Rapid generation of an anthrax immunotherapeutic from goats using a novel non-toxic muramyl dipeptide adjuvant

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Abstract

Background: There is a clear need for vaccines and therapeutics for potential biological weapons of mass destruction and emerging diseases. Anthrax, caused by the bacterium Bacillus anthracis, has been used as both a biological warfare agent and bioterrorist weapon previously. Although antibiotic therapy is effective in the early stages of anthrax infection, it does not have any effect once exposed individuals become symptomatic due to B. anthracis exotoxin accumulation. The bipartite exotoxins are the major contributing factors to the morbidity and mortality observed in acute anthrax infections.

Methods: Using recombinant B. anthracis protective antigen (PA83), covalently coupled to a novel non-toxic muramyl dipeptide (NT-MDP) derivative we hyper-immunized goats three times over the course of 14 weeks. Goats were plasmapheresed and the IgG fraction (not affinity purified) and F(ab')₂ derivatives were characterized in vitro and in vivo for protection against lethal toxin mediated intoxication.

Results: Anti-PA83 IgG conferred 100% protection at 7.5 μg in a cell toxin neutralization assay. Mice exposed to 5 LD₅₀ of Bacillus anthracis Ames spores by intranasal inoculation demonstrated 60% survival 14 d post-infection when administered a single bolus dose (32 mg/kg body weight) of anti-PA83 IgG at 24 h post spore challenge. Anti-PA83 F(ab')₂ fragments retained similar neutralization and protection levels both in vitro and in vivo.

Conclusion: The protection afforded by these GMP-grade caprine immunotherapeutics post-exposure in the pilot murine model suggests they could be used effectively to treat post-exposure, symptomatic human anthrax patients following a bioterrorism event. These results also indicate that recombinant PA83 coupled to NT-MDP is a potent inducer of neutralizing antibodies and suggest it would be a promising vaccine candidate for anthrax. The ease of production, ease of covalent attachment, and immunostimulatory activity of the NT-MDP indicate it would be a superior adjuvant to alum or other traditional adjuvants in vaccine formulations.
Background

*Bacillus anthracis*, the causative agent of anthrax, has been the focus of much research and attention following the release of spores through the US mail system in 2001. 22 cases of infection resulted in 5 deaths, causing much concern regarding treatment, therapeutics and vaccine efficacy. Recently, the CDC discontinued the administration of the current anthrax vaccine (Anthrax Vaccine Adsorbed -AVA) due to adverse side effects observed in a large percentage of volunteers. This revocation of available vaccine has left healthcare workers, laboratory personnel and first responders with only limited means of protection following potential exposures to anthrax spores.

In humans, the anthracis bacilli can cause three types of infections: cutaneous via abrasions in the skin, gastrointestinal through ingestion of spores in contaminated meat and inhalation when spores less than 5 μm are deposited into the lungs [1]. The mortality rates vary between each form of the disease with cutaneous anthrax presenting as a self-limiting and treatable infection with only a 20% case fatality rate. When left untreated gastrointestinal infections can progress rapidly and have over 80% case fatality rates. Inhalation anthrax infections are rare but have a high case fatality rate (over 75%) even with antibiotic treatment.

Treatment options for patients presenting with symptoms of inhalational anthrax infections are limited and are generally ineffective at reducing mortality. Although antibiotic therapy is effective in the early stages of infection, it does not have any effect on the bipartite exotoxins, which are the major contributing factors to the mortality observed in acute anthrax infections [1]. The current lack of an approved, available vaccine puts laboratory workers, military personnel and first responders at an increased risk of inhalational anthrax should another terrorist event, similar to the anthrax mailings in 2001, occur. Clearly there is a need for an effective vaccine as well as a well-tolerated, economical, post-exposure therapeutic for the treatment of human anthrax infections.

