplantation experiments, Fan et al. concluded that pDNA vaccines may enter the skin through the hair follicles [27]. Therefore, one possibility for the adjuvant effect from CT may be that CT can somehow enhance the access of pDNA via the hair follicles. Also, it is possible that the CT may be a danger signal to produce an inflammatory response, thereby causing APCs like DCs to migrate to the hair follicle sites [18]. Finally, it should be pointed out that, despite the fact that very little is known about the mechanisms of the adjuvanticity, the synergistic effect of immunization with pDNA-coated nanoparticles with CT warrants more attention. It is well known that non-invasive DNA immunization on skin with 'naked' pDNA alone is very inefficient in inducing immune responses [9,17,28,29]. In six independent immunization studies in Balb/C mice by topical application of 'naked' pDNA alone (4–100 μg) on skin, average specific total IgG titer with geometric means below or close to 100 were observed, with most of the mice being non-responders [9,28,29]. This observation agreed with other reports in the literature [17]. However, in the present studies, after topical immunization with pDNA-coated nanoparticles with CT (100 μg) on shaved mouse skin, specific total IgG titer with a geometric mean of 24,000 was obtained, strongly indicating that a therapeutically relevant level of serum IgG may be achievable. Due to its strong toxicity, administration of CT by the parenteral, oral, or nasal routes was precluded. However, it is likely that this toxicity issue can be avoided by administering CT non-invasively on skin. Based on these studies, it was concluded that a combination of CT with other pDNA delivery systems, such as the cationic nanoparticles, may have potential for future non-invasive DNA immunization on skin.

3.2. Effect of co-administration of LA on DNA immunization by s.c. injection

Shown in Fig. 3 is the specific total IgG titer in the sera of mice immunized with either 'naked' pDNA alone or pDNA-coated nanoparticles, with or without LA (50 μg) by s.c. injection. Immunization with pDNA-coated nanoparticles led to more than 16-fold enhancement in total serum IgG titer over immunization with 'naked' pDNA alone ($P = 0.038$), which agreed well with previous reports [7]. Co-administration of LA with 'naked' pDNA also resulted in close to 16-fold enhancement in serum total IgG titer ($P = 0.029$) over immunization with pDNA alone. Specifically, the total IgG titer from mice immunized with pDNA-coated nanoparticles and LA was 16-fold ($P < 0.05$) higher than that from mice immunized with pDNA-coated nanoparticles alone, and over 250-fold ($P = 0.0002$) greater than that from mice immunized with 'naked' pDNA alone without LA. These results strongly demonstrated that pDNA-coated nanoparticles and LA, when administered together, synergistically enhanced the resulting antibody responses.

Table 2 shows the in vitro cytokine release from isolated splenocytes after stimulation with β-galactosidase protein. A one-way ANOVA analysis showed no statistical difference in the IL-4 levels among all groups tested ($P = 0.31$). However, both immunization with the pDNA-coated nanoparticles and immunization with 'naked' pDNA with LA led to significantly enhanced IFN-γ release, compared to immunization with 'naked' pDNA alone. Again, splenocytes isolated from mice immunized with pDNA-coated nanoparticles with LA released the highest amount of IFN-γ after stimulation. Co-administration of LA also led to more positive cases of proliferation and a greater extent of proliferation of isolated splenocytes than immunization without LA for both 'naked' pDNA and pDNA-coated nanoparticles (Table 3).

Earlier studies with LA demonstrated that its adjuvant activity is related to its potential to activate macrophages and its ability to induce IFN-γ and IL-2, both known to be

