POTENTIATION OF AN ANTHRAX DNA VACCINE WITH ELECTROPORATION

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Abstract

DNA vaccines are a promising method of immunization against biothreats and emerging infections because they are relatively easy to design, manufacture, store and distribute. However, immunization with DNA vaccines using conventional delivery methods often fails to induce consistent, robust immune responses, especially in species larger than the mouse. Intramuscular (i.m.) delivery of a plasmid encoding anthrax toxin protective antigen (PA) using electroporation (EP), a potent DNA delivery method, rapidly induced anti-PA IgG and toxin neutralizing antibodies within two weeks following a single immunization in multiple experimental species. The delivery procedure is particularly dose efficient and thus favorable for achieving target levels of response following vaccine administration in humans. These results suggest that EP may be a valuable platform technology for the delivery of DNA vaccines against anthrax and other biothreat agents.

Keywords: DNA vaccine, biodefense, anthrax, electroporation

Abbreviated article title: Electroporation-based DNA vaccine for anthrax

Abbreviations: EP: electroporation; GMT: geometric mean titer; i.m.: intramuscular; LF: lethal factor; PA: protective antigen; TA: *tibialis anterior* muscle

1. Introduction

The development of medical countermeasures for biothreats and emerging infections has become a public health priority [1]. This will undoubtedly include the development of relevant vaccines [2]. Such vaccines should provide rapid induction of protective immune response with a minimal number of immunizations. They should also be compatible with multi-antigen strategies, straightforward to design, manufacture and deploy, stable upon storage, based on a non-immunogenic vector, and safe for all subjects and the environment [2]. Plasmid DNA vaccines appear to be a promising platform for such applications because they are straightforward to design and manufacture, and exhibit a good stability profile, thereby allowing rapid deployment in response to novel biothreats. Since they are not based on an immunogenic vector, there is no concern of reduction of vaccine potency upon re-administration. Finally, DNA immunization is a favorable platform for the development of vaccines against a wide variety of targets because DNA vaccines can induce both humoral and cellular immune responses, and are conducive to multivalent / multi-agent strategies. Despite all of these promising characteristics, clinical development of novel DNA vaccines has been hampered by the inability to induce consistent, high-level immune responses in humans [3]. Since a necessary condition for DNA immunization is the intracellular delivery of the DNA plasmid to the target cells, the inefficient uptake associated with conventional injection is considered a key limitation [4]. Therefore, it is thought that methods for enhancing intracellular delivery of DNA vaccines could improve immunogenicity and enable the technology for clinical use [4]. In that regard, electroporation (EP) appears promising, because it is a potent *in vivo* method for intracellular gene delivery [5] as well as a very effective means for DNA vaccination [6-9].

Using anthrax protective antigen (PA) as a model antigen, we have investigated the potential of EP based DNA immunization for biodefense. PA is the cell-binding component of the *Bacillus anthracis* toxin, which oligomerizes to form heptamer rings at the surface of target cells and allows intracellular entry of the enzymatic components lethal factor (LF) and edema factor (EF), thereby causing intoxication [10]. PA was selected for these studies because the development of an effective humoral immune response directed against PA can confer protection against anthrax [11-15], and because PA is the major protective immunogen in AVA [16, 17], an anthrax vaccine currently licensed in the United States. In these initial studies, we have assessed the characteristics of immune responses induced by a DNA vaccine candidate encoding anthrax PA delivered by EP in a variety of experimental species.

2. Materials and methods

2.1 Animals

Female Swiss-Webster ND4 mice (8-12 weeks) and Sprague-Dawley rats (200-250 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Experiments were approved by Ichor's Institutional Animal Care and Use Committee (IACUC) and were conducted under the guidelines set forth by the National Institutes of Health in the *Guide for Care and Use of Laboratory Animals*. Female New Zealand White rabbits (2.5-3.0 kg) were housed at LAB International (San Diego, CA). Experiments were approved by LAB International's IACUC and were conducted under the regulations set forth by the USDA.

2.2 Plasmids

The plasmid used for vaccination, called pIMS-120, was constructed at Ichor. The nucleotide sequence encoding the mature 83kDa full length PA protein (without the 29 aminoacid prokaryotic secretory signal sequence) (GenBank accession number AF306782) was codon optimized by GeneArt (Regensburg, Germany). A construct consisting of the codon optimized PA gene fused with a nucleotide sequence encoding the TPA leader peptide was produced and cloned into the mammalian expression vector pVAX1 (Invitrogen, Carlsbad, CA). The plasmid was transferred into TOP10 chemically competent *E.Coli* (Invitrogen, Carlsbad, CA) and grown on kanamycin selective antibiotic plates. Plasmid batches were prepared using the Qiagen Endofree Plasmid Giga kit (Qiagen, Valencia, CA) according to manufacturer's instructions and dissolved in 1X calcium and magnesium free PBS (Mediatech Inc., Herndon, VA).

