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ORIGINAL PAPER

A DNA vaccine candidate for *B. anthracis* immunization, pcDNA3.1+PA plasmid, induce Th1/Th2 mixed responses and protection in mice

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Abstract The protective antigen (PA) of *Bacillus anthracis* (*B. anthracis*) is a potent immunogen and a candidate subunit vaccine. To address the question whether antibodies raised against PA following injection of pcDNA3.1+PA plasmid, encoding PA, can protect against virulent *B. anthracis* two different regimens of PA based vaccines (DNA and live spore) were used. The groups of BALB/c mice that received live spores of the Sterne strain, naked pcDNA3.1 and naked pcDNA3.1+PA were compared to control groups. All groups were injected three times with 30-day intervals. Two weeks after the last immunization, all mice were subjected to challenge with a pathogenic strain of *B. anthracis* (C2). Blood samples were taken before each injection and challenge. Evaluation of the sera by ELISA method showed that DNA immunization using pcDNA3.1+PA plasmid resulted in an antibody profile representative of a mixed Th1 and Th2 response, with a skewing to a Th1 response. The group which received the naked pcDNA3.1+PA had a survival rate of >80%. This challenge assay revealed that antibodies raised following DNA vaccination against PA can confer strong protection, and resistance against virulent species of *B. anthracis*.

Keywords DNA vaccine · *Bacillus anthracis* · pcDNA3.1

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Introduction

Most vaccines use live-attenuated strains, bacterial supernatants or protein subunit approaches (Arnon and Ben-Yedidia 2003). Recently, novel DNA vaccine approaches as a safe and effective vaccine have been proposed (Davis 1997; Gurunathan et al. 2000; Hasan et al. 1999; Mor 1998).

Anthrax is an epizootic (zoonotic) disease caused by *Bacillus anthracis* (*B. anthracis*) mainly affecting domesticated and wild animals, primarily herbivores, including goats, sheep, cattle, horses, and swine (Lew 2000). Humans usually become infected by contact with infected animals or contaminated animal products. *B. anthracis* is a spore-forming, large Gram-positive, rod-shaped, and non-motile bacillus. Anthrax is found worldwide and transmitted primarily through spores that are highly resistant to heat, drought, and many disinfectants (Dixon et al. 1999). Vaccination programs must be continued considering the fact that no eradication can be anticipated for *B. anthracis*.

The capsule and the toxin complex are the two known virulence factors of *B. anthracis*. Fully virulent strains of *B. anthracis* contain two plasmids, pXO1 and pXO2, that encode for two binary exotoxins (PA + LF, PA + EF), and a poly-γ-D-glutamic acid capsule, respectively. PA is a component of anthrax toxin, by itself it is not toxic. The

earliest indication of its function came from a series of experiments using partially purified toxin components (Sirard et al. 1994). Anthrax has become one of the best characterized bacterial pathogens in regard to the mechanism of toxicity and vaccine development.

Since PA is the only antigen known to induce protective antibodies against anthrax, the protein has become the main focus of anthrax vaccine research. The primary goal of anthrax vaccination is to produce neutralizing antibodies against PA (Ivins and Welkos 1988; Singh et al. 1998). PA, in the absence of LF and EF, has been shown to be capable of producing effective protection both as a purified protein and when is used in a recombinant or attenuated vaccine (Skowera et al. 2005). Although PA is clearly the major immunogen, there are additional immunogens in the spore or in vegetative cells that contribute to protective immunity to anthrax.

A clear role for antibody subclasses in protection against anthrax has not been demonstrated, although some vaccination regimens are associated with higher proportions of IgG1 (in mice), usually correlated with predominantly a Th2 response (Flick-Smith et al. 2005; Skowera et al. 2005).

Immunization with a recombinant vector plasmid containing a fragment of the protective antigen has been studied. It has been demonstrated that immunizations with plasmid DNA encoding the *B. anthracis* PA induce specific antibody responses in mice and also can confer protection against a challenge with *B. anthracis* lethal toxin (Galloway et al. 2004; Gu et al. 1999; Price et al. 2002; Williamson et al. 2002).

We have previously demonstrated a DNA construct encoding the PA63, pcDNA3.1+PA construct, to be effective in inducing an immune response to this protein in BALB/c mice, following manual delivery by the intramuscular route (Vahedi et al. 2005). Our results indicated that the pcDNA3.1+PA eukaryotic expressing vector could express PA antigen in mice and successfully induce an antibody response (Vahedi et al. 2005).

In the current study, we have investigated the competency of pcDNA3.1+PA plasmid, a DNA vaccine candidate, in protecting BALB/c mice after a lethal challenge with spore from the C2 strain and also have compared its efficacy with live attenuated vaccine, Sterne strain, in protection and antibody isotype profiles.