Passive immunotherapy is a non-chemical therapeutic providing immediate immunity to infectious agents and toxins. This treatment option has been shown to be effective against many diseases including anthrax [2-6] and other bioterror agents [7,8]. Several approaches have been used previously for the production of immunotherapeutics specific for *B. anthracis* although they all have significant drawbacks. The pooling of immune serum from previously vaccinated volunteers yields highly protective anti-sera in very small quantities, limiting its use as a source of therapeutics for the Strategic National Stockpile or as a commercially available product. Monoclonal antibodies are highly specific, limiting their application to a single antigenic target and have a high cost associated with their development further limiting their feasibility for mass production and stockpiling. In the past animal vaccination has successfully been used to generate immunotherapeutic antisera specific for infectious and toxic agents including snake venom, botulism toxin and Ebola virus [9-12] but limitations in quantity and safety have prevented their widespread use in the development of human therapeutics. Horses can provide large amounts of antisera but are costly to maintain. Mice, rabbits and guinea pigs are inexpensive to maintain but yield limited volumes of anti-sera. Goats provide a renewable source of plasma and serum; however they have not been traditionally used in the generation of passive immunotherapeutics. We have plasmapheresed hyper immunized goats to successfully produce liters of GMP-grade antisera following a short immunization schedule (3 immunizations over 14 weeks), with minimal cost.

*Bacillus anthracis* produces two separate exotoxins, edema toxin (EdTx) and lethal toxin (LeTx). The two exotoxins utilize a common cell binding component termed protective antigen (PA83, 83 kDa) which binds to the ubiquitous anthrax toxin receptor (ATR) found on most cell surfaces. Once PA83 is bound to the host cell surface, a furin-like protease cleaves the full-length, inactive protein into the active form, PA63 (63 kDa), thereby exposing the binding sites for the catalytic components of the exotoxins (edema factor, EF or lethal factor, LF). A heptamer composed of PA63 + three LF/EF moieties [13,14] forms on the cell surface and is internalized via receptor mediated endocytosis. The subsequent decrease in pH within the endosome causes conformational changes in PA63, so that it inserts into the endosomal membrane, forming a protease-stable pore; formation of this pore allows EF and LF to enter the cell and exert their toxic effects [15]. LeTx is formed when PA63 is combined with LF, and is responsible for the most severe intoxicative effects of anthrax infection. EF is an adenylate cyclase capable of causing severe disregulation of cellular cAMP levels [16]. LF has been shown to be a zinc-dependant metallopeptide with specificity for mitogen-activated protein kinase kinases (MAPKKs) capable of disrupting several cell signaling cascades; however, its specific mode of action is still unclear [17,18]. Disruption of the binding of PA to ATR or LF would disrupt internalization of functional LeTx and would thereby prevent toxin-mediated death of the host following rapid multiplication of the bacilli.

Here we immunized goats with recombinant PA83, coupled to a novel non-toxic muramyl dipeptide derivative (NT-MDP) capable of inducing both innate and humoral immunity and does not induce clotting even when administered at high concentrations. The resulting polyclonal anti-sera conferred protection against *in vitro* and *in
**Methods**

**Recombinant anthrax toxin proteins**
High-purity, histidine-tagged rLF and rPA83 were supplied by the Northeast Biodefense Center Protein Expression Core. Functional lethal toxin (LeTx) was formed by the combination of purified rLF and rPA83 at a 1:1 (w/w) ratio diluted in sterile PBS.

**Caprine antisera**
Purified rPA83 was supplied to Virionyx Corporation Ltd (Auckland, NZ) for caprine immunizations as follows. A novel muramyl dipeptide adjuvant (NT-MDP) was oxidized with sodium metaperiodate (0.5 M) for 1 h and excess sodium meta periodate was removed by centrifugation followed by a water wash. 1 mg of rPA83 in sodium carbonate buffer (0.1 M, pH 9.5) was added to 10 mg of activated NT-MDP and incubated overnight at room temperature. The resulting Schiff’s base was reduced by the addition of sulfuric acid and absorbance was measured at 492 nm. Anti-PA83 IgG titers were measured and expressed as the reciprocal of the antibody dilution which produced an absorbance value equal to 50% maximum absorbance.

**Anti-sera titer determination**
ELISAs were performed in microtiter plates coated with rPA83 (10 nM) in 10 mM carbonate/bicarbonate buffer (pH 8.5) with a final coating volume of 50 μl. Plates were coated for 1 h then washed in water and blocked with 5% non-fat milk powder. Antibody titers were measured by reacting (2 h) serially diluted anti-PA83 IgG with the rPA83-coated microtiter wells. The wells were then washed with water and reacted (2 h) with horseradish peroxidase-labeled rabbit anti-goat IgG. Following one water wash, the wells were reagent (30 min) with the substrate, orthophenylenediamine. The reaction was stopped by the addition of sulfuric acid and absorbance was measured at 492 nm. Anti-PA83 IgG titers were measured and expressed as the reciprocal of the antibody dilution which produced an absorbance value equal to 50% maximum absorbance.

