Construction of a Eukaryotic Plasmid Encoding *Bacillus anthracis* Protective Antigen, a Candidate for DNA Vaccine

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ABSTRACT

Background: DNA immunization with plasmid DNA encoding bacterial, viral, parasitic and tumor antigens has been reported to trigger protective immunity**. Objective:** To evaluate the use of a DNA immunization strategy for protection against anthrax, a plasmid was constructed. **Methods:** The partial sequence of protective antigen of *Bacillus anthracis*, amino acids 175-764, as a potent immunogenic target was selected. The DNA encoding this segment was utilized in the construction of pcDNA3.1+PA plasmid. After intramuscular injection of rats with pcDNA3.1+PA plasmid, the expression of PA was assessed by RT-PCR and immunohistochemistry at RNA and protein levels, respectively. We also evaluated the presence of anti-PA antibodies in sera of immunized mice with pcDNA3.1+PA construct using immunoblotting. **Results:** The integrity of pcDNA3.1+PA construct was confirmed with restriction analysis and sequencing. The expression of PA was detected at RNA and protein levels. The presence of anti-PA antibodies in immunized mice with pcDNA3.1+PA construct was also confirmed. **Conclusion:** Our results indicate that pcDNA3.1+PA eukaryotic expressing vector could express PA antigen, induce antibody response and may be used as a candidate for DNA vaccine against anthrax.

Keywords: *Bacillus anthracis*, Protective Antigen (PA), DNA Vaccine

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INTRODUCTION

Anthrax caused by *Bacillus anthracis* (*B. anthracis*) is a historical disease of animals and humans. Bacteria belong to the genus Bacillus which is a Gram-positive rod and forms a single spore (1). Due to the extremely high durability and longevity of *B. anthracis* spores, no eradication can be expected. Outbreaks or epidemics are a constant threat for endemic regions because spores can persist in soil for long periods of time (2). Thus it remains to be a world wide problem. Control of anthrax can be accomplished with the proper use of vaccines (3).

There are two major virulent factors of *B. anthracis*; a tripartite protein exotoxin encoded by the plasmid pX01 (184kb) and a poly D-glutamic acid capsule encoded by the plasmid pX02 (97 kb). The three components of the exotoxin are protective antigen (PA, 83 kDa), lethal factor (LF, 90kDa) and edema factor (EF, 89 kDa). PA combines with LF and EF to form lethal toxin and edema toxin (EdTx), respectively. None of the proteins are toxic alone (4). LF and EF are the catalytic components of the toxin, whereas PA83 is the receptor–binding component, which mediates the entry of LF and EF to the cytosol of mammalian cells by a mechanism associated with its ability to heptamerize. The transmembrane pore is formed after cleavage of PA83 into PA63 by furin-like structure which is present at the exterior of cells. Following binding of EF or LF to PA63, the protein complex is endocytosed and trafficked to the endosome. LF is a zinc metalloprotease that disrupts signal transduction events by cleavage of several isoforms of MAP-kinase-kinase. The EF protein, a calmodulin-dependent adenylate cyclase, leads to clinical manifestations such as edema, by deregulation of cellular physiology. PA63 is the most immunogenic of the three and hence serves as a major component of all vaccines against anthrax (5). Protection against anthrax is associated with a humoral immune response directed against PA63 (6).

During the last decade, DNA vaccines have been developed against several viral, bacterial and parasitic infections (7-10). For construction of DNA vaccines, non-viral vectors (plasmids) are preferred because they have many advantages over viral vectors, e.g. superior targeting, low immunogenicity, reliable and large-scale production at an acceptable cost (11).

Researchers have examined the possibility of inducing protection against anthrax toxin by immunizing with PA or LF encoding DNA vaccines (11-15).

In the present study, we have constructed a PA encoding DNA vaccine using pcDNA3.1+ non-viral vector and determined its potency for antibody production in mice.

