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# Evaluation of *Bacillus anthracis* extractable antigen for testing anthrax immunity

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## ABSTRACT

Three extractable *Bacillus anthracis* cell-wall-associated antigens were evaluated for potential use as skin testing agents, and as possible candidates for in-vitro diagnosis of anthrax immunity. Anthraxin and a partially purified extractable antigen (EAP) were produced from avirulent *B. anthracis* strain 34F2 (Sterne). The thermoextractable antigen used for the Ascoli reaction was obtained commercially. Guinea-pigs were immunised and boosted several times subcutaneously with the Sterne live veterinary anthrax vaccine. Four weeks after the last booster dose, animals were skin-tested with the three antigens. Serum antibody levels were also determined by ELISA, and the in-vitro T-cell response was evaluated by [$^3$H]-thymidine incorporation. EAP was the most active antigen in both the serological and cellular reactions. EAP also elicited a distinct positive skin reaction in animals immunised with *B. anthracis*. The data obtained in this preliminary study indicated that extractable cell-wall antigens obtained from the vegetative form of *B. anthracis* may be used for skin tests and in-vitro testing of specific humoral and cell-mediated anthrax immunity.

**Keywords** Anthrax, *Bacillus anthracis*, diagnosis, infection, immunity

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## INTRODUCTION

Anthrax has recently re-emerged as a potential biological weapon. Following a hypothetical attack in which a large population is exposed to aerosolised *Bacillus anthracis*, certain individuals will have been exposed to the pathogen in various doses, while others may be unexposed or protected. Yet treatment for the entire population at risk is identical, consisting of antibiotic therapy for 60 days and repeated anthrax vaccinations [1,2].

Long-term antibiotic therapy may be associated with increased costs, risk of adverse events, and the development of antibiotic resistance in *B. anthracis* and commensals. Currently, the shortage of anthrax vaccine does not allow the duration of antibiotic treatment or prophylaxis to be reduced. As a result, it might be important to differentiate between individuals who have been exposed to *B. anthracis*, and therefore need a full vaccination schedule and prolonged antibiotic therapy or post-exposure prophylaxis, and those who have not been exposed. Since antibiotics kill *B. anthracis* rapidly, diagnostic tests that are based on a microbiological detection of the organism may not be relevant in a population that has been treated with antibiotics, even for a very short duration [3].

The anthraxin skin test, which measures cell-mediated immunity to anthrax antigens, has been used as a diagnostic tool in the former USSR and in Russia [3–6]. Skin testing has also been recommended by the World Health Organisation for evaluation of the immunological memory against anthrax in humans and animals [7]. Skin test screening can be applied successfully with antigens extracted from the anthrax bacillus cell-wall, but not by using the protective antigen, which is the major antigen of the anthrax vaccine used in the USA and western Europe [8]. Therefore, the anthraxin skin test can differentiate between exposed and unexposed individuals, even if they

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have been immunised. However, as it is a skin test, an individual may become sensitised following repeated testing, and a false-positive reaction may occur. An in-vitro test of cell-mediated and humoral immune responses might circumvent this problem.

In the present study, three different extractable *B. anthracis* antigens were evaluated for potential use as skin test agents, and for the in-vitro detection of cell-mediated and humoral immunity in guinea-pigs immunised with the Sterne live veterinary anthrax vaccine; these were a partially purified extractable antigen, anthraxin, and a thermoextractable antigen used for the Ascoli reaction.

## MATERIALS AND METHODS

The avirulent *B. anthracis* strain 34F2 (Sterne) was obtained from the Onderstepoort Bioproduct Institute (Onderstepoort, South Africa). Thermoextractable antigen (ATE; Bioveta, Ivanovice na Hané, Czech Republic) was used as the routine positive control for the Ascoli reaction. The concentration of the protein in ATE was determined as 14.28 mg/L.