2.3 Immunization procedure

Mice and rats were anesthetized with isoflurane gas anesthesia. Rabbits were anesthetized either by injection of a ketamine/xylazine cocktail or with isoflurane gas anesthesia. Then, fur was removed over the treatment site and aseptically swabbed. Vaccine delivery was performed by an i.m. administration of plasmid DNA using Ichor's TriGrid™ EP technology as previously reported [7]. The intraelectrode spacing of the TriGrid electrode array used in mice, rats, and rabbits was 2.5 mm, 3.0mm, and 6.0mm respectively.

Mice and rats were injected into one *tibialis anterior* (TA) muscle. Mice were injected with 20 µl and rats were injected with 10 µl using a 3/10cc U-100 Insulin syringe (Becton-Dickinson, Franklin Lakes, NJ). Unless otherwise indicated, rabbits were injected with 0.4ml into the *vastus lateralis* of one quadriceps muscle using a 1cc syringe (Becton-Dickinson) with a 23 gauge

needle. DNA dose was as indicated in the figures. Injection of DNA was immediately followed by electrical stimulation at an amplitude of 250 volts/centimeter of electrode spacing. The total duration of electrical stimulation was 40mS, applied over a 400mS interval (a 10% duty cycle). After completion of pulsing, the integrated TriGrid administration device was removed and the animal was transferred to warm recovery. Controls were treated by injecting conventional i.m. injection of the DNA vaccine or by EP based delivery of the vector backbone without gene insert ('empty vector').

2.4 Assessment of anti-PA antibody response

At various times following immunization, blood was collected by retro-orbital bleed in mice, saphenous bleed in rats or central auricular artery bleed in rabbits. Serum was recovered by centrifugation. Anti-PA IgG responses were measured by ELISA. Briefly, serial dilutions of serum samples were added to 96 well plates coated with 100ng/well recombinant PA. Recombinant PA was obtained from List Biological Laboratories (Campbell, CA). Biotinylated anti-mouse IgG (KPL, Inc., Gaithersburg, MD), anti-mouse IgG_1, anti-mouse IgG_{2a}, anti-mouse IgG_{2b}, anti-mouse IgG_3 (Southern Biotechnology Associates, Birmingham, AL), anti-rat IgG or anti-rabbit IgG (KPL), as well as streptavidin-horseradish peroxidase conjugate (Zymed Laboratories, Inc., South San Francisco, CA) and SureBlue TMB microwell peroxidase substrate (KPL) were used for detection. OD reading at 450nm was performed using a Model 550 microplate reader (BioRad, Hercules, CA). Antibody titer was calculated as the reciprocal of the sample dilution yielding an OD_{450} of 0.600. Total serum IgG concentration was assessed on some samples using an ELISA kit from Bethyl (Montgomery, TX). On selected time points, anti-

PA antibody titers in rabbits were also assessed by ELISA as μg anti-PA IgG per ml as previously described [15].

2.5 Neutralizing antibody assay

Neutralizing antibody titers were assessed as previously described [18]. Briefly, purified anthrax toxin subunits PA and LF were incubated for an hour together with various dilutions of serum from immunized animals. Recombinant LF was obtained from List Biological Laboratories. The mixture was then added in triplicate to J774A.1 cells (American Type Culture Collection, Manassas, VA) and incubated for three hours at 37^{o}C. Under the conditions used (30,000 cells per well, 20ng PA and 8ng LF per well, 96 well flat bottom plate, 100 μl final volume), the toxin kills virtually all J774A.1 cells. Neutralizing antibodies protect J774A.1 cells from being killed by the toxin. Cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay kit (Promega, Madison, WI). Cells that survive exposure to the toxin are able to reduce MTT to an insoluble purple pigment. After incubation for two hours at 37^{o}C in a cell culture incubator, the pigment was solubilized by the addition of 100μl of solubilization buffer according to the manufacturer instructions. OD_{570} was assessed using the BioRad Model 550 Microplate reader. The OD reading provides a proportional measure of cell viability. Antibody titer was calculated as the reciprocal of the sample dilution yielding an OD_{570} equal to twice the background. On selected time points, neutralizing antibody titers in rabbits were also assessed as ED50 as previously described [15].

2.6 Determination of antibody avidity

The avidity of anti-PA antibodies in serum was estimated using the thiocyanate elution method as previously described [19, 20]. The avidity index is defined as the molarity of thiocyanate required to elute 50% of antibody under conditions of antigen excess. It is measured as the molarity of thiocyanate required to decrease by 50% the absorbance reading as compared to reading in samples in the absence of thiocyanate, assuming that the absorbance reading in the absence of thiocyanate represents total binding of specific antibody [19].