MATERIALS AND METHODS

Construction of pcDNA3.1+PA Plasmid

PA gene fragment encoding amino acids 175 to 764 was PCR amplified from *B. anthracis* 34F2 Sterne strain using specific primer and *pfu Taq* polymerase. Primers were designed according to the deposited sequences of *B. anthracis* in GenBank database and synthesized by TIB Mol. Biol., Germany. The primers sequences were as follow: The forward primer (PCPA5):

5´ACAAGTAAGCTTACCATGGTTCCAGACCGTGAC3´ with a *Hind* III restriction site. The reverse primer (PCPA3): 5´CTCGAGCTTCAATTACCTTATCCT3´ with an *Xho* I restriction site.

Cycling profile for amplification was one starting cycle at 95°C for 5 min, followed by 33 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. The final primer extension at 72°C was performed for 10 min. PCR products were electrophoresed on 1 % (w/v) agarose gel, the corresponding band was cut and the PCR product was purified from agarose gel using DNA extraction kit (GENECLEAN II, Q-Biogene). Restriction enzyme digestion was performed on the purified PCR product using *Hind* III and *Xho* I. The pcDNA3.1+plasmid was amplified in *E. coli* DH5α cells and the amplified plasmid was purified by alkaline method. The purified plasmid was digested with *Xho* I and *Hind* III. The products were separated on 1% (w/v) agarose gel and the fragments were purified from the gel by DNA extraction kit. The purity and concentration of DNA fragments were verified using gel electrophoresis and spectrophotometer. The pcDNA3.1+Vector and PA DNA fragments were ligated at ratio of approximately 3:1 by T4 DNA ligase at 20°C for 30 min. Competent *E. coli* DH5α cells was transformed with 5 µl ligation reaction mixture and plated on LB agar containing 50 µg/ml ampicillin. The colonies were then transferred to LB medium and incubated overnight at 37°C under shaking conditions to obtain a saturated culture. Recombinant plasmid DNA was extracted and purified and analyzed. Restriction enzyme analysis was employed to confirm the presence of the PA fragment in pcDNA3.1+PA vector. Then selected colonies were also sequenced (TIB Mol. Biol, Germany).

Plasmid Preparation for Injection

Selected plasmids were purified from transformed *E. coli* DH5α cells by anion exchange chromatography (Endofree plasmid Mega kit, Qiagen, Germany) as instructed by the manufacturer. The purified plasmids were dissolved in sterile endotoxin free PBS, pH 7.2 and stored at -20°C. The integrity of the DNA plasmids was checked by agarose gel electrophoresis after digestion with appropriate restriction enzymes. The DNA concentration was determined by measuring the optical density at 260 nm.

***In vivo* Expression at RNA Level**

Four rats received 100 µg of pcDNA3.1+PA construct by multispot intramuscular injection in the hind leg muscles to examine the capability of *in vivo* expression. Total RNA was isolated from this muscle after 72 hours post inoculation using Tri Pure Kit (Roche). The samples were analyzed by RT-PCR with specific PA upstream and downstream primers and β-actin primers as an internal control.

***In vivo* Expression at Protein Level**

Antibodies bound in situ to PA were analyzed by using indirect immunohistochemistry staining. The muscle sections from immunized rats were paraffinized. Samples from non immunized rats were also prepared as negative control. Indirect immunohisto-chemistry staining was performed according to DAKO LSAB2 System Peroxidase kit, using anti PA monoclonal antibody (KOMA Co., Korea), after deparaffinization in ethanol series. The antibody stains were developed upon addition of diaminobenzidine (DAB) and the nuclei were stained by hematoxylin.

Determination of the Expressed Protein Antigenecity

Ten female BALB/C mice were divided into, control and experimental groups (five mice per group). Each experimental mouse received 100 µg pcDNA3.1+PA plasmid

in the hind leg muscle as described above. Control mice were injected in the same manner with equal volumes of pcDNA3.1+ plasmid. All animals received their second injection as booster three weeks later. Sera were obtained from mice two weeks after second injection. Following SDS-PAGE, the PA protein was electrically transferred onto nitrocellulose and blocked with 5% BSA/PBS for 30 min. The nitrocellulose was probed with collected mouse sera raised against PA for 16 h at 4°C. Bound antibody was detected using an anti-mouse IgG monoclonal antibody conjugated to horseradish peroxidase (Serotec, Norway) and visualized with the diaminobenzidine tetrahydrochloride.