Materials and methods

Plasmids

The plasmids used for vaccination were pcDNA3.1(+) (Invitrogen, Carlsbad, CA) without the insert as a negative control and pcDNA3.1+PA (GenBank accession number: EF550208) encoding the PA63 gene of the toxigenic *B. anthracis* Sterne strain (Vahedi et al. 2005). This plasmid is targeting PA63 to the endoplasmic reticulum of the cell without any secretion by the eukaryotic cells. This plasmid does not contain a secretion signal sequence to facilitate secretion of the protein (Chen and Okayama 1987).

Preparation of plasmids

Maxi preparation of pcDNA3.1 and pcDNA3.1+PA plasmids were done using phoenix TM maxi prep kit (QBiogene, Inc, CA), as per the manufacturer's protocol for high copy plasmids.

Integrity of purified plasmids were analysed with restriction mapping and electrophoresis on agarose gels. Using a NanoDrop ND-100 spectrophotometer, the concentration and purity of the purified plasmids were evaluated. Levels of Endotoxin were assessed by a colorimetric assay, by the QCL-1000 Limulus Ameobocyte Lysate Kit (BioWhittaker, Walkersville, MD, USA), following the manufacturer's protocol.

Immunization and challenge

Thirty female BALB/c mice, 6–8 weeks of age were provided from animal house of Razi vaccine and serum research institute (RVSRI), Iran. They were divided into five groups of 6 mice each. Groups 1 and 2 were injected on days 1, 30 and 60 with 100 μg of the plasmid. Group 3 was vaccinated with 200 μl of Sterne vaccine, the live veterinary vaccine (Razi vaccine and serum research institute, Iran). All of the injections were down intramuscularly (I.M.). One group was sham-inoculated with sterile PBS and one group was without any injection (Table 1).

Collection of blood was carried out before each injection or challenge from the tail vein and the sera were stored in −20°C.

Immunized groups were infected intradermally (I.D.) with 1×10^3 spores of a pathogenic strain of *B. anthracis* (C2) 14 days after the last immunization. The PBS control group was also included. The animals were checked every day for mortality during next 10 days.

Table 1 Description of BALB/c mice groups

Group	Description
A	pcDNA3.1+PA, Days 0, 30, 60, each time 100 μg
B	Sterne vaccine, Days 0, 30, 60, each time 200 μl
C	pcDNA3.1, Days 0, 30, 60, each time 100 μg
D	PBS, Days 0, 30, 60, each time 100 μl
E	No injection

Detection of Anti-PA IgG antibodies by ELISA

Flat-bottom 96-well microtiter plates (Corning, Finland) were each coated with 100 µl of purified rPA at the concentrations of 1 and 10 µg/ml in PBS, pH 7.4 overnight at 4°C. After removing rPA solution from wells, 150 µl blocking agent containing 4% BSA and 0.05% Triton X-100 in PBS were added per well and plates were incubated at 37°C for 2 h. Antigen-coated and blocked plates were then washed three times with washing solution, PBS containing 0.1% Tween 20 and blotted by inversion on clean paper towels. Purified monoclonal mouse anti-PA *B. anthracis* (4 mg/ml, KOMA Co, Korea) was prepared at the appropriate dilutions in normal mouse sera. Normal serum from BALB/c mice without monoclonal anti-PA antibody was used as negative control. Plates were incubated for 2 h at 37°C, and the plates were then washed three times with washing solution. F(ab′)2 goat anti-mouse IgG: HRP were prepared at three different dilutions and added to the wells. The plates were incubated for 1 h at 37°C. Washing was done as in previous steps. Bound conjugates were detected colorimetrically by using 100 µl of OPD/H_2O_2 substrate. Colour development was over 30 min and was stopped by addition of 50 µl solution, H_2SO_4 3 N to all wells. OD values were read with a multiskan microtitre plate reader (Lab2system, Finland) at a wavelength of 492 nm. This procedure was then repeated for goat anti-mouse IgG2b: HRP conjugated for what was the best dilution of conjugated.

All collected sera were diluted 1/20 in PBS. Detection of anti-PA total IgG was done using the best dilution of antigen (rPA) and F(ab′)2 goat anti-mouse IgG: HRP determined as described above. All sera were tested in duplicate. In addition, normal mouse serum as a negative control and PBS as a blank were used in duplicate on each plate.

Goat anti-mouse IgG1: HRP, goat anti-mouse IgG2a: HRP and goat anti-mouse IgG2b: HRP were used in dilutions according to manufacturer's recommendation for detection of anti-PA IgG1, IgG2a, and IgG2b antibodies, respectively. Endpoint titres were defined as the reciprocal at 492 nm of greater than 0.1.

