

The Effect of Cationic Liposomes Encapsulating pcDNA3.1+PA Plasmids on Humoral Immune Response in Mice

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ABSTRACT

Background: DNA vaccines are third generation vaccines which have made promises to combat infectious diseases. Cationic liposomes are used as effective delivery systems for DNA vaccines to generate stronger immunity. **Objective:** Encapsulation of pcDNA3.1+PA plasmid, encoding protective antigen (PA) of *Bacillus anthracis* (*B. anthracis*) into cationic liposomes, and evaluation of its effect on specific humoral immunity against PA were aimed. **Methods:** The liposomes containing pcDNA3.1+PA plasmids were prepared with phosphatidylcholine (PC), dioleoyl phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) using dehydration-rehydration method. BALB/c mice were immunized by intramuscular (IM) injection to investigate the immunogenicity of the formulations. The resulting specific antibodies against PA, total IgG, IgG1, IgG2a and IgG2b isotypes, were evaluated by enzyme linked immunosorbent assay (ELISA) method. **Conclusion:** A higher concentration of specific IgG against PA was found in sera of a group immunized with the encapsulated plasmid compared with the naked plasmid alone. This difference was significant for IgG1 isotype.

Keywords: *Bacillus Anthracis*, Cationic Liposome, DOPE- DOTAP, DNA Vaccine, Mice, pcDNA3.1+PA

INTRODUCTION

The use of plasmid DNA as a type of vaccination has been developed in several trials and it is proposed as a new promising approach in combating infectious diseases and cancers in recent years (1). DNA vaccination involves the inoculation of a gene encoding a relevant antigen, against which an immune response is required. This gene is under the control of a promoter that will permit its expression in eukaryotic cells (2).

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DNA vaccines possess potentially remarkable advantages over conventional vaccines, e.g. it may provide a much wider stimulation of the immune system, including the stimulation of a cytotoxic T cell response (3). They trigger certain events in the immune system resulting in the generation not only the antibodies, but also of cellular immune responses (1-4). In addition to the avoidance of a breakthrough of diseases occurring from infectious agents, the simpler manufacturing of a plasmid DNA and a greater stability is considered as advantages of DNA vaccines (4). The magnitude of immune response to DNA vaccines is generally modest when DNA plasmids are used in the naked form (5). Intramuscular (IM) route has been used in most DNA immunization trials. It is proposed that much of the DNA is degraded by interstitial nucleases and the remaining DNA is taken up by myocytes or antigen presenting cells (APCs) after injection (6).

To overcome this problem, delivery systems such as liposomes have been utilized (7, 8). Liposome-encapsulated DNA has been shown to increase the potency of DNA vaccines by direct delivery to APCs, protecting plasmids from nuclease degradation and facilitate the uptake of plasmids by muscle cells (9). Delivery systems based on fusogenic liposomes can be used to preferentially induce humoral and cellular immune responses which are well-tolerated in clinical trials (10-13). Fusogenic cationic liposomes are made of positively charged lipids which interact with negatively charged DNA and fuse with the surface membrane of a target cell, releasing the particle contents into the cytoplasmic compartment of the cell (14-15).

We have recently designed a DNA vaccine encoding the protective antigen (PA) of *Bacillus anthracis* (*B. anthracis*) pcDNA3.1+PA (GeneBank accession number: EF550208 whose efficacy to induce protective immunity was approved following its naked IM injection into mice (16-17).

The main objective of this study was to intrap pcDNA3.1+PA plasmids into cationic liposomes and find out the effect of this formulation on the immune response against anthrax.

MATERIALS AND METHODS

Animals: Twenty-four female BALB/c mice (6 weeks old) were provided from the animal house of Razi vaccine and serum research institute (RVSRI), Iran. The mice were divided into four groups (A, B, C and D), housed in the animal care facility of RVSRI located in Karaj.