**Cell lines and media**
Murine macrophage-like cells, J774A.1, were obtained from the American Type Cell Culture Collection (ATCC TIB-67). Cells were cultured in complete medium: Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, Glutamax, and penicillin/streptomycin at 37°C with 5% CO₂.

**In vitro cytotoxicity and protection assays**
Macrophage-like cells were harvested by gentle scraping (no trypsin) and were seeded in 96-well plates at a density of 6 × 10⁴ cells/well in 100 μl of complete medium. Cells were incubated for 18–24 h or until > 90% confluence had been achieved. Medium was removed, and cells were washed once in sterile PBS before addition of toxin or anti-sera. For toxicity assays, 100 μl of LeTx was added to the cells at final concentrations of 1000 ng, 100 ng, 10 ng and 0.1 ng (data not shown). For protection assays, 50 ng of LeTx (2 TCEC₅₀) was combined with varying dilutions of anti-PA83 IgG or F(ab’)₂ and incubated at 37°C, while shaking for 1 h prior to the addition of 100 μl per well. Cells with LeTx alone or in combination with anti-sera were incubated at 37°C and 5% CO₂ for 4 h. Cell viability was determined using Sigma’s Cell Growth Determina-
tion Kit, an MTT-based assay. Briefly, 10 µl of MTT dye was added to cells and incubated for 15 h at 37°C and 5% CO₂. 100 µl of solubilization solution was added to each well after removal of media, and cell viability was measured at 570 nm. Percent relative cell viability was calculated as the ratio between LeTx-treated cells (LeTx) and untreated control cells (100 µl PBS). Percent protection conferred by caprine anti-PA83 IgG or F(αb')₂ was measured as follows:

\[
\left(1-\frac{\text{PBS} - \alpha \text{PA83 IgG}}{\text{PBS} - 50 \text{ ng LeTx}}\right) \times 100.
\]

**In vivo protection assays**

**Lethal toxin challenge**

Female Balb/c mice (average weight 17.5 g) were injected with 100 µg LeTx in 200 µl saline via intraperitoneal injection (5 per group). Five minutes following toxin injection mice were injected on the opposite side with 8 mg/kg anti-PA83 IgG or F(αb')₂ in 200 µl saline. Control mice (3 in group) received LeTx followed by saline injections. Mice were observed for signs of illness and distress for 11 days at which point all surviving mice were sacrificed.

**Virulent B. anthracis spore intranasal challenge**

Female Swiss Webster mice (average weight 25.2 g) were infected with approximately 5 × 10^4 B. anthracis Ames spores (5 LD₅₀) by 20 µl installations in each nares. Groups of 10 mice received saline at 1 hour post-infection or anti-PA83 IgG at 24 h post-infection (32 mg/kg) by intraperitoneal injection. Mice were monitored twice daily for 14 d for signs of illness and death. To evaluate synergistic effects of antibiotic treatment post-exposure, low-dose Ciprofloxacin was administered twice daily at 0.9 mg/day via intraperitoneal injection for the first six days post spore challenge.

**Statistical Analysis of in vivo results**

Statistical analysis (logrank test) of the in vivo survival data was performed using GraphPad Prism (version 4.03), GraphPad Software, San Diego, CA.

**Results and Discussion**

**Anthrax lethal toxin activity**

Purified rLF (90 kDa) and rPA83 (83 kDa) showed high product purity, with no significant breakdown products by SDS PAGE, trypsin digestion and mass spectroscopy (> 95% purity for both, data not shown). In vitro bioactivity of LeTx was confirmed by treating J774A.1 murine macrophage-like cells with varying doses of LeTx (10 – 0.001 ng/µl), and cell viability determined via toxin neutralization assay. Cell viability experiments established a TCEC₅₀ of 25 ng LeTx (equivalent to 2.85 nM, data not shown). This dose of LeTx is within the range of previously reported TCEC₅₀ [22-25]. Based on this data, all subsequent in vitro protection assays were performed at 2× TCEC₅₀ equivalent to a total of 50 ng LeTx per well.