Statistical analysis methods and software

ELISA data were subjected to a mixed model repeated measures analysis of variance. The live bacterial challenge data were subjected to one-way analysis of variance. An overall significance level of $P < 0.05$ was accepted. SPSS software, (Version 11.1.1; Statistical Product and Service Solutions, Inc, Chicago, III) was used for these analyses.

Results

Maxi-preparation and analysis of purified plasmids for injection

At optimized conditions ~3.5 µg DNA/ml LB medium were obtained. About 50 µg pcDNA3.1+PA plasmid and 30 µg pDNA3.1 plasmid were purified from 1.5 l cultures of each clone containing the above plasmids. Purified plasmids were checked by spectrophotometery and agarose gel electrophoresis and their size was confirmed. Also restriction digestion showed the integrity of plasmids (Fig. 1). Endotoxin levels in the prepared plasmids were confirmed to be less than 5 pg/ml by the QCL-1000 Limulus Ameobocyte Lysate Kit.

Challenge

Ten days after challenging the mice with virulent *B. anthracis* bacteria, the rate of survival was determined (Fig. 2). Group A (pcDNA3.1+PA), showed a survival rate of 83.3% and group B (Sterne Vaccine) showed a rate of

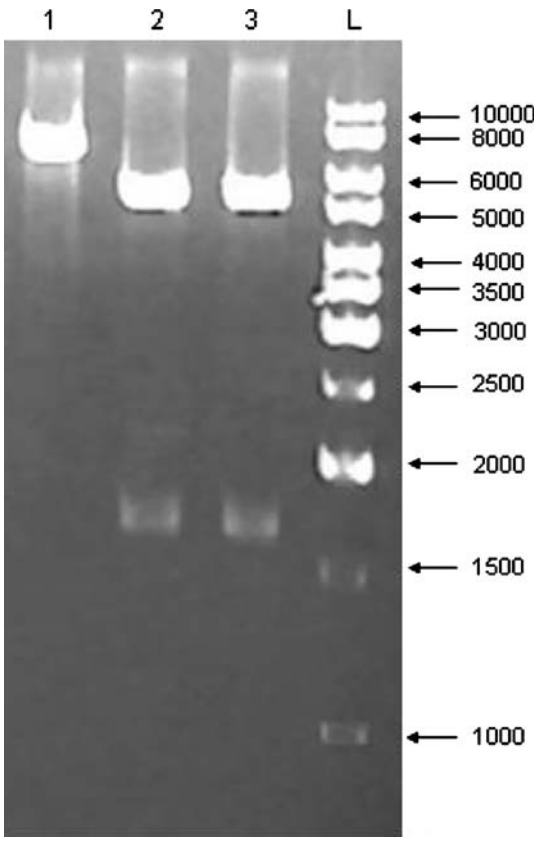

Fig. 1 Agarose gel electrophoresis of restriction digestion analysis of pcDNA3.1+PA plasmid. Lane L: DNA markers (bp); 1Kb: Fermentas, lane 1: plasmid digested with *Xho* I; linearized pcDNA3.1+PA is seen as a 7063-bp fragment, lanes 2 and 3: plasmid digested with *Xho* I and *Hind* III; 1710-bp and 5353-bp fragments are seen

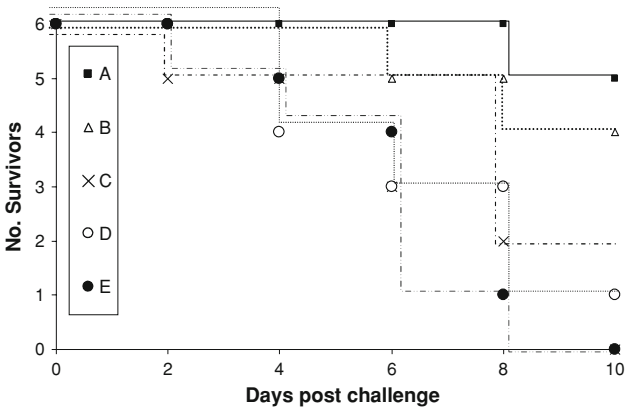

Fig. 2 Survival of BALB/c mice following a lethal challenge with anthrax spores. A: pcDNA3.1+PA; B: Sterne Vaccine; C: pcDNA3.1; D: PBS; and E: no injection

66.7%. But in groups C, D and E, the survival rate was low and differed significantly from groups A and B ($P < 0.01$).

ELISA

To assess the humoral responses induced by DNA immunization, each mouse sera was tested for PA-specific IgG, as well as PA-specific IgG1, IgG2a, IgG2b and total IgG was assayed by ELISA. The ELISA assays were optimized for antigen and conjugate concentrations.

