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# BclA and toxin antigens augment each other to protect NMRI mice from lethal *Bacillus anthracis* challenge

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ABSTRACT

While proving highly effective in controlling Anthrax in farm animals all over the world currently attenuated live anthrax vaccines employed in a veterinary context suffer from drawbacks such as residual virulence, short term protection, variation in quality and, most importantly, lack of efficacy if administered simultaneously with antibiotics. These limitations have stimulated the development of non-living component vaccines which induce a broad spectrum immune response capable of targeting both toxaemia (as in the case of PA based vaccines) and bacteraemia. To contribute to this several new approaches were tested in outbred NMRI mice for antibody titres and protectiveness. Plasmids encoding a recombinant toxin derived fusion peptide and a spore surface derived peptide were tested as DNA-vaccines in comparison to their protein counterparts utilising two adjuvant approaches and two DNA-vector backbones. The combination of two plasmids encoding LFD1PAD4-mIPS1 and TPA-BclAD1D3-LAMP1, when delivered by GeneGun, protected 90% of the animals against a lethal challenge with 25$LD_{50}$ spores of the Ames strain of *Bacillus anthracis*. Single applications of either antigen component showed significantly lower protection rates, indicating the beneficial interaction between anti-spore and anti-toxin components for an acellular vaccine formulation.



## 1. Introduction

Anthrax is caused by *Bacillus anthracis*, a Gram-positive, spore forming, rod-shaped bacterium [1]. Spores gain access via cutaneous, oral or inhalational routes where they germinate and develop into vegetative bacilli which then replicate and produce toxins which eventually kill the host [2]. The pathogen expresses two major plasmid encoded virulence factors, a gamma-linked poly-D-glutamic acid capsule (pX02 [3]) and a tripartite toxin (pX01 [4]) comprised of Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) [5,6].

Current live attenuated veterinary anthrax vaccines are less than ideal. They can cause problems in sensitive animals such as goats and llamas, protection is short term, variation in vaccine quality can cause vaccine failure and most importantly the live nature of the vaccine prevents its efficacy if delivered at the same time as antibiotics [7,8]. These limitations have stimulated the development of non-living, component vaccines capable of inducing a broad spectrum immune response which targets both toxaemia and bacteraemia.

The strong correlation between toxin neutralising activity (tna) of PA-specific antibodies and protection [9] has prompted efforts to develop vaccines based solely on domains which stimulate antibodies with tna [10–12]. One such study which employed a fusion protein comprised of domain 4 of PA (receptor binding site) and domain 1 of LF (PA binding site) protected mice against a subsequent lethal challenge with *B. anthracis* spores [13]. To further assess the immunogenic value of this protein we administered it as a DNA-vaccine in two different vectors and compared its activity to that seen against full length rPA83.

In addition to neutralising the action of toxins the spore can also be targeted to prevent the pathogen from gaining a foothold in the infected individual [14,15]. Vaccination experiments with live nonvirulent or formaldehyde-inactivated spores have shown that spore specific immune responses can enhance the level of protection when given in combination with PA [16].

One such component is the *Bacillus* collagen like protein of *anthracis* (BclA) which forms hair-like structures projecting from the spore surface and represents a major spore immunogen [17,18]. The removal of the collagen-like region (CLR, domain 2) from BclA has no detrimental effect on immunogenicity and

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results in a smaller peptide which is easier to incorporate into a multicomponent vaccine [19,20]. In this study we determined the immunogenicity of a CLR-deficient version of BclA called rBclAD1D3 when administered as a DNA-vaccine in two different vectors.

For the DNA vaccine studies we employed two different plasmid backbones (pDNAVaccUltra and NTC7382) which varied with regards to intracellular routing signals and immune stimulatory elements [21]. To improve in vivo antigen presentation we utilised intracellular routing signals which directed vaccine peptides to the MHC I and MHC II pathways. To target the MHC II pathway [22] we employed tissue plasminogen activator (TPA) which routes newly expressed proteins to the secretion pathway [23] and lysosome-associated membrane protein (LAMP1) which directs proteins to the endosome [24,25]. To enhance MHC I presentation we employed ubiquitin which directs the associated protein to the proteasome [26,27].

To enhance the immunogenicity of the expressed proteins we investigated the utility of two molecular adjuvants. Mouse interferon-ß promoter stimulator 1 (mIPS-1) incorporated into the backbone of the antigen encoding plasmid significantly induces type I interferon and interferon-stimulated genes in a TLR-independent matter [28–30]. Mouse class II MHC transactivator (CIITA) up-regulates MHC expression [31,32] and was co-administered on a separate plasmid.

In comparison to the DNA vaccines, full length rPA and rBclA were tested as proteins alone and in combination in the presence of a previously tested and approved lipopeptide adjuvant comprising Pam3Cys-SKKKK, a TLR2/1 activator admixed with $Pam_3Cys$ conjugated to the promiscuitive T-helper-cell epitope of the sperm whale myoglobin SFISEAIIHVLHSRHPG [33,34].