3. Results

3.1 Enhancement of magnitude and kinetics of antibody responses in multiple species

Swiss-Webster mice were immunized with EP or by conventional i.m. injection of 10μg of the pIMS-120 plasmid in a volume of 20μl. Blood was collected at various times following immunization for determination of anti-PA antibody titers in serum. As shown in Figure 1, both anti-PA IgG (Fig. 1A) and toxin neutralizing antibodies (Fig. 1B) were induced within two weeks following a single immunization with EP and remained elevated for the six months duration of the study in the absence of any boost immunizations. Anti-PA IgG were also induced in mice immunized with conventional i.m. injection; however, the titers were lower, by approximately one order of magnitude and response peaked later, at approximately six weeks. Importantly, in marked contrast to animals immunized using EP, neutralizing antibodies were undetectable in animals immunized with conventional i.m. injection. As shown in Table 1, total levels of serum IgG were not affected by either immunization methods. This shows that the augmentation in antibody titers following EP based immunization was not due to a massive non-specific increase in total serum IgG.

Immune responses to the vaccine delivered by EP or conventional i.m. injection were then assessed in Sprague-Dawley rats using the same dose of DNA (10μg) as that used in mice. Anti-PA IgG (Fig. 1C) and neutralizing antibodies (Fig. 1D) were rapidly induced following a single injection of vaccine with EP. Antibody titers remained high over the five month duration of the experiment. In contrast, animals immunized with conventional i.m. injection exhibited inconsistent anti-PA IgG responses approximately two orders of magnitude lower than with EP. Consistent with the results in mice, conventional i.m. immunization did not induce detectable neutralizing antibodies.

A third immunogenicity study was conducted in New Zealand White rabbits. Animals were immunized with 300μg of DNA using EP or conventional i.m. injection. Consistent with the findings in mice and rats, EP was associated with the rapid induction of anti-PA IgG (Fig. 1E) and neutralizing antibodies (Fig. 1F) in all subjects following a single immunization. Antibody titers remained elevated for the seven month duration of the experiment. In contrast, anti-PA IgG titers in animals immunized with conventional i.m. injection were lower (by more than one order of magnitude) than with EP. No neutralizing antibodies could be detected in these animals.

Differences were noted in the kinetics of the immune response between the three animal models, most notably in the toxin neutralizing antibody titers. Additionally, the variation of the ELISA titers between individual animals (standard deviation) was greater at all time points in animals in which EP was not used.

3.2 Dose efficiency

The studies described above indicate that EP is an appropriate delivery method to enhance the magnitude of antibody responses to the vaccine candidate. We next assessed whether these

characteristics were maintained over a broad range of vaccine doses. As shown in Figure 2, when the DNA was delivered by EP, a five fold dose reduction in DNA dose (from 10μg to 2μg) did not result in decreased magnitude of anti-PA IgG and neutralizing antibody responses in mice, remaining significantly higher than the response to 10μg of DNA administered with conventional i.m. injection. At a dose of 0.4μg of DNA administered with EP, the magnitude of the response trended lower. Although the geometric mean titer (GMT) was still slightly higher than with 10μg of DNA administered with conventional i.m. injection, the difference was not statistically significant. Administration of empty vector with EP did not induce an anti-PA IgG response. Overall, these findings suggest that EP mediated delivery enhances the potency of the PA DNA vaccine by between one and two orders of magnitude as compared to conventional i.m. injection.

In order to assess the dose response of EP mediated DNA immunization in a species of larger body mass, rabbits were immunized at vaccine doses of 50, 150 and 500μg. Total DNA amount was kept equal to 500μg by adding the necessary amount of empty vector (pVAX1). Anti-PA IgG and neutralizing antibodies were assessed for 28 weeks following a single immunization with EP. As shown in Figure 3, delivery with EP enabled high magnitude anti-PA IgG and neutralizing antibody responses to be rapidly and consistently induced in all subjects, even when as little as 50μg of PA encoding DNA was administered.

3.3 Further comparison of immune responses induced by EP or conventional injection

To evaluate the induction of immunological memory and further assess the immunogenicity of the PA DNA vaccine delivered, a boost immunization was administered 36 weeks after the initial immunization to the mice described in section 3.1 and shown in Fig. 1 (A, B). As shown in Figure 4, anti-PA IgG titers were increased in all animals after the second immunization with

either delivery method, indicating that the vaccine induced long-term immune memory. After the second immunization, titers in animals immunized by conventional i.m. injection were comparable to titers measured following a single EP mediated administration. Interestingly, in contrast to the animals immunized with EP (where anti-PA IgG titers of this magnitude correlated with induction of neutralizing titers in all subjects), neutralizing antibodies were detectable only in some of the animals immunized by conventional i.m. injection. This remained the case in subsequent bleeds (data not shown). These results indicate that the magnitude of anti-PA IgG response is not the sole determinant of induction of neutralizing antibodies.