Validation of the ELISA determined that an anti-mouse IgG: HRP conjugate dilution of 1:500 and an antigen concentration of 10 μg/ml were appropriate ($P < 0.01$). This result was repeated for anti-mouse IgG2b: HRP conjugate. The best results were obtained using conjugate at dilution of 1:500 and antigen at 10 μg/ml (Fig. 3). For finding the optimized dilution of sera three dilutions of negative and positive sera (1, 1/20 and 1/100) were evaluated using 1 μg/ml and 10 μg/ml of antigen. The best dilution of sera was found to be 1/20 ($P < 0.01$) at two different concentrations of antigen (Fig. 4).

At first bleeding there was no significant difference between groups and we did not find any specific total IgG anti-rPA in sera ($P < 0.01$). On the second bleeding, 30 days after the first injection, levels of anti-rPA total IgG were found to be higher in groups A and B, however in comparison with negative groups these differences were not found to be significant. The serum levels of anti-rPA total IgG of groups A and B showed a significant increase at the third bleeding, before the third injection. Assessment of sera before challenge showed a more significant increase in PA-specific total IgG level ($P < 0.01$) (A > B).

In Fig. 5 variation of the PA-specific total IgG level is shown. After each injection the levels of PA-specific total IgG have increased. No significant differences were found between the groups before the first injection. Before the

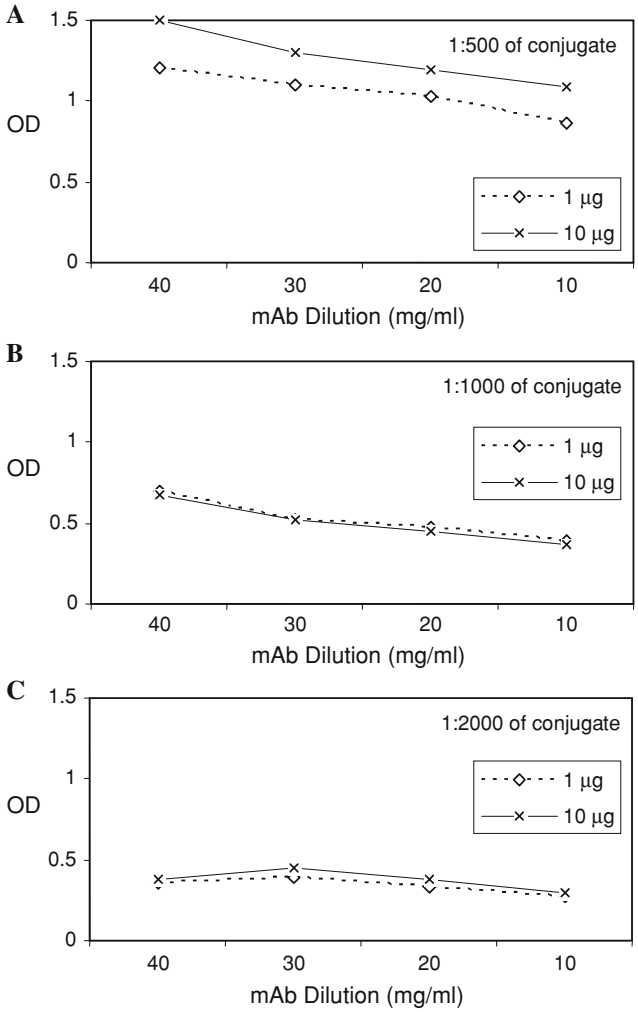

Fig. 3 Optimization of ELISA assay for determination of specific PA total IgG. Two different concentrations of PA were evaluated against anti-mouse IgG: HRP conjugate at (**a**) 1:500; (**b**) 1:1000; and (**c**) 1:2000

second injection, groups A and B (A > B) showed an increase in PA-specific IgG1 levels respectively, but only in group A this increase was significant ($P < 0.01$). Before the third injection, PA-specific IgG1 were found in groups A and B. This increase was significant ($P < 0.01$). Before challenge high levels of PA-specific IgG1 were found in these groups with a significant increase ($P < 0.01$). Figure 5 represents variation of PA-specific IgG1 subclass over time.

The first bleeding did not reveal significant difference between groups and no specific IgG2a anti-rPA was found in sera. On second bleeding, 30 days after first injection, substantial levels of anti-rPA IgG2a were found in groups B and A. But in comparison with the negative groups these differences were not found to be significant ($P < 0.01$). Similar result was found after the second injection. The serum levels of anti-rPA IgG2a of groups A > B showed a significant increase before challenge ($P < 0.01$). In Fig. 5, variation of PA-specific IgG2a during time is shown.