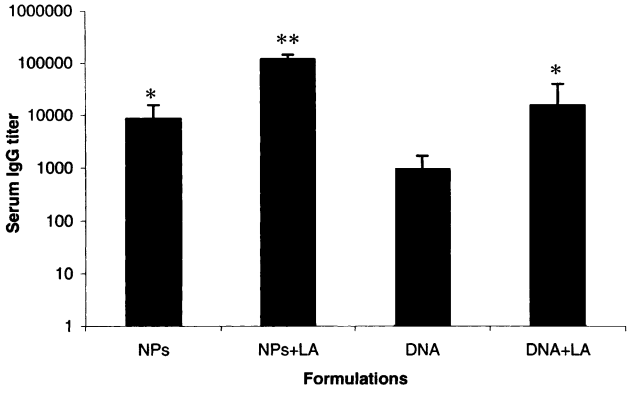

Fig. 3. Antigen-specific total IgG titer in sera to expressed β-galactosidase 28 days after s.c. immunization. Mice ($n = 6$/group) were immunized with 'naked' pDNA (CMV-β-gal, 5 μg) mixed with either 0 μg LA (DNA) or 50 μg LA (DNA + LA), or with pDNA-coated nanoparticles mixed with either 0 μg LA (NPs) or 50 μg LA (NPs + LA) on days 0, 7, and 14. Data reported are the geometric mean ± standard deviation of $n = 5$–6. One-way ANOVA of the four mean serum IgG titers resulted in a P value of 0.0002. ** indicates that the result for NPs + LA was significantly different from that from the other groups. * indicates that the results for the NPs and DNA + LA were significantly different from that of the DNA. The results for NPs and DNA + LA were not significantly different ($P = 0.46$).

Table 2
In vitro cytokine release profiles from isolated splenocytes[a]

	IFN-γ (pg/ml)	IL-4 (pg/ml)
Naive	1155 ± 70	60 ± 7
NPs	2008 ± 395	73 ± 2
NPs + LA	3159 ± 230*	79 ± 4
DNA	1025 ± 50	62 ± 10
DNA + LA	2056 ± 537**	83 ± 12

[a] Mice were immunized s.c. with either 'naked' pDNA mixed with 0 μg LA (DNA) or 50 μg LA (DNA + LA), or with pDNA-nanoparticles mixed with 0 μg LA (NPs) or 50 μg LA (NPs + LA). Naive mice were not treated. Splenocyte preparation and cytokine release studies were completed as mentioned in Section 2. Data reported are the mean ± standard deviation ($n = 3$). A one-way ANOVA revealed no significant difference between all the IL-4 data ($P = 0.31$). However, for the IFN-γ data, a P value of 0.013 was obtained after one-way ANOVA analysis. * indicates that the IFN-γ result for NPs + LA was statistically different from the IFN-γ results for all other groups. ** indicates that the IFN-γ level for DNA + LA was statistically different from that for DNA. Also, except for the DNA, the IFN-γ concentrations from all other immunized groups were statistically different from the naive group.

essential for the induction of Th1-type cell-mediated immune responses [11,25]. In 1997, Sasaki et al. studied the effect of co-administration of monophosphoryl LA with a DNA vaccine encoding HIV-1 *env* and *rev* genes on the resulting immune responses and hypothesized that the LA could help to further boost the Thl-type cytokine release [19]. The authors reported that the serum from mice immunized by intramuscular injection with the LA preparation revealed 60–500-fold higher HIV-1 specific IgG titer than the sera from mice immunized without LA. HIV-1 specific IgG subclass analysis showed that LA tends to facilitate IgG2a production, suggesting enhancement of a predominant Th1-type response [19]. These observations agreed well with the results reported in the present studies. The specific IgG titer in the sera of the mice immunized with 'naked' pDNA with LA was over 16-fold higher than that in the mice immunized without LA. Also, in vitro cytokine release studies revealed that the enhancement was biased towards a Th1-type response.

Table 3
In vitro proliferation of isolated splenocytes[a]

	Positive cases of proliferation	Extent of proliferation (%)
Naive	0 (3)	N/A
NPs	1 (3)	43
NPs + LA	3 (3)	44–145
DNA	1 (3)	29
DNA + LA	3 (3)	8–49

[a] Mice were immunized s.c. with either 'naked' pDNA mixed with 0 μg LA (DNA) or 50 μg LA (DNA + LA), or with pDNA-coated nanoparticles mixed with 0 μg LA (NPs) or 50 μg LA (NPs + LA) on days 0, 7, and 14. Naive mice were not treated. On day 28, the mice were sacrificed and their spleens were removed. Two spleens from the same group were pooled together so that each treatment had three splenocyte preparations. Isolated splenocytes (5×10^6/well) were incubated with either 0 or 3.3 μg/well of β-galactosidase protein for 94 h. Cell proliferation results were reported as the % increase of the OD_{490} of the stimulated cells over their corresponding unstimulated cells.