The overall aim of this study was to determine the ability of BclA to confer additional protectiveness when given together with a toxin-specific vaccine.

## 2. Materials and methods

### 2.1. Antigen preparation

E. coli BL21-CodonPlus-RIL cells (Stratagene, La Jolla, CA) harboring the plasmid pREP 4 (Qiagen, Venlo, Netherland) and pQE-30 (Qiagen) encoding either rPA83, rBclA or rLF were grown and purified as described previously [35]. Proteins used for ELISA received no further treatment while proteins used for vaccination were tested for endotoxin using the Limulus Amoebocyte Lysate Endochrome-K test kit (Charles River, Wilmington, MA) as described by the manufacturer. Endotoxin removal was performed via EndoTrap blue endotoxin removal system (Hyglos, Bernried, Germany).

### 2.2. Preparation of DNA-vaccines

Providing of vector-backbones (pDNAVaccUltra and NTC7382) including signal sequences, cloning of respective gene sequences and purification of the constructs was undertaken by the Nature Technology Corporation (Lincoln, NE). The sequence for murine CIITA (Mn01492) was acquired from GeneCopoeia (Rockville, MD) and the sequence of mIPS-1 was used as given for pUNO1-mIPS1 (GenBank: NM_144888.2). Antigens used comprised of rPA83 (2208 bp) [35], LFD1PAD4 (1300 bp) [13] and BclAD1D3 (480 bp) [20]. Vaccines were prepared and applied as described previously [35].

### 2.3. Administration of vaccines and challenge

Trials were performed using 8-12 weeks old female outbred NMRI mice (Charles River). Immunisation groups comprised of 10 animals, while groups which only received an adjuvant (lipopeptide or CIITA) contained 5 individuals.

Mice were immunised 3 times at intervals of 2 weeks. Challenge was performed 3 weeks after the last immunisation and mice were monitored for survival for up to 4 weeks. Blood was taken prior to immunisation, before challenge and after the end of the challenge. All data presented is referring to sera taken before the challenge unless stated otherwise. Preparation, vaccination and challenge of the mice was performed under anesthesia with Isofluran (Actavis, Weiterstadt, Germany) using a ventilated Box.

Mice immunised with protein received a 200 μl dose s.c. in the neck containing 25 μg of each antigen and 50 μg of the lipopeptide adjuvant (EMC microcollections, Tuebingen, Germany) diluted in sterile endotoxin free PBS (Sigma–Aldrich, St. Louis, MO). Mice immunised with DNA-vaccines were shaved 1–2 days before the immunisation. Each mouse received 2 cartridges containing a total of 3 μg of DNA (6 μg for the combination) per immunisation applied via GeneGun (Table 1). For the challenge, a dose of 200 μl of a fully virulent Ames strain containing ~1000 spores ($25LD_{50}$) for all DNA-vaccines and ~2000 spores ($50LD_{50}$) for all protein vaccines was administered s.c. in the neck. The increased challenge dose for the protein vaccines was utilised due to the residual protectiveness of the lipopeptide alone observed when challenged with lower spore doses (data not shown), thus normalising its effect to allow for a better discrimination between different antigens and applications. Mice that died during the challenge or were killed via $CO_2$ after the end of the challenge were dissected to prepare liver and spleen which were then plated on blood agar to verify the presence of the pathogen.

### 2.4. Serology

ELISA was performed as described previously [35]. Secondary antibodies comprised of horseradish peroxidase conjugated polyclonal goat anti-mouse IgG (Sigma–Aldrich), IgG1 and IgG2a (Acris, San Diego, CA). Endpoint titres were defined as the reciprocal of the highest serum dilution that resulted in an absorbance greater than two standard deviations above the average of the negative control serum sample (pooled naive sera of the according group) with a minimum $OD_{414nm}$ value of 0.1.

Sera were analysed for neutralising antibody titres via in vitro toxin neutralising assay (TNA) as published previously [35]. The neutralisation titre (NT) corresponds to the inverse serum dilution at which the toxin neutralisation yielded 50% ($NT_{50}$). The $NT_{50}$ was estimated using the SigmaPlot regression wizard (4-parameter sigmoid regression curve). Detailed Titres are only described for sera taken before the challenge.

### 2.5. Statistics

Estimated antibody titres of different groups were compared via Mann–Whitney *U*-test. Survival rates were analysed through log rank test, taking full days survived into consideration. Correlations between titres and titres and survival were estimated via spearman-rank correlation test. For all statistical purposes a *P*-value of 0.05 and smaller was considered significant, resulting in a critical value of 0.564 for $r_s$ for quantities of 10.

## 3. Results and discussion

### 3.1. Addition of rBclA to rPA83 increased the level of protection when applied together as proteins

Groups of mice vaccinated with either rPA83, rBclA or a combination of both together with lipopeptide adjuvant induced significant IgG antibody titres with a strong IgG1 emphasis against their respective antigens (Figs. 1 and 2). The measured antibody