**Generation and evaluation of anti-PA83 caprine immunoglobulin**

One goal of this study was to produce large volumes of high titer, hyper-immune goat sera in a short period of time. Goats were immunized four times (days 0, 14, 28, 56) over a period of 56 days and subsequently plasmapheresed (day 94). Total IgG was purified from plasma and rPA83 specificity was confirmed by Western blot and ELISA (data not shown), validating the efficacy of the immunogen/adjuvant, immunization schedule, and IgG purification methods established previously with the anti-HIV immunotherapeutic [19-21]. Specific rPA83 titers were obtained from immunized goats on days 0, 27, 40, 54, 67, and 94. Antibody titers were measured by ELISA by reacting serially diluted anti-PA83 IgG with 10 nM rPA83. Anti-PA83 IgG demonstrated significant titer (> 10,000, calculated as the reciprocal of the dilution producing 50% maximum absorbance) within 2 weeks (27 d post-immunization), and reached a maximum of ~16,000 after the fourth immunization (Fig. 1). High titer polyclonal antisera could be generated in as little as 42 days thus establishing that rapid production of target-specific caprine goat anti-PA83 IgG titer

**Figure 1**

Goat anti-PA83 IgG titer. Serially diluted goat anti-PA83 IgG reacted with 10 nM rPA83 in a microplate ELISA. Titer calculated as the reciprocal of the dilution producing 50% maximum absorbance. Day 0 is 1st immunization with PA83-NT-MDP, asterisks indicate timings of 2nd (day 14), 3rd (day 28) and 4th (day 56) booster immunizations. Purified anti-PA83 IgG was obtained from plasmapheresed goats on day 94 (time point designated by a square).
immunotherapeutics using the novel NT-MDP adjuvant is achievable.

**Anti-PA83 IgG and F(ab')2 protect cells against LeTx-induced cytotoxicity**

The protective efficacy of the anti-PA83 IgG and the F(ab')2 derivative was evaluated in the J774A.1 LeTx in vitro model. Cells were exposed to 0.5 ng/μl of LeTx and dilutions of anti-PA83 IgG or F(ab')2. MTT-based cell viability assays were used to determine percent protection as described in Materials and Methods. Control included untreated cells (i.e., PBS substituted for LeTx), cells treated with IgG alone (7.5 μg α PA83 Ig with no LeTx), or cells treated with 0.5 ng/μl LeTx alone (LeTx). LeTx treated cells demonstrated a statistically significant decrease in cell viability (p < 0.001) as compared to the untreated PBS control cells, while standard concentrations of anti-PA83 IgG (7.5 μg) had no effect on cell viability (data not shown). The use of higher concentrations of anti-PA83 IgG (up to 250 μg) produced no significant differences in cell viability (data not shown). These results confirm that caprine IgG exhibits no inherent cytotoxic effects in vitro and does not interfere with the observed cytotoxicity of the recombinant LeTx.

Cells treated with varying concentrations of anti-PA83 IgG exhibited protection from LeTx cytotoxicity in a dose-dependent manner (Fig. 2A). Cells were exposed (five separate assays each with four replicates) to varying doses of anti-PA83 IgG and 50 ng LeTx for 4 h. 7.5 μg anti-PA83 IgG fully protected cells against LeTx mediated cell death, while 0.95 μg offered minimal protection (35%) over the LeTx treated control cells (Fig. 2A). Treatment of LeTx exposed cells with anti-PA83 F(ab')2 demonstrated equivalent protection at 7.5 μg compared to anti-PA83 IgG (Fig. 2B). At lower doses, there was an observable diminished protection afforded by the anti-PA83 F(ab')2 compared to whole IgG. These data confirm that rapidly produced caprine immunotherapeutics, either whole IgG or despeciated F(ab')2 fragments, elicit complete protection against LeTx-mediated cytotoxicity in vitro.