LA has been shown to have adjuvant activity when used alone, or in combination with other immunostimulants and delivery systems [22,23,25]. For example, Newman et al. [23] reported that following s.c. immunization, incorporation of monophosphoryl LA in ovalbumin (OVA)-loaded PLGA microspheres resulted in increased production of IFN-γ, when compared to OVA-loaded PLGA microspheres without the incorporation of LA. Also, immunization with OVA-loaded PLGA microspheres without incorporated LA resulted in increased IFN-γ production compared to immunization with OVA alone [23]. Although our studies used a DNA vaccine and nanoparticles, the results agreed well with the observations by Newman et al. using a protein-based vaccine. Similar to our previous reports, the present studies demonstrated that immunization with pDNA-coated nanoparticles led to enhanced Th1-type cytokine release compared to immunization with 'naked' pDNA alone. Moreover, co-administration of LA with the pDNA-coated nanoparticles further enhanced IFN-γ release over immunization with the pDNA-coated nanoparticles alone. By intramuscular and s.c. injection, DNA vaccines are known to favor the production of Th1-type responses, which are important for the induction of cell-mediated immune responses [2]. One of the reasons for the lack of effective vaccines for HIV, malaria and tuberculosis is that most of the current vaccines fail to induce cellular immune responses, which are thought to be equally as critical as inducing neutralizing antibodies for successful prevention of these pathogens [2]. DNA vaccines are thought to be promising for the development of effective vaccines for these pathogens. Therefore, the strategy of combining LA with a nanoparticle-based delivery system may have potential to elicit both enhanced antibody production and Th1-biased immune responses.

Although the toxicity associated with LA may preclude its further use, this may be avoided by using the detoxified monophosphoryl LA (MPL®), which has proven to be as effective as the original LA in enhancing immune responses, while at the same time being less toxic than LA (100–1000-fold) [25]. However, further work needs to be carried out to prove whether MPL® is as effective as LA when co-administered with these pDNA-coated nanoparticles.

In conclusion, in the present studies, we reported for the first time that CT can perform as an effective adjuvant in non-invasive topical DNA immunization. The use of CT resulted in enhanced antibody and more Th1-biased immune responses. In addition, co-administration of our previously reported cationic nanoparticle-based DNA vaccine delivery system with known adjuvants, either CT or LA, synergistically enhanced the resulting immune

responses from a model DNA vaccine. For example, topical non-invasive immunization of mice with the pDNA-coated nanoparticles with 100 μg of CT led to over a 300-fold increase in antigen specific IgG titer compared to immunization with 'naked' pDNA alone. Also, a greater than 250-fold enhancement in IgG titer was observed when mice were s.c. immunized with the pDNA-coated nanoparticles with 50 μg of LA compared to immunization with 'naked' pDNA alone. The results suggested that the combination of known adjuvants with the delivery system warrants additional investigation.

References

[1] M.A. Liu, M.R. Hillerman, R. Kurth, DNA vaccines: a new era in vaccinology, Ann. N. Y. Acad. Sci. 772 (1995) 1–294.

[2] S. Gurunathan, D.M. Klinman, R.A. Seder, DNA vaccines: immunology, application, and optimization, Annu. Rev. Immunol. 18 (2000) 927–974.

[3] R.J. Mumper, H.C. Ledebur Jr., Dendritic cell delivery of plasmid DNA: application for controlled nucleic acid-based vaccines, Mol. Biotech. 19 (2001) 79–95.

[4] D.T. O'Hagan, Recent advances in vaccine adjuvants for systemic and mucosal administration, J. Pharm. Pharmacol. 49 (1997) 1–10.

[5] M. Singh, M. Briones, G. Ott, D. O'Hagan, Cationic microparticles: a potent delivery system for DNA vaccines, Proc. Natl. Acad. Sci. USA 97 (2000) 811–816.

[6] J. Kazzaz, J. Neidleman, M. Singh, G. Ott, D.T. O'Hagan, Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1, J. Control. Release 67 (2000) 347–356.

[7] Z. Cui, R.J. Mumper, Genetic immunization using nanoparticles engineered from microemulsion precursors, Pharm. Res. 19 (2002) 939–946.

[8] Z. Cui, R.J. Mumper, Intradermal immunization with novel pDNA-coated nanoparticles via a needle-free injection device, AAPS Annual Meeting and Exposition, Toronto, Ontario, Canada, 2002.

[9] Z. Cui, R.J. Mumper, Topical immunization using nanoengineered genetic vaccines, J. Control. Release 81 (2002) 173–184.