Preparation of Plasmids. pcDNA3.1+PA plasmids were prepared using Phoenix TM maxi prep kit (QBiogene, Inc, CA), following the manufacturer's directions. Determination of the concentration and purity of the plasmids were made using a NanoDrop ND-100 spectrophotometer.

Liposome Preparation and Encapsulation of pcDNA3.1+PA. Liposomes containing pcDNA3.1+PA plasmids were prepared as dehydration-rehydration vesicles (DRV) and composed of phosphatidylcholine (PC), dioleoyl phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) in a molar ratio of 4:2:1 as described previously (18). Briefly, the lipid phase was dissolved in chloroform: methanol; 2:1, v/v in a round bottom flask. The solvent was removed by rotary evaporation resulting in the deposition of a thin lipid film on the walls. This lipid film

was then freeze-dried overnight to ensure total removal of the solvent. The lipid film was hydrated with distilled water at 55°C and vortexed for 30 min. The resulting multilamellar vesicles (MLVs) were converted to small unilamellar vesicles (SUVs) using an extruder with 1000, 400 and 100 nm filters. The empty SUVs were mixed with an appropriate amount of pcDNA3.1+PA plasmids, froze at -70°C for 2 hours and freeze-dried overnight. The dried broken liposome powder was rehydrated at 55°C for 30 min with distilled water, using a volume equivalent to one-tenth of the total SUVs. Rehydration was carried out by gentle vortexing. The liposomes were then diluted with phosphate buffered saline (PBS). The resulted oligolamellar liposomes were separated from untrapped pcDNA3.1+PA plasmids using centrifugation at 20000 g for 10 min and washing 3 times with PBS. The supernatant was separated and the concentration of DNA was measured by spectrophotometry. The morphological features of the liposomes were studied using light microscopy.

Detection of Encapsulation Efficiency. The efficiency of incorporation (% entrapment) of the plasmids into the liposome preparation was determined by measuring the absorbance at 260 nm of the supernatants following the PBS washing and using the following formula:

% entrapment= [(total amount of plasmid added to liposomes - amount of plasmid recovered in the supernatants) / total amount of plasmid added to the liposomes] × 100.

Immunizations: Different groups of mice, 6 mice per group, were immunized 3 times IM at 1 month intervals with one of the following formulations: group A with 100 µg of the plasmid/100 µl PBS/mouse, group B with 100 µg plasmid entrapped in liposomes/100 µl PBS/mouse, group C with sterile PBS and group D with empty liposome. Groups C and D were used as controls.

Evaluation of Anti-PA IgG Antibodies by ELISA. Blood samples were collected from the mice before each injection or challenge, 4 times, and the sera were used to detect anti-PA IgG1, IgG2a, and IgG2b antibodies as was described previously (17). Briefly, 96-well microtiter plates (Corning, Finland) were coated with 100 µl of 10 µg/ml purified PA overnight at 4 °C. Plates were washed and blocked with 4% bovine serum albumin (BSA) in PBS Tween 20. Serum samples were diluted 1:20 with PBS- Tween 20 and applied to the plates. Anti mouse horseradish peroxidase (HRP) conjugated antibodies (total IgG, IgG1, IgG2a, and IgG2b), Sigma, were used and optical densities were determined at 492 nm as reference wavelength.

Protection Assessment by Challenge. The resistance of animals was verified by a challenge 2 weeks after the last immunization with a C2 strain of *B. anthracis* as described previously (17). The control groups were also included. The daily mortality of the mice was recorded.

Statistical Analysis: The data were analyzed using GraphPad software (GraphPad Software Inc., San Diego, CA, USA). One-way ANOVA, Tukey and student T- tests were performed. A probability level of $P < 0.05$ was considered significant.

RESULTS

Liposome Characterization. The liposomes were morphologically MLVs, as found under light microscope. Purified pcDNA3.1+PA plasmids were entrapped in liposomes

successfully and the encapsulation efficacy of plasmids into liposomes was determined to be 78%.