In an attempt to identify other factors that could also contribute to the induction of neutralizing antibodies by EP, we assessed the relative avidities of anti-PA antibody induced after a single immunization by EP and conventional i.m. injection, and we also measured the IgG subclasses induced by each immunization method. The results of representative avidity measurements are shown in Table 2. As indicated in the table, there was no significant difference in antiserum avidity for PA between the cohorts immunized with EP and conventional i.m. injection. Examination of IgG subclasses (Fig. 5A) induced by immunization revealed that the subclasses induced by conventional i.m. injection of the vaccine were mainly IgG_1 and IgG_{2a}. EP based vaccination markedly enhanced both IgG_1 and IgG_{2a} with a statistically significant induction of the GMT of 11.3 fold for IgG_1 and a slightly lower induction of 6.7 fold for IgG_{2a}. The ratio of IgG_1 titers to IgG_{2a} titers in individual animals appeared consistent between the two immunization methods since we found no statistically significant difference between the two immunization groups (Fig. 6B). This indicated that the relative induction of the two subclasses was comparable between the two immunization methods. IgG_{2b} titers were relatively modest as compared to IgG_1 and IgG_{2a} titers; they were found elevated in the group immunized with EP as

compared to the group immunized with conventional i.m. injection, although the induction was more limited (2.6 fold) than with IgG_1 or IgG_{2a}. This difference in IgG_{2b} induction was statistically significant between the two immunization groups in the experiment presented in Fig.6, but not in a repeat study (not shown). Anti-PA IgG_3 were not detectable in animals immunized by either method. Overall, there did not appear to be a notable difference in the composition of antigen specific IgG subclasses following immunization with EP or conventional i.m. injection.

3.4 Potential for induction of protective immunity

Antibody responses obtained in rabbits following a single immunization with EP or conventional i.m. injection were re-assessed on selected time points using the same assays that have been used previously to assess protective antibody titers for inhalation anthrax in the rabbit model [15]. The results of this evaluation, including anti-PA IgG and neutralizing antibody are shown in Figure 6. The results are consistent with above results showing the potentiation of immune responses to PA using EP. Importantly, anti-PA IgG and neutralizing antibody titers induced using EP were comparable to titers previously reported [15] in rabbits immunized with rPA and found protected against inhalation anthrax.

4. Discussion

When compared to conventional i.m. injection, EP enhanced multiple aspects of immune responses to DNA vaccination for anthrax PA in several species. The magnitude of anti-PA IgG responses was increased by approximately one order of magnitude in mice and by one to two orders of magnitude in rats and rabbits. In addition, in all three species, EP based vaccination

exhibited more rapid response and lower inter-subject variability in anti-PA IgG titers than conventional i.m. injection. In all species tested, EP based immunization induced neutralizing antibodies in 100% of the subjects within 2-3 weeks following a single immunization. This was in marked contrast to conventional i.m. injection which did not induce a detectable neutralizing antibody response in the vast majority of animals. EP also greatly improved the dose efficiency of the PA DNA vaccine, inducing consistent, high magnitude immune responses even at relatively low dose levels. Although there was some variation in the kinetics of response among the three species tested, it is notable that EP mediated delivery of the vaccine induced comparable levels of toxin neutralizing antibodies in mice, rats and rabbits. This represents an absolute comparison since the exact same assay was used with all three species. Assessment of quantitative anti-PA IgG titers indicated that peak anti-PA IgG titers in sera from rabbits immunized with EP were greater than 100μg anti-PA IgG per ml serum. These titers are comparable to titers previously measured using the same method at the peak of antibody responses in rabbits immunized with recombinant PA (rPA) adsorbed to aluminum hydroxide and resistant to challenge with live anthrax spores (Figure 3 in ref. [15]). Anthrax toxin neutralizing antibodies were also induced in rabbits immunized with EP with ED50 values comparable to those previously found to be protective in rabbits immunized with rPA (Figure 4 in ref. [15]). These results are significant since antibody responses to PA are considered a correlate for protection against inhalation anthrax [12, 13, 15].

We cannot completely explain the difference between the two immunization methods for induction of neutralizing antibodies. It is likely that several mechanisms contribute to the ability of EP to enable the rapid induction of neutralizing antibodies. As discussed above, even though the magnitude of anti-PA IgG response is an important determinant of the magnitude of the

neutralizing antibody response, there is not a perfect correlation between the two responses. We found no difference between the two immunization methods with regards to antibody avidities and IgG subclass distribution, two elements that we thought could have played a role in neutralizing antibody induction. One could speculate about other factors may contribute to the difference. For instance, recent reports have shown that EP can induce reactivity against a broader range of epitopes than conventional vaccine administration. Thus far, this has been demonstrated only with T cell epitopes [7-9]. If this is also the case for B cell epitopes, this could include epitopes that are critical for neutralizing activity.

Regardless of the method of administration, our anthrax PA DNA vaccine candidate preferentially induced anti-PA IgG of the IgG_1 subclass over the IgG_{2a} subclass, which indicates a Th2 skew of the response. Although DNA vaccines are generally considered strong inducers of Th1 responses, previous observations suggest that this may not be the case for all antigens [21]. In fact, preferential induction of the IgG_1 over the IgG_{2a} subclass has been noted previously with vaccines for anthrax PA, including protein [22] and other DNA [23] based vaccines. In that latter study, direct measurement of T cell cytokine indicated that despite the stronger induction of IgG_1 than IgG_{2a}, both Th1 and Th2 cytokines were induced to a comparable extent [23], which suggests that DNA vaccination elicited a mixed Th1 / Th2 response. Overall, this response profile may be favorable to induce protection against anthrax. Since the majority of anti-PA monoclonal antibodies capable of toxin neutralization produced so far are of the IgG_1 subclass [24], vaccines inducing this subclass may be favorable for toxin neutralization. On another hand, preserving a Th1 component in the response may help in other aspects of protective immunity [25].