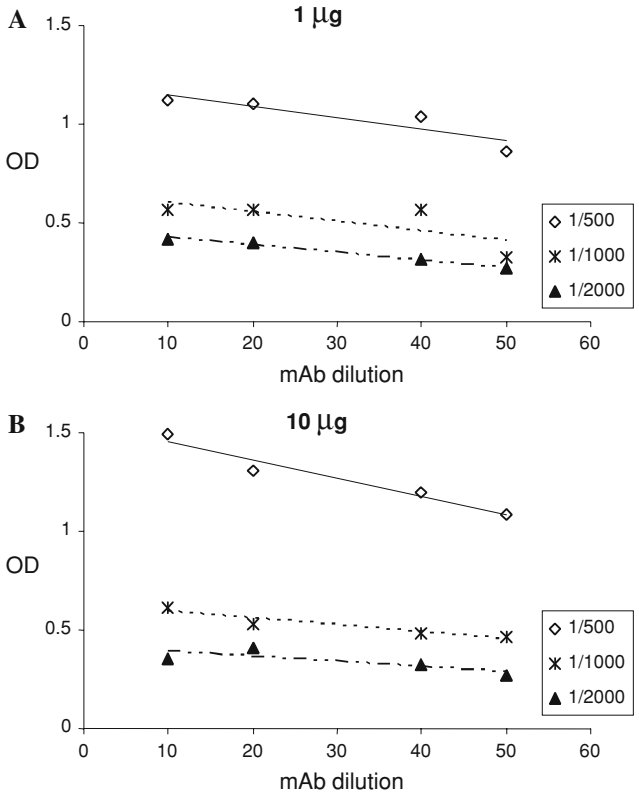

Fig. 4 Optimization of ELISA assay for determination of specific PA IgG2b subclass. (**a**) Using 1 µg Ag, three dilutions of anti-mouse IgG2b: HRP conjugate were tested. (**b**) Using 10 µg Ag, three dilutions of anti-mouse IgG2b: HRP conjugate were tested

For IgG2b no significant difference between groups was found before the first injection. Before the second injection, groups B and A (B > A) showed a significant increase in PA-specific IgG2b level. Before the third injection, significant increase in PA-specific IgG2b level was found in these groups with A > B ($P < 0.01$). Before challenge high levels of PA-specific IgG1 were found in both groups with group A still being greater than the group B and both having a significant increase in response ($P < 0.01$). Figure 5 represents variation of PA-specific IgG2b subclass during time.

Discussion

In the present study the use of pcDNA3.1+PA plasmid as DNA immunization strategy for protection against anthrax, was evaluated. Previous results indicated that the pcDNA3.1+PA eukaryotic expressing vector could express the PA antigen, induce an antibody response and may be used as a candidate for a DNA vaccine against anthrax (Vahedi et al. 2005). Our main objective in this study was to confirm the induction of protection after immunization of mice with the pcDNA3.1+PA plasmid, and make a comparison of the antibody profiles between this vaccine candidate and the Sterne strain vaccine.

Murine CD4+ T cell clones have been classified into at least two subsets, Th1 and Th2, on the basis of their distinct