Antibody Response. To determine the type of immune response generated in immunized mice, the anti-PA-specific IgG, IgG1, IgG2a and IgG2b antibodies were assessed after immunization and challenge by ELISA. These results have been summarized in Fig. 1.

The sera from mice immunized with naked (A) and encapsulated (B) plasmids showed significantly higher levels of total IgG antibody compared to the control groups ($p < 0.05$) after the second and the third injections, respectively. Significant increased level of PA-specific IgG1 was found only in the encapsulated (B) plasmids ($p < 0.05$).

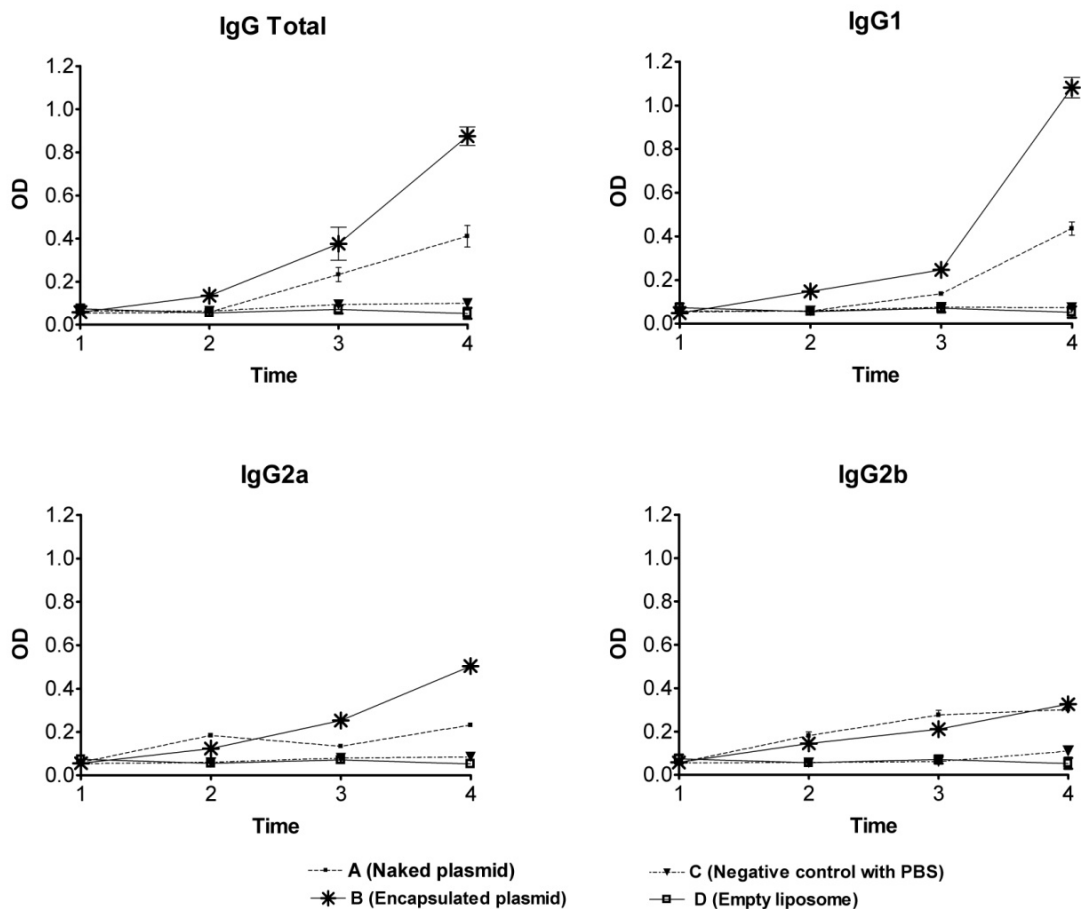


Figure 1. Anti-PA IgG levels in the sera as determined by ELISA. Time 1: before first immunization, Time 2: before second immunization, Time 3: before third immunization, Time 4: before challenge immunization. Six mice were included in each group. A: naked pcDNA3.1+PA plasmid; B: Encapsulated plasmid; C: PBS injected as the negative control group. The differences in total IgG and IgG1 isotype were found to be significant in groups A and B ($P < 0.05$) before challenge.