RESULTS

Construction of pcDNA3.1+PA

We selected the partial sequence of PA protein (amino acid 175-764) as the target gene. This sequence was used for construction of pcDNA3.1+PA vector after ligation of PA gene in the *Hind* III and *Xho* I restriction sites of the eukaryotic expression vector, the pcDNA3.1+ plasmid (Figure 1). Integrity of construct was confirmed by restriction enzyme analysis and sequencing (Figure 2). The sequence reported in this paper has been deposited in the GenBank database (Accession No. AY921578).

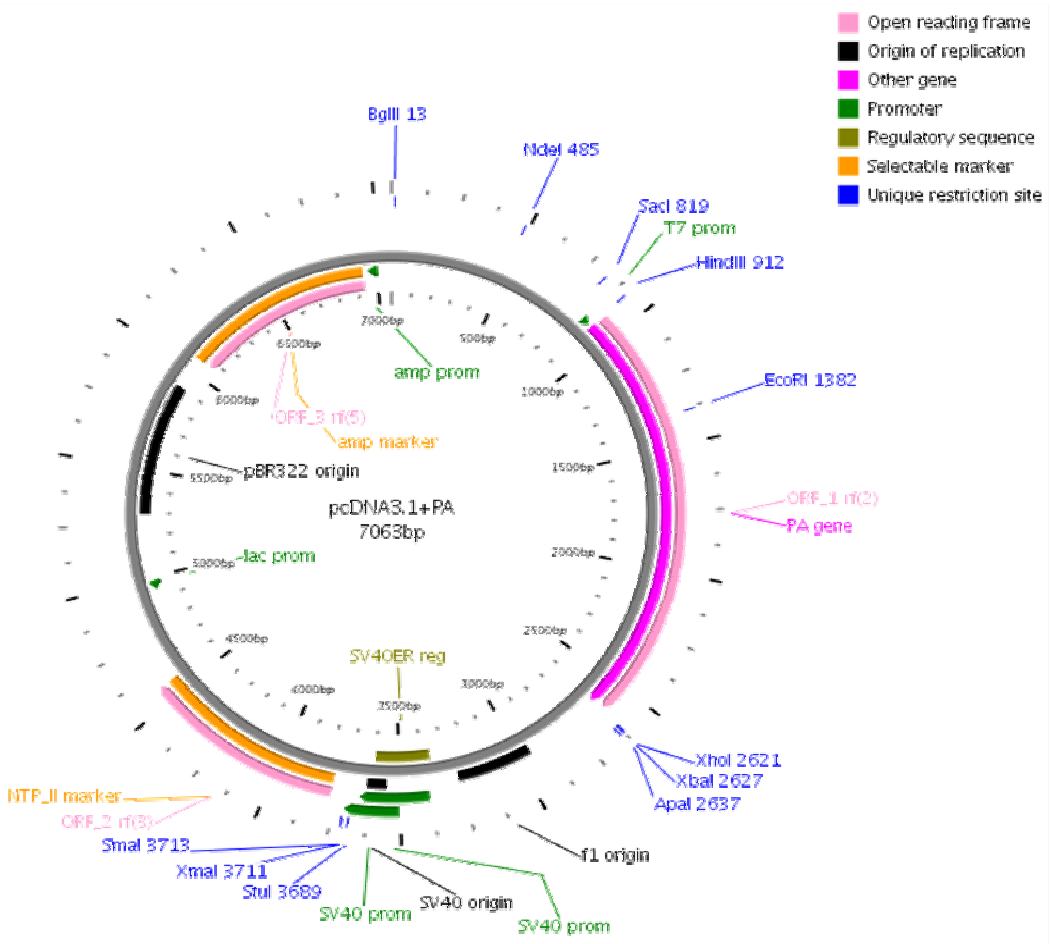

Figure 1. pcDNA3.1+PA vector. PA gene was cloned into the multiple cloning sites (between *Hind* III and *Xho* I) in pcDNA3.1+.