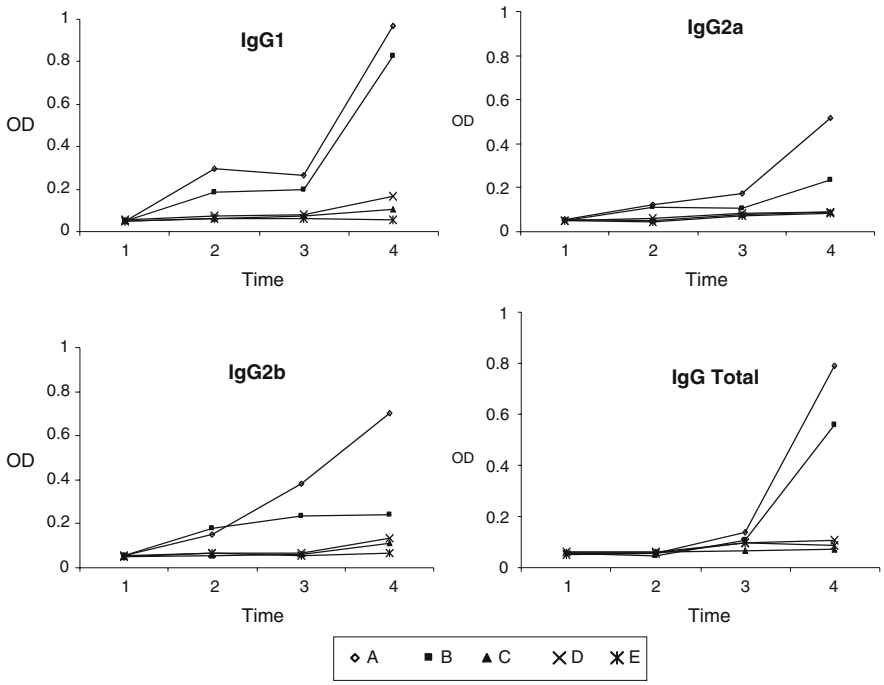

Fig. 5 Anti-PA total IgG levels in the sera immediately prior to each immunization and challenge were determined by ELISA. Time 1: before first immunization, Time 2: before second immunization, Time 3: before third immunization, Time 4: before challenge immunization. A: pcDNA3.1+PA; B: Sterne Vaccine; C: pcDNA3.1; D: PBS; and E: no injection

lymphokine secretion profiles and functions (Chang et al. 1990). Since production of IgG1 (Th2), IgG2a and IgG2b (Th1) are markers for the type of immune response generated in mice, it is possible to define the T cell phenotype induced by immunization based on these imunoglobulins (Lipford et al. 1997). The extrinsic factors which influence differential activation of Th0 cells to become either Th1 or Th2 cells include the mode of infection, type and concentration of antigen, and the microenvironment in which the reaction occurs. Not only is immune activation biased towards one pathway, but the memory response (Th1 or Th2) produced following activation of immunity tends to activate the immune response in either pathway in the long term. It is imperative therefore that the initial exposure to a vaccine triggers the appropriate pathway of immunity (Krieg 2000; Sato et al. 1996).

While immunization with vector DNA alone generated no humoral response against PA, high titers of IgG antibodies were detected in sera collected from mice immunized with DNA vaccines. For pcDNA3.1+PA, IgG1, IgG2a, and IgG2b isotypes were generated. But IgG1 production was predominant. Overall, we can conclude that DNA immunization raised a mixed pattern of Th1 and Th2 responses with a skewing towards the Th2 phenotype.

It is notable that using the pcDNA3.1(+) vector for construction of the encoding PA plasmid, has primed a Th2 like profile. It is also possible that the delivery via the intramuscular route may target specific subsets of dendritic cells, which in turn preferentially may prime Th2 responses. Additionally, the nature of the antigen may skew the type of Th response. In previous studies it has been reported that the PA protein and the live attenuated Sterne strain can shift immune response to a Th2 type response (Aloni-Grinstein et al. 2005; Baillie et al. 1999; Garmory et al. 2003). One of the most prevalent hypotheses for polarization of T cells are based on the dendritic cells are capable of eliciting both types of responses depending on the nature of the antigen (Pulendran et al. 1999). Comparative analyses of plasmid DNA, adenovirus and vaccinia virus vaccines have demonstrated that each vector system is associated with a unique outcome following immunization (Mor et al. 1996). Thus, to design an effective genetic vaccine, one must consider both the biology of the vaccine vector/antigen combination and the biology of the pathogen (Aarts et al. 2002). The antibody isotype results clearly suggest that the T-cell phenotype induced by DNA immunization is largely determined by the characteristics of the specific antigen being expressed from the DNA vaccine. This preferential induction of Th1 or Th2 responses by specific antigens may be due to one or more of the following: the protein half-life, the availability of the antigen, the location of antigen processing, the type of antigen presenting cell that processes the antigen, or the adjuvant properties of the antigen (Kowalczyk and Ertl 1999; Sutherland et al. 2001). The effects of Th1 promoting specific motifs in the DNA backbone may possibly explain the skewing towards a mixed Th phenotype in the current study (Klinman et al. 1997). The presence of a CpG motif contained in the PA gene sequences between base 3676 was recognized (AACGTT). Such motifs have been recognized as immunostimulatory (Welkos et al. 1988). The bacterial DNA, of which DNA vaccines are composed, contains immune enhancers. In contrast to vertebrate DNA, bacterial DNA is unmethylated and typically exhibits a near random distribution of CpG dinucleotides and flanking bases (Ballas et al. 1996; Storl et al. 1978).

It is worth nothing that the expressed PA protein after DNA immunization was not expected to be secreted thus it could be argued about why a Th2 response was predominant. In spite of the apparent lack of secretion of PA, the antibody response was observed. It is apparently not necessary to attach a secretion signal sequence to PA in order to obtain an antibody response to PA. Possible ways for induction of antibody responses without secretion have been postulated previously (Barry and Johnston 1997).