[10] Z. Cui, R.J. Mumper, Intranasal administration of plasmid DNA-coated nanoparticles results in enhanced immune responses, J. Pharm. Pharmacol. 54 (2002) 1195–1203.

[11] R.K. Gupta, G.R. Siber, Adjuvants for human vaccines – current status, problems and future prospects, Vaccine 13 (1995) 1263–1276.

[12] R. Edelman, Vaccine adjuvants, Rev. Infect. Dis. 2 (1980) 370–383.

[13] N. Lycke, The mechanism of cholera toxin adjuvanticity, Res. Immunol. 148 (1997) 504–520.

[14] G.M. Glenn, M. Rao, G.R. Matyas, C.R. Alving, Skin immunization made possible by cholera toxin, Nature 391 (1998) 851.

[15] S.A. Hammond, C. Tsonis, K. Sellins, K. Rushlow, T. Scharton-Kersten, I. Colditz, G.M. Glenn, Transcutaneous immunization of domestic animals: opportunities and challenges, Adv. Drug Del. Rev. 43 (2000) 45–55.

[16] G.M. Glenn, D.N. Taylor, X. Li, S. Frankel, A. Montemarano, C.R. Alving, Transcutaneous immunization: a human vaccine delivery strategy using a patch, Nat. Med. 6 (2000) 1403–1406.

[17] D. Tang, Z. Shi, D.T. Curiel, Vaccination on bare skin, Nature 388 (1997) 729–730.

[18] R.K. Gupta, E.H. Relyveld, E.B. Lindblad, B. Bizzini, S. Ben-Efraim, C.K. Gupta, Adjuvants – a balance between toxicity and adjuvanticity, Vaccine 11 (1993) 293–306.

[19] S. Sasaki, T. Tsuji, K. Hamjima, J. Fukushima, N. Ishii, T. Kaneko, K. Xin, H. Mohri, I. Aoki, T. Okuo, K. Nishioka, K. Okuda, Monophosphoryl lipid A enhanced both humoral and cell-mediated immune responses to DNA vaccination against human immunodeficiency virus Type 1, Infect. Immunol. 65 (1997) 3520–3528.

[20] D.L. Lodmell, N.B. Ray, J.T. Ulrich, L.C. Ewalt, DNA vaccination of mice against rabies virus: effect of the route of vaccination and the adjuvant monophosphoryl lipid A (MPL®), Vaccine 18 (2000) 1059–1066.

[21] S. Sasaki, K. Hamajima, J. Fukushima, A. Ihata, N. Ishii, I. Gorai, F. Hirahara, H. Mohri, K. Okuda, Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine, Infect. Immunol. 66 (1998) 823–826.

[22] L.F. Fries, D.M. Gordon, R.L. Richard, J.E. Egan, M.R. Hollingdale, M. Gross, C. Silverman, C.R. Alving, Liposomal malaria vaccine in humans: a safe and potent adjuvant strategy, Proc. Natl. Acad. Sci. USA 89 (1992) 358–362.

[23] K.D. Newman, J. Samuel, G. Kwon, Ovalbumin peptide encapsulated in poly(*d*,*l*-lactic-co-glycolic acid) microspheres is capable of inducing a T helper type 1 immune response, J. Control. Release 54 (1998) 49–59.

[24] D. Wang, D.R. Robinson, G.S. Kwon, J. Samuel, Encapsulation of plasmid DNA in biodegradable poly(D,L-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery, J. Control. Release 57 (1999) 9–18.

[25] J.R. Baldridge, R.T. Crane, Monophosphoryl lipid A (MPL) formulations for the next generation of vaccines, Methods 19 (1999) 103–107.

[26] M.L. Kapsenberg, J.D. Bos, Skin, contribution to immunity, in: I.M. Roitt, P.J. Delves (Eds.), Encyclopedia of Immunology, Academic Press, San Diego, CA, 1992.

[27] H. Fan, Q. Lin, G.R. Morrissey, P.A. Khavari, Immunization via hair follicles by topical application of naked DNA to normal skin, Nat. Biotech. 17 (1999) 870–872.

[28] Z. Cui, R.J. Mumper, Chitosan-based nanoparticles for topical genetic immunization, J. Control. Release 75 (2001) 409–419.

[29] Z. Cui, W. Fountain, M. Clark, M. Jay, R.J. Mumper, Novel ethanol-in-fluorocarbon microemulsions for topical genetic immunization, Pharm. Res. (2002) in press.