The strong enhancement of immune responses by EP provides encouragement that EP-based immunization may be useful for the development of DNA vaccines. Several characteristics of EP based DNA immunization observed in the present study are of interest for the development of vaccines for biodefense. First, the dose efficiency associated with EP is conducive to the inclusion of more than one plasmid, and thus to the development of multivalent / multi-agent vaccines. Second, EP is associated with a fast onset of immune responses with few immunizations, which is favorable for applications where a rapid response is required. Recent epidemiological studies on the 2001-2002 anthrax attack in the US [26, 27] showed that antibiotic treatment provided sub-optimal protection against the disease and indicated that a multi-modality treatment combining antibiotics with another intervention capable of providing rapid protection (within less than 4 weeks) could significantly improve treatment outcome following anthrax exposure. These studies indicate that the earlier the onset of protection, the more beneficial the additional treatment. From a practical and logistical perspective, the intervention would ideally require a minimal number of administrations to maintain protection.

The results we have obtained using anthrax PA as a model antigen are potentially relevant to other biothreats for which the major component of protection is the generation of an antibody response. In particular, neutralizing antibodies against antigens that play a key role in pathogenesis, such as viral envelope glycoproteins and bacterial toxins, are considered correlates for immunity for a number of agents. Based on studies by Ichor (unpublished) and others [28], EP appears to favor the induction of neutralizing antibodies with other antigens as well. Of note, DNA vaccines are known to induce strong T cell responses. T cell responses increasingly appear as an important protective mechanism, even with agents like anthrax for which antibody responses are the only identified correlate for immunity [25]. Therefore, EP based DNA

vaccination may offer the additional benefit of inducing strong cellular immune responses [7-9, 29, 30].

Based on its ability to promote the rapid induction of broad immune responses with relatively few immunizations, this platform technology may facilitate the development of vaccines against agents for which traditional vaccination methods are either inefficient or ineffectual. Preparations are now underway for further development of this anthrax vaccine, including efficacy and safety studies designed to support human clinical studies. In addition, the promising results observed with anthrax DNA vaccines have led to the initiation of pre-clinical investigations of EP based DNA immunization against a variety of other bacterial and viral agents relevant to biodefense.

Acknowledgements

We thank Dr. Stephen Leppla (NIAID, NIH) for reagents and advice, and Dr. Edwin Ades (CDC, Atlanta, GA) for helpful discussions and advice. We also thank Katie Bernard, Jessica Hankins, Michael Lao, Elizabeth Lung, Pamela Rahn and Rafael Villanueva for technical assistance, and Lacey Tichenor for outstanding administrative assistance. This work was supported in part by the SBIR grant program from NIH (grants #AI052670 and AI053936).

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Table 1: Total serum IgG levels in mice immunized with EP or conventional i.m. injection

Treatment	Pre-immune	10µg DNA/EP	2µg DNA/EP	0.4µg DNA/EP	10µg DNA/no EP
Total serum IgG (mg/ml)* ± 1 SD	0.88 ± 0.10	1.15 ± 0.29	0.75 ± 0.10	0.89 ± 0.24	0.95 ± 0.12
*Assessment at day 14 following a single immunization					

Table 2: Relative anti-PA IgG avidities in serum of mice immunized with EP or conventional i.m. injection

Immunization method	Avidity index measured in individual animals (expressed in mM NH_4SCN)*	Average (± 1 SD)	p value **
EP	746, 797, 847, 932, 983, 1254	927 (±182)	p>0.05
Conventional i.m. injection	424, 457, 525, 729, 814, 1254	701 (±312)	
*Assessment at day 42 following a single immunization with 10µg of DNA **Based on one-tailed Student's *t* test			

Figure Legends

Figure 1: Induction of anti-PA IgG and toxin neutralizing antibody responses following a single EP based immunization in mice, rats and rabbits

Swiss-Webster mice (A, B) and Sprague-Dawley rats (C, D) were immunized at day 0 with 10 μg pIMS-120 plasmid in one TA muscle. New Zealand White rabbits (E, F) were immunized at day 0 with 300 μg pIMS-120 plasmid in one quadriceps. Vaccine was administered with EP (filled diamonds, solid lines) or conventional i.m. injection (empty diamonds, broken lines). Anti-PA Ig titers (A, C, E) and toxin neutralizing antibodies (B, D, F) were assessed in serum at various times. Error bars represent one standard deviation.