Gu et al. found that the predominant antibody type produced as a result of these immunizations is of the IgG1 subclass, although significant levels of subclass IgG2 antibodies were also produced (Gu et al. 1999). This result was consistent with a previous report by Price et al. that the majority of the immune response after PA DNA based vaccine was a Th2 type response (Price et al. 2002). In these two studies, the immunization methods were different. In the first study, plasmid immunization was conducted via intramuscular injection, whereas in the Price study plasmids were delivered by means of a gene gun. It has been reported that intramuscular vaccination with DNA generally produces a Th2 response (Price et al. 2001). The administration route was a key factor in determining the Th phenotype. The effect of the DNA delivery route on immunity and clinical protection has been analyzed (De Rose et al. 2002). In the Gu and Price studies, like our study, BALB/c mice were used. BALB/c mice in contrast to most other strains, develop a strong Th2 response to infectious pathogens like *L. major* (Infante-Duarte and Kamradt 1999; Mills et al. 2000). Furthermore, these data and considering a variety of factors contribute in production of immune response, raised the question about which type of immune response, Th1 or Th2, is the most protective.

The definitive test of an anthrax vaccine is the ability to protect against a lethal challenge in vivo. Injection of a lethal dose of spores into mice results in a course of infection resembling the pathogenesis of disease of fully virulent spores (Welkos et al. 1986). In this study, protection was measured by reduced mortality. The

immunized mice were challenged by a virulent strain of *B. anthracis*. Injection of $\sim 10^4$ spores results in an 80–90% mortality. Ten days past challenge nearly all of mice in the control groups had died. However, in the other groups those that received the pcDNA3.1+PA plasmid and the live attenuated Sterne strain vaccine had survival rates of 83.3% and 66.7%, respectively.

This study has shown that immunization of mice with PA as a DNA vaccine could be a good candidate for vaccination against anthrax and strongly suggest that a protective humoral response was stimulated by pcDNA3.1+PA. The potential of achieving protective immunity by DNA vaccination against the *B. anthracis* warrants further studies to optimize the production and administration of DNA encoding PA and investigate the factors that could affect this protective efficacy.

References

Aarts WM, Schlom J, Hodge JW (2002) Vector-based vaccine/cytokine combination therapy to enhance induction of immune responses to a self-antigen and antitumor activity. Cancer Res 62:5770–5777

Aloni-Grinstein R, Gat O, Altboum Z, Velan B, Cohen S, Shafferman A (2005) Oral spore vaccine based on live attenuated nontoxinogenic *Bacillus anthracis* expressing recombinant mutant protective antigen. Infect Immun 73:4043–4053

Arnon R, Ben-Yedidia T (2003) Old and new vaccine approaches. Int Immunopharmacol 3:1195–1204

Baillie LWJ, Fowler K, Turnbull PCB (1999) Human immune responses to the UK human anthrax vaccine. J Appl Microbiol 87:306–308

Ballas ZK, Rasmussen WL, Krieg AM (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol 157:1840–1845

Barry MA, Johnston SA (1997) Biological features of genetic immunization. Vaccine 15:788–791

Chang TL, Shea CM, Urioste S, Thompson RC, Boom WH, Abbas AK (1990) Heterogeneity of helper/inducer T lymphocytes. III. Responses of IL-2- and IL-4-producing (Th1 and Th2) clones to antigens presented by different accessory cells. J Immunol 145:2803–2808

Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745–2752

Davis HL (1997) Plasmid DNA expression systems for the purpose of immunization. Curr Opin Biotechnol 8:635–640

De Rose R, Tennent J, McWaters P, Chaplin PJ, Wood PR, Kimpton W, Cahill R, Scheerlinck JP (2002) Efficacy of DNA vaccination by different routes of immunisation in sheep. Vet Immunol Immunopathol 90:55–63

Dixon T, Meselson M, Guillemin J, Hanna PC (1999) Anthrax. N Engl J Med 341:812–815

Flick-Smith HC, Waters EL, Walker NJ, Miller J, Stagg AJ, Green M, Williamson ED (2005) Mouse model characterisation for anthrax vaccine development: comparison of one inbred and one outbred mouse strain. Microb Pathog 38:33–40

Galloway D, Liner A, Legutki J, Mateczun A, Barnewall R, Estep J (2004) Genetic immunization against anthrax. Vaccine 22:1604–1608

Garmory HS, Titball RW, Griffin KF, Hahn U, Bohm R, Beyer W (2003) Salmonella enterica serovar typhimurium expressing a chromosomally integrated copy of the *Bacillus anthracis* protective antigen gene protects mice against an anthrax spore challenge. Infect Immun 71:3831–3836

Gu ML, Leppla SH, Klinman DM (1999) Protection against anthrax toxin by vaccination with a DNA plasmid encoding anthrax protective antigen. Vaccine 17:340–344