Protection Assessment. The efficacy of the naked and liposome-encapsulated pcDNA3.1+PA plasmids in protecting animals is shown in Fig. 2. Five of the six mice in group C (negative control, PBS injected) and all of the mice in group D (empty liposomes) died 10 days after challenge infection, but in groups A and B only 1 and 2 death was found, respectively showing a significant difference with the control groups (Fig. 2).

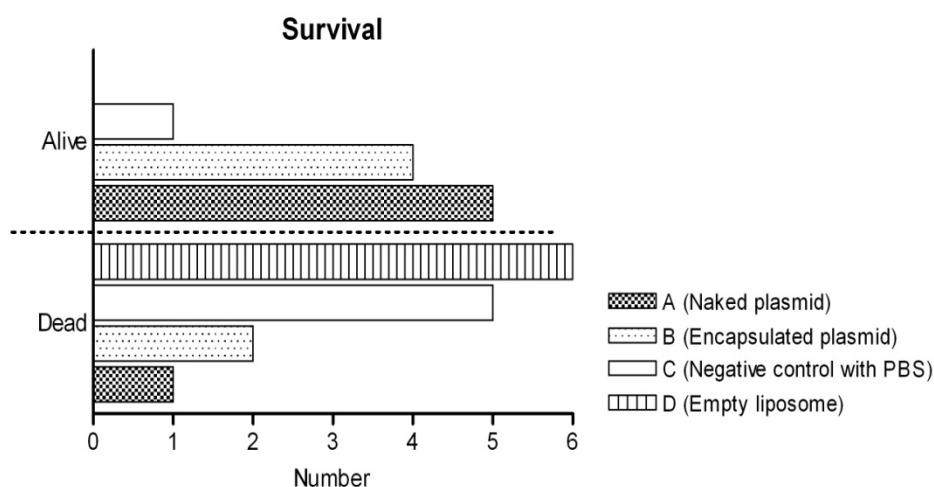


Figure 2. Survival number of mice following a lethal challenge, fourteen days after the last immunization with a pathogenic strain of *B. anthracis* (C2). A: naked pcDNA3.1+PA plasmid; B: Encapsulated plasmid; C: PBS injected as negative control group. There was no significant difference protection in the efficiency of groups A and C.

DISCUSSION

The advantages of DNA plasmid immunization has resulted in the birth of a new generation of vaccines, namely DNA vaccines. Many efforts are under way to optimize this promising approach and it is a clearly needed to optimize the potency of DNA vaccines (19).

The use of fusogenic cationic liposomes is thought to enhance the potency of DNA vaccines and improve both innate and adaptive immune responses, possibly by protecting entrapped plasmid from cellular DNAase and/or facilitating the uptake of plasmids by APCs. (6, 9).

In this work, immunization with cationic liposomes containing pcDNA+PA plasmids produced a higher level of IgG1, indicating that cationic liposomes may be a more

effective approach compared with the naked liposomes in generating humoral responses.

We had previously shown that DNA immunization using naked pcDNA3.1+PA plasmid resulted in an antibody profile representative of a mixed Th1 and Th2 response, to shift towards a Th1 response (17). However, our new finding shows that the liposomes may shift the immune response profile to Th2 type especially with regard to the evaluation of IgG1, IgG2a and IgG2b isotypes. This effect is being more significant after the third injection. The IgG1 isotype titer was found to be higher in group B that were injected with encapsulated DNA.

Immunization studies with DNA encapsulated into microspheres and gel forms have been described previously and the success with micro encapsulation has been shown in mice with different antigens (18, 20).

Encapsulation and weak binding of DNA to biodegradable polymers may act as a continuous antigen release system, potentially reducing the number of immunizing doses required to elicit a protective immune response (21).

The effect of using cationic liposomes on immunologic memory will be evaluated in our future studies.

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