Figure 2: Dosing study in mice

Swiss-Webster mice were immunized at day 0 by injecting in one TA muscle 10 μg (filled diamonds), 2 μg (filled squares), or 0.4 μg (filled triangles) pIMS-120 with EP or 10 μg (empty diamonds) pIMS-120 plasmid by conventional i.m. injection. Controls received empty vector with EP (cross). Preimmune levels are indicated by a circle. Six weeks after immunization, serum was collected, and anti-PA Ig titers (A) and toxin neutralizing antibodies (B) were assessed. Horizontal bars represent the geometric mean titers. Statistical difference of each EP group relative to the conventional i.m. injection group are shown as * ($p<0.05$) and ** ($p<0.01$) using a one sided Student's *t* test assuming a lognormal distribution.

Figure 3: Dosing study in rabbits

New Zealand White rabbits were immunized at day 0 by injecting in one quadriceps 500µg pIMS-120 (diamonds), 150µg pIMS120 + 350µg pVAX.1 (squares), or 50µg pIMS-120 + 450µg pVAX.1 (triangles) in a volume of 500µl with EP. Anti-PA Ig titers (A) and toxin neutralizing antibodies (B) were assessed in serum at various time points. Error bars represent one standard deviation.

Figure 4: Comparison of immune responses following one and two immunizations in mice

Swiss-Webster mice were immunized at day 0 by injecting in one TA muscle 10 µg pIMS-120 with EP (filled diamonds) or conventional i.m. injection (empty diamonds). A second identical immunization was administered in the contralateral TA at week 36 following the initial immunization. Anti-PA IgG (A) and neutralizing antibodies (B) were assessed at 6 weeks and 38 weeks. Horizontal bars represent the geometric mean titers. Statistical difference of each EP group relative to the conventional i.m. injection group are shown as * ($p<0.05$) and ** ($p<0.01$) using a one sided Student's *t* test assuming a lognormal distribution.

Figure 5: Determination of IgG subclasses in mice

Swiss-Webster mice were immunized at day 0 by injecting in one TA muscle 10 µg pIMS-120 with EP (grey) or conventional i.m. injection (white). Anti-PA IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 titers were assessed in serum at 16 weeks. (A) Geometric mean titers are shown in grey for the group immunized with EP and in white for the group immunized by conventional i.m. injection. Error bars represent one standard deviation. (B) Ratios of IgG_1 titers to IgG_{2a} titers in each individual animal were calculated for the EP group (black diamonds) and conventional i.m. injection group

(white diamonds). Statistical difference of each EP group relative to the corresponding conventional i.m. injection group are shown as * ($p<0.05$) and ** ($p<0.01$) using a one sided Student's *t* test assuming a lognormal distribution.

Figure 6: Reassessment of anti-PA IgG and toxin neutralizing antibody responses following a single EP based immunization in rabbits using a standardized assay

New Zealand White rabbits were immunized as indicated in Figure 1. Anti-PA IgG and neutralizing antibody were assessed on selected serum samples using standardized assays that have been used to determine immune correlates for protection against inhalation anthrax in rabbits [15]. Error bars represent one standard deviation.