Gurunathan S, Klinman DM, Seder RA (2000) DNA Vaccines: Immunology, Application, and Optimization. Annu Rev Immunol 18:927–974

Hasan U, Abai A, Harper D, Wren B, Morrow W (1999) Nucleic acid immunization: concepts and techniques associated with third generation vaccines. J Immunol Methods 29:1–22

Infante-Duarte C, Kamradt T (1999) Th1/Th2 balance in infection. Springer Semin Immunopathol 21:317–338

Ivins BE, Welkos SL (1988) Recent advances in the development of an improved, human anthrax vaccine. Eur J Epidemiol 4:12–19

Klinman DM, Yamshchikov G, Ishigatsubo Y (1997) Contribution of CpG motifs to the immunogenicity of DNA vaccines. J Immunol 158:3635–3639

Kowalczyk DW, Ertl HCJ (1999) Immune responses to DNA vaccines. Cell Mol Life Sci 55:751–770

Krieg AM (2000) The role of CpG motifs in innate immunity. Curr Opin Immunol 12:35–43

Lew DP (2000) Bacillus anthracis (anthrax). In: Mandell GL, Bennett JE, Dolin R (eds) Principles and practice of infectious diseases, 5th edn. Churchill Livingstone, Philadelphia, NY, pp 2215–2220

Lipford GB, Sparwasser T, Bauer M, Zimmermann S, Koch ES, Heeg K, Wagner H (1997) Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. Eur J Immunol 27:3420–3426

Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM (2000) M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol 164:6166–6173

Mor G (1998) Plasmid DNA: a new era in vaccinology. Biochem Pharmacol 55:1151–1153

Mor G, Yamshchikov G, Sedegah M, Takeno M, Wang R, Houghten RA, Hoffman S, Klinman DM (1996) Induction of neonatal tolerance by plasmid DNA vaccination of mice. J Clin Invest 98:2700–2705

Price BM, Liner AL, Park S, Leppla SH, Mateczun A, Galloway DR (2001) Protection against anthrax lethal toxin challenge by genetic immunization with a plasmid encoding the lethal factor protein. Infect Immun 69:4509–4515

Price BM, Barten Legutki J, Galloway DR, Von Specht BU, Gilleland LB, Gilleland HE, Staczek J (2002) Enhancement of the protective efficacy of an oprF DNA vaccine against Pseudomonas aeruginosa. FEMS Immunol Med Microbiol 33:89–99

Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E, Maliszewski CR (1999) Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. Proc Natl Acad Sci USA 96:1036–1041

Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen MD, Silverman GJ, Lotz M, Carson DA, Raz E (1996) Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. Science 273:352–354

Singh Y, Ivins BE, Leppla SH (1998) Study of immunization against anthrax with the purified recombinant protective antigen of *Bacillus anthracis*. Infect Immun 66:3447–3448

Sirard JC, Mock M, Fouet A (1994) The three *Bacillus anthracis* toxin genes are coordinately regulated by bicarbonate and temperature. J Bacteriol 176:5188–5192

Skowera A, de Jong EC, Schuitemaker JH, Allen JS, Wessely SC, Griffiths G, Kapsenberg M, Peakman M (2005) Analysis of

anthrax and plague biowarfare vaccine interactions with human monocyte-derived dendritic cells. J Immunol 175:7235–7243

Storl HJ, Simon H, Barthelmes H (1978) Antibodies elicited by defined oligodeoxyribonucleotide sequences. Nucleic Acids Res 5:4919–4931

Sutherland RM, McKenzie BS, Corbett AJ, Brady JL, Lew AM (2001) Overcoming the poor immunogenicity of a protein by DNA immunization as a fusion construct. Immunol Cell Biol 79:49–53

Vahedi F, Taiebi Meibody N, Kianizadeh M, Mahmoudi M (2005) Construction of a Eukaryotic Plasmid Encoding *Bacillus anthracis* Protective Antigen, a Candidate for DNA Vaccine. Iran J Imunol 3:134–140

Welkos SL, Keener TJ, Gibbs PH (1986) Differences in susceptibility of inbred mice to *Bacillus anthracis*. Infect Immun 51:795–800

Welkos SL, Lowe JR, Eden-McCutchan F, Vodkin M, Leppla SH, Schmidt JJ (1988) Sequence and analysis of the DNA encoding protective antigen of *Bacillus anthracis*. Gene 69:287–300

Williamson ED, Bennett AM, Perkins SD, Beedham RJ, Miller J, Baillie LW (2002) Co-immunisation with a plasmid DNA cocktail primes mice against anthrax and plague. Vaccine 20:2933–2941