Expression of PA in Myocytes

To confirm that the construct pcDNA3.1+PA is functional and can direct expression of PA in mammalian cells, it was transfected into myocytes, an *in vivo* study. Plasmid pcDNA3.1+ was used as a negative control. The expression of PA was detected as RNA extracted from local tissue after 72 h of injection. We have shown the presence

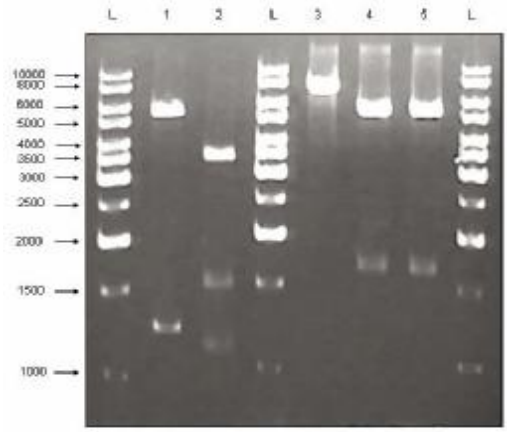

Figure 2. Agarose gel electrophoresis of restriction digestion analysis of pcDNA3.1+PA plasmid. Lanes L, DNA size marker; lane 1, plasmid digested with *EcoR* I, *Hind* III and *Xho* I ; lane 2, plasmid digested with *EcoR* I and *Pvu* II; lane 3 plasmid digested with *EcoR* I ; lanes 4 and 5 plasmid digested with *Xho* I and *Hind* III.

of bands about 1700 bp following RT-PCR on RNA extracts indicating that recombinant PA mRNA is transcribed in the myocytes (Figure 3).

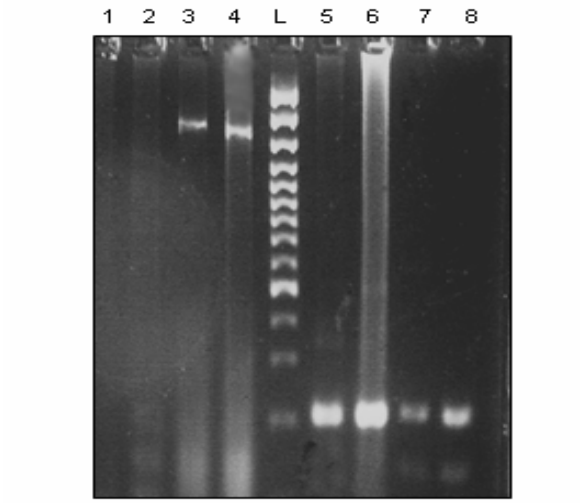

Figure 3. Analysis of expressed products by the mRNA level of pcDNA3.1+PA using RT-PCR. Lane L, DNA size marker; Lanes 1 to 4 β-actin amplification (220 bp) as a control for cDNA synthesis; lanes 5 and 6 indicate the expression of PA in muscles from two individual immunized mice. Lane 7 indicates no expression of PA in normal mice by RT-PCR; lane 8 is a direct PCR on RNA extract which indicates no contamination of RNA extract with pcDNA3.1+ plasmid.

Indirect immunohistochemistry analysis confirmed that anti PA monoclonal antibody could bind to in situ generated PA from transfected cells (Figure 4). This result and RNA assay data indicated that pcDNA3.1+PA was delivered into myocytes and expressed successfully. Hence, pcDNA3.1+PA appeared to direct the synthesis of PA protein by mammalian cells *in vivo*.

Immunogenicity of Expressed PA Gene

To examine the immunogenicity of PA gene, mice were immunized with pcDNA3.1+PA plasmid construct or pcDNA3.1+ plasmid without the insert. Two weeks after the last immunization, sera were obtained from their tails. SDS-PAGE

and Western-blot analysis were performed to assess the presence of anti-PA antibody. In immunoblotting analysis, a pool of sera from 5 mice immunized with pcDNA3.1+PA reacted strongly with PA-63 KDa protein band (Figure 5). In contrast, antibodies recognizing PA antigen were absent in the sera of mice injected with control plasmid pcDNA3.1+. This indicates the potency of the expressed protein as immunogen to elicit antibody production.