Figure 1

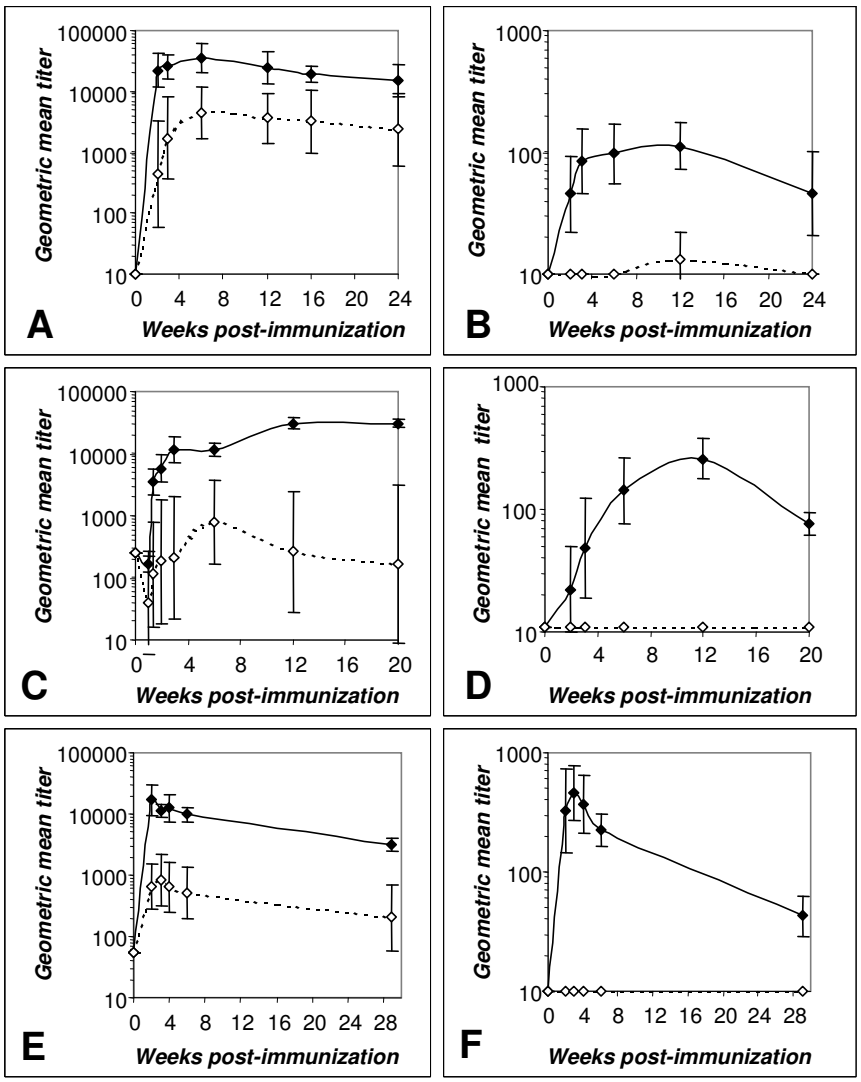


Figure 2

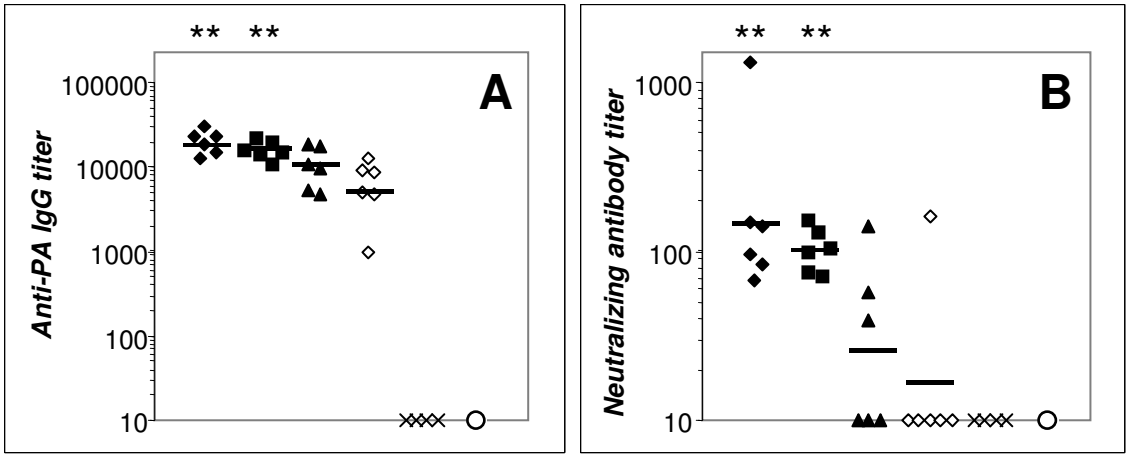


Figure 3

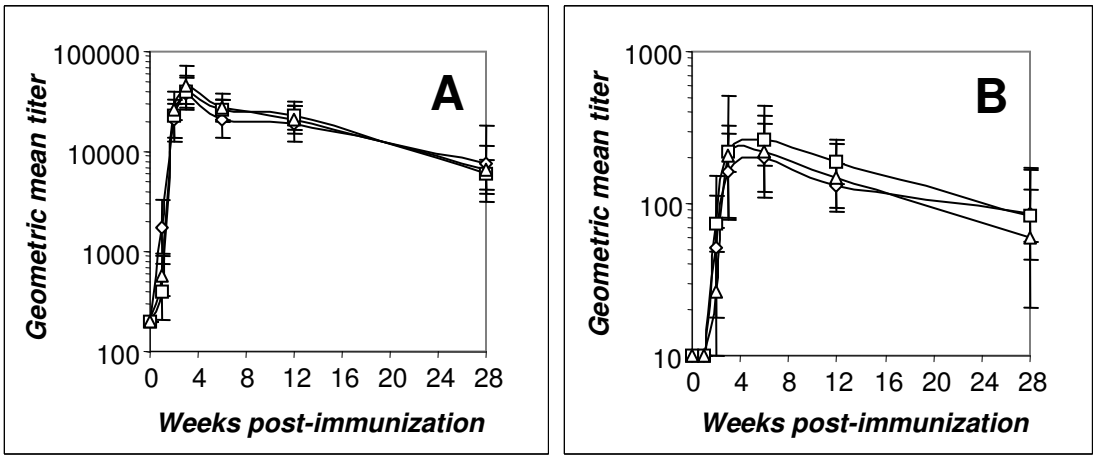


Figure 4

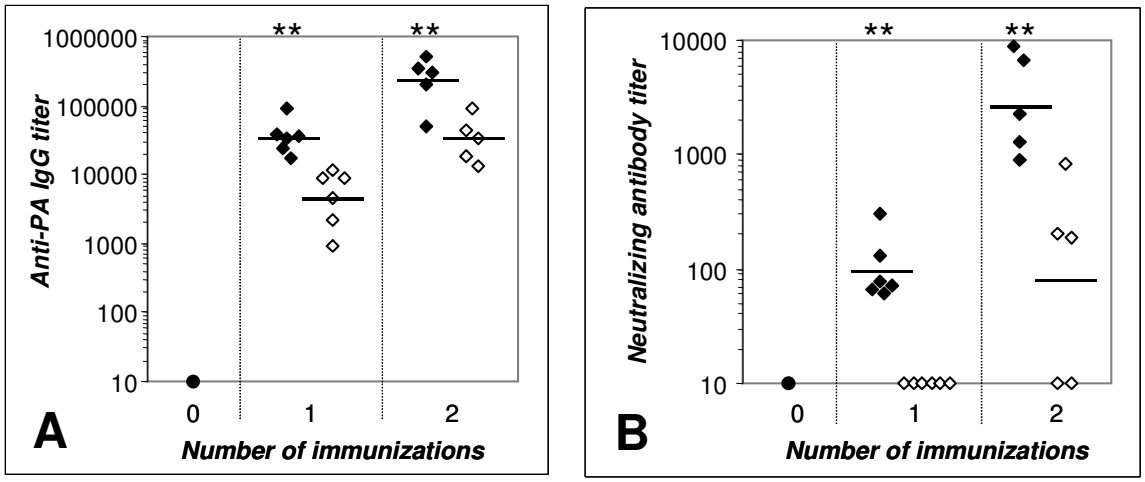


Figure 5

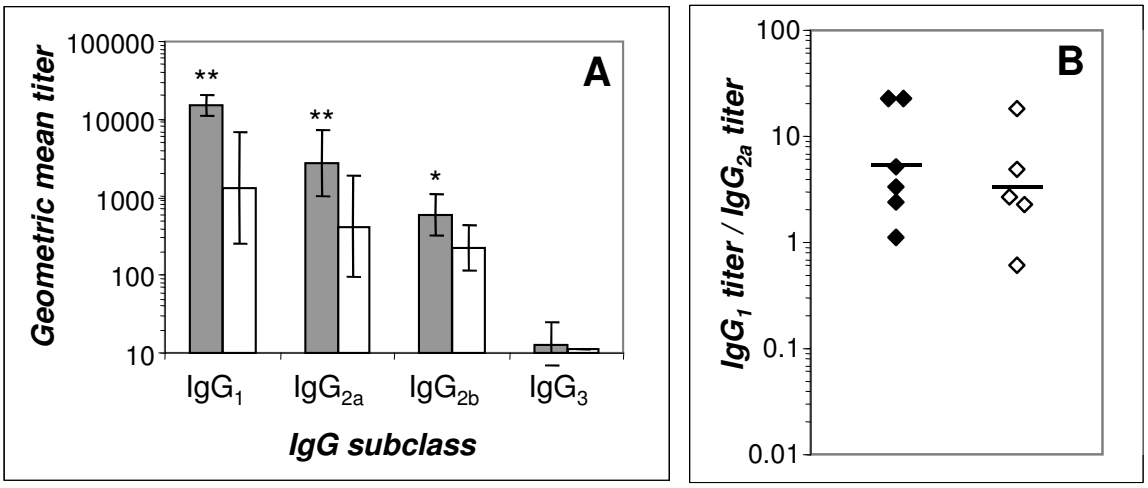