**In vivo protection of mice following LeTx challenge**

Efficacy for the anti-PA83 IgG and F(ab')2 immunotherapeutics was established in an intraperitoneal LeTx-challenge mouse model (Fig. 3). The LeTx -challenge mouse model simulates a post-exposure, symptomatic patient. Mice were first injected with 2LD100 (200 μg LeTx) of recombinant LeTx on the left side of the abdomen. This dose of LeTx has been confirmed to be fatal to 100% of mice within 48 h post challenge (data not shown). After five minutes, mice were injected with approximately 8 mg/kg anti-PA83 IgG or F(ab')2 immunotherapeutics on the right side of the abdomen. Control mice received 200 μl of PBS instead of IgG or F(ab')2. Control mice succumbed to LeTx by day 2 while IgG and F(ab')2 treated groups showed 80% and 100% survival, respectively. F(ab')2-treated group survival rates declined to 80% on day 3 and remained there throughout the 11 d study. The IgG-treated group also showed 80% protection for the remainder of the study. The ability for the goat derived passive immunotherapeutic to protect against an in vivo LeTx challenge suggests its potential for use as a therapeutic intervention in humans. Since this model simulates a symptomatic patient, we speculated that the anti-PA83 immunotherapeutics could be used efficaciously post-exposure to prevent mortality.
Passive protection of mice 24 hours post-infection with Ames spores

To evaluate post-exposure efficacy of the anti-PA83 IgG, a mouse model of inhalational anthrax was used. Female Swiss Webster mice were challenged with virulent *B. anthracis* spores via an intranasal infection route. Mice received 5 LD$_{50}$ *B. anthracis* Ames spores in 20 µl instillations into each nares. Control mice received saline at 1 h post-challenge. Twenty-four hours post-challenge, test groups received 32 mg/kg caprine anti-PA83 IgG by intraperitoneal injection. At 4 d post-infection (p.i.), only 20% of control mice survived, while 70% of mice treated with anti-PA83 IgG were still alive (Fig. 4A). By day 6, another 10% of the mice in each group had succumbed to disease and no further mortality was observed through the remaining 14 d study. One test group also received low-dose Ciprofloxacin to examine synergistic effects of post-exposure treatments (Fig. 4B). Mice treated with antibiotics alone exhibited a 50% survival rate out to the end of the study (14 d p.i.). Survival of IgG treated mice dropped to 60% by day 6 p.i. and remained there through the completion of the study. Comcomitant administration of Ciprofloxacin (twice daily on days 1–6) and anti-PA83 IgG (single bolus at 24 h p.i.) completely protected mice for 6 days (Fig. 4B) while Ciprofloxacin was administered. When Ciprofloxacin treatment was stopped, survival decreased to levels comparable to anti-PA83 IgG treatment alone. These results confirm the potential for passive transfer of immunity up to 24 hours post-exposure to *B. anthracis* spores and suggest parallel treatment with antibiotics can significantly enhance survival.

Many groups have shown the efficacy of polyclonal, animal-derived sera for use as a passive immunotherapeutic against anthrax infections, however these groups have relied on smaller animal models (e.g., mice, rabbits, guinea pigs) to generate the antisera [3,4,26,27]. Smaller animals are typically terminally bled in order to produce larger volumes of serum. Yields from a terminal bleed typically range from 0.5 ml for mice up to 200 ml for terminally bled rabbits. The large number of animals required to produce the therapeutic quantities needed for a useful medical countermeasure stockpile (e.g., the SNS) makes these animal models prohibitively expensive. Caprine plasmapheresis does not require the animals to be euthanized/terminally bled in order to generate large volumes of antisera. Additionally, the goats can be plasmapheresed up to four times per year for several years making for a nearly endless source of antisera. Plasmapheresis of three goats generated liters of anti-PA83 serum within a very short time frame. Additionally, the goats used to produce this material are part of a certified pathogen-free herd and the antisera produced are of GMP grade. Comparably produced IgG against HIV has been previously approved for clinical trials in humans [19-21].

The previously approved AVA anthrax vaccine required a series of six immunizations followed by annual boosts. The use of a novel non-toxic MDP adjuvant enabled the generation of extremely high-titer antiserum following only two immunizations although for the current study, IgG was isolated from goats immunized four times. With further optimization of the immunization regimen, we may be able to generate an efficacious immunotherapeutic with fewer immunizations, thus shortening the production time and cost. It should also be emphasized that the data presented here used non-affinity-purified IgG or F(ab')$_2$. Studies are underway to evaluate the efficacy of the affinity purified materials, which may significantly reduce the amount of material required to offer significant protection in both animals and humans.