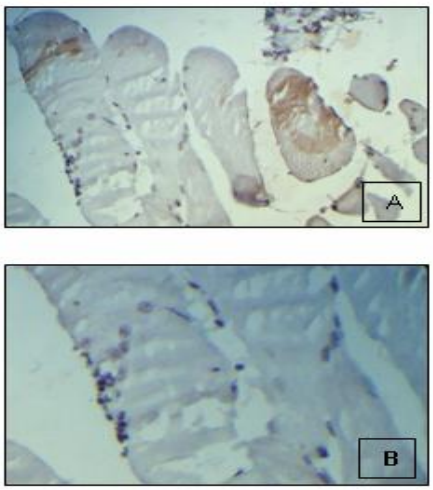

Figure 4. Analysis by indirect immunohistochemistry of muscle sections from rat, muscle sections were fixed and were reacted with the monoclonal anti PA antibody as the first antibodies and stained with goat anti-mouse IgG conjugated with Horse Radish Peroxidase (X100). (A) Rat immunized with pcDNA3.1+PA plasmid (B) Rat without any injection (negative control).

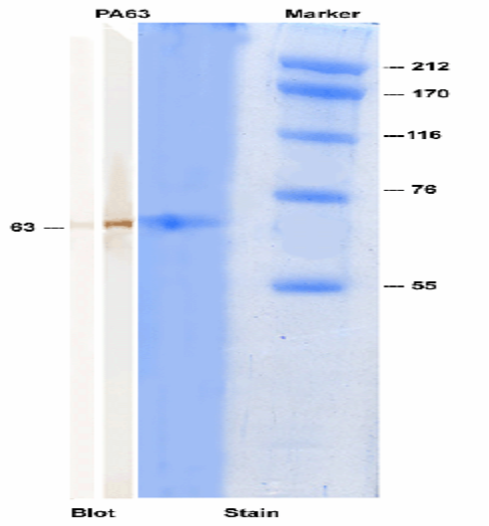

Figure 5. SDS-PAGE of *B. anthracis* PA63, PA63 was run on a 10% SDS-PAGE gel which was western blotted and probed with immunized mouse sera. Molecular weight of proteins is shown in KDa.

DISCUSSION

DNA vaccines have been widely used in laboratory animals and non-human primates to induce humoral and cellular immune responses. Clinical trials have shown that DNA vaccine is safe and well tolerated and appear to offer certain advantages, such as ease of construction, low cost of mass production, high level of temperature stability, and the ability to elicit both humoral and cell-mediated immune responses (16-18).

In this study, we constructed a plasmid encoding PA gene of *B. anthracis*, a most potent immunogenic target, as a candidate DNA vaccine for anthrax. First, the partial PA gene fragment was amplified from total extracted DNA of *B. anthracis*. Then purified PA gene was cloned into eukaryotic expression vector pcDNA3.1+. Both the enzyme digestion and sequencing confirmed the successful construction of a recombinant plasmid pcDNA3.1+PA. Competent *E. coli* DH5α cells were transformed successfully with plasmid pcDNA3.1+PA. Expression of PA protein was demonstrated both at RNA level using RT-PCR and at protein level by immunohistochemistry.

RT-PCR is an easy and efficient way to detect gene expression at RNA level (19). It is also demonstrated *in vivo* that the myocytes transfected with pcDNA3.1+PA could express the PA protein, but the myocytes transfected by pcDNA3.1+ plasmid could not express this protein. The expression of pcDNA3.1+ plasmid is controlled by a strong eukaryotic promoter such as human cytomegalovirus (CMV) promoter (20).

These results suggest that pcDNA3.1+PA plasmid may be used as a DNA vaccine against anthrax. DNA vaccines provide a valuable technology for rapid development of safe and efficient vaccines needed for emerging infectious diseases. Further studies are necessary to determine whether this form of vaccination is effective against live anthrax.

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