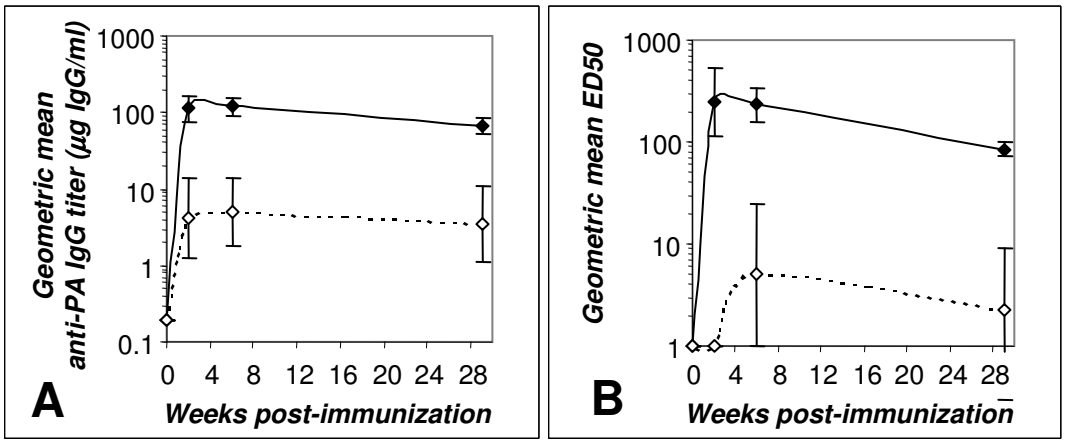


Figure 6


Geometric mean anti-PA IgG titer (µg IgG/ml)
1000
100
10
1
0.1
0 4 8 12 16 20 24 28
Weeks post-immunization
A
Geometric mean ED50
1000
100
10
1
0 4 8 12 16 20 24 28
Weeks post-immunization
B