F(ab')$_2$ antibodies have been used for the treatment of rattlesnake bites [28,29], bee stings [30] and evaluated for their potential to treat several infectious diseases including respiratory syncitial virus (RSV) [31]. Many monoclonal antibodies (MAbs) have been generated that are specific for the anthrax protective antigen. The majority of these MAbs do not demonstrate significant protection post-exposure and appear to require a blend of several MAbs in order to reduce the mortality associated with anthrax infections [32,33]. A recent study using a monoclonal antibody against the anthrax protective antigen demonstrated a requirement for the Fc portion of the antibody in order to retain neutralizing capabilities [25]. Our polyclonal immunotherapeutic retained similar neutralizing levels both in vitro and in vivo after removal of the Fc region by pepsin.

Figure 3

*In vivo* protection against LeTx cytotoxicity. Percent survival of female Balb/c mice treated with 100 µg LeTx by i.p. injection followed 5 minutes later with 8 mg/kg anti-PA83 IgG or F(ab')$_2$ antibodies in 200 µl (5 per group). Control mice (Saline, 3 in group) received 100 µg LeTx followed by 200 µl Saline. All mice were observed twice daily for signs of illness or distress and all surviving mice were euthanized at day 11 post-challenge. P < 0.03 by the logrank test.
digestion. These findings are consistent with data from other polyclonal antiserum, which indicate most F(\(ab'\))\(_2\) retain comparable neutralizing and protective abilities to full length IgG [26,29,30,34]. The utility of F(\(ab'\))\(_2\) antisera derived from goats will reduce the potential for side-effects associated with patients who have a pre-existing sensitivity to goat proteins. In addition, patients requiring multiple treatments with an animal derived therapeutic may also be at increased risk of developing allergic hypersensitivity, so the use of F(\(ab'\))\(_2\) antibody fragments will decrease this risk and increase the overall safety of this immunotherapeutic for multiple uses within a large population.

**Conclusion**

This work has shown that pharmaceutical-grade goat polyclonal immunotherapeutics specific for the anthrax protective antigen can be rapidly produced in large quantities. Three goats immunized four times over a 56 day period produced liters of GMP grade, high titer antisera that was capable of neutralizing anthrax lethal toxin both in vitro and in vivo. More importantly the passive transfer of the goat-derived antibodies 24 h post-exposure to virulent anthrax spores provided mice with a substantial survival advantage over untreated mice. A synergistic effect was seen with concomitant antibiotic treatment although levels of protection returned to the levels observed with IgG treatment alone once antibiotic therapy was discontinued. This indicates that a combined treatment approach for patients presenting with clinical signs of anthrax infection could overall increase in survival rates associated with symptomatic disease. Additionally, this immunotherapeutic can be easily produced in quantities large enough to fulfill the requirements for a national medical countermeasures stockpile. The non-toxic MDP adjuvant developed is easily produced; amenable to covalent attachment of antigens, and importantly, renders toxins and pathogens inactive once coupled to the molecule. The use of this novel adjuvant should improve vaccine development and quality control in addition to eliciting significantly higher immune responses than standard adjuvants.

**Competing interests**

Portions of these studies were funded by Virionyx Corporation Ltd who hold patent rights to the non-toxic MDP adjuvant.
Authors’ contributions
CDK performed all in vitro and in vivo B. anthracis lethal toxin assays and was primary author on this manuscript.
CO and FBG provided NT-DMDP, immunized goats, purified IgG fractions, isolated F(ab’), fractions, and contributed to writing this manuscript. JWP and LES performed B. anthracis infectious murine in vivo assays. NMC provided study designs and contributed to writing this manuscript.

Acknowledgements
Funding for the intranasal mouse study was provided by the National Institutes of Allergy and Infectious Diseases contract with the University of Texas Medical Branch, Contract # N01-AI-30065. CDK received support from the SUNY Albany Foundation through a Ford Foundation IFW Women in Science Fellowship. Thanks to the Northeast Biodefense Center Protein Core Laboratory for the production and purification of recombinant proteins. We are grateful to Jim Hengst and Michelle Ferrier-Jacobi for their technical assistance.

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