### Anthraxin preparation

The Sterne strain was grown for 18–20 h at 37 °C on Brain Heart Infusion agar (Oxoid, Basingstoke, UK), supplemented with yeast extract. Before harvesting, the culture was tested for homogeneity and purity, i.e., microscopically there were *c.* 80% vegetative cell filaments and 20% bacillus forms, with few spores present. The bacterial mass was washed twice with phosphate buffered saline pH 7.3 (PBS), and centrifuged at 800 *g* for 30 min. The pellet was autoclaved for 30 min at 120 °C, dispensed into sterile dishes and dried at room temperature. The dried pellet was triturated in a sterile mortar and de-fatted with ether and chloroform in a Soxsclet apparatus. The resulting powder was hydrolysed by mixing 1 g with 100 mL of acetic acid 1% v/v. The suspension was boiled at 100°C for 3 h with periodic shaking, cooled to room temperature and placed at 4°C overnight. The supernatant was centrifuged twice at 3000 *g* and cleared by filtration through a paper filter type 595 (Schleicher and Schull, Keene, NH, USA), repeated until a clear yellowish liquid was obtained. The filtrate was adjusted to pH 7.4 with NaOH 10% w/v, and the protein concentration was determined with a BCA protein kit (Pierce Biotechnology, Rockford, IL, USA). The immunochemical specificity of the product obtained was evaluated by the Ouchterlony test, using a monospecific anthrax-precipitating immune serum (Bioveta) to yield one line of precipitation after overnight incubation. The anthraxin preparation was dispensed in vials, autoclaved for 30 min at 120°C and stored at 4–8 °C.

### Preparation of extractable antigenic protein (EAP)

Intact *B. anthracis* Sterne strain cells (10 g) were suspended in 80 mL of buffer containing 0.1 M Tris-HCl pH 8.0, 5 mM EDTA, 5 mM iodacetamide, and 5 mM amino-caproic acid, vortexed at 4°C for 2 h, and centrifuged at 3500 *g* for 15 min. This was repeated three times. The final cell pellet was suspended in 20 mL of 0.1 M Tris-HCl pH 8.0 containing N-lauryl sarcosine (ICN Pharmaceuticals, Costa Mesa, CA, USA) 3% w/v and extracted as described above. The supernatants were pooled and dialysed overnight at 4 °C against double-distilled water. At 4 °C, 12 mL of sepharose CL-4B (Amersham, Uppsala, Sweden) was added to 60 mL of the supernatant, while stirring. Ammonium sulphate was added to achieve 70% saturation and the mixture stirred on ice for 1 h. The sepharose was collected and the mixture put on a sintered glass filter and washed under vacuum with 0.5 L saturated (70% w/v) ammonium sulphate solution, prepared in 5 mM $K_2HPO_4$ pH 6.8. The combined eluate, containing EAP, was dialysed overnight at 6 °C against 30 volumes of 5 mM $K_2HPO_4$–$KH_2PO_4$ pH 8.0 containing 5 mM EDTA, 5 mM iodacetamide, and 5 mM amino-caproic acid. The partially purified EAP (15.7 mL) was loaded on to a hydroxylapatite column equilibrated with 5 mM $K_2HPO_4$–$KH_2PO_4$ pH 6.8 at 6 °C. Under these conditions, EAP bound to the column, which was then washed with the same buffer. EAP was eluted as a single peak with a non-linear 3.3 column volume gradient (0.078–1 M $K_2HPO_4$ – $KH_2PO_4$ pH 5.7). The collected fractions were analysed by SDS–PAGE. Fractions (98 kDa) containing pure EAP were pooled and dialysed against double-distilled water overnight. The protein concentration was determined with the BCA protein assay reagent, after which the preparation was lyophilised. The serological activity and purity of the EAP were evaluated by the Ouchterlony test, using precipitating sera for the Ascoli reaction (Bioveta; Omsk Biofactory, Omsk, Russia; State Veterinary Institute, Bucharest, Romania) to give a single precipitating line. These sera were prepared from different animals (donkeys, horses, mules), immunised with a variety of virulent and attenuated *B. anthracis* strains.

### Immunisation of guinea-pigs

Ten white male guinea-pigs (250–300 g) were kept in quarantine for 1 week before subcutaneous immunisation with the Sterne live anthrax veterinary vaccine (Colorado Serum Company, Denver, CO, USA) containing $5.6 \times 10^6$ spores/mL. The schedule was $2 \times 10^6$ spores on day zero, $4 \times 10^6$ spores on day 15, and $5.6 \times 10^6$ spores on day 30. Ten non-immunised male guinea-pigs were used as controls.

### Skin testing of guinea-pigs

On day 28 after the last *B. anthracis* booster dose, nine vaccinated and five control animals were tested with anthraxin, EAP and ATE. Intradermal injections (0.1 mL) were spaced 2–4 cm apart on both sides of the previously epilated skin of the trunk. Reading of the skin reactions was performed after 24 h. Erythema and skin infiltration, if present at the site of injection, were graded 1–4. Briefly, grade 0 was a negative skin reaction (no erythema, no induration), and grade 0.5 was assigned to erythema without induration. Grade 1 was skin erythema with a diameter of 8–15 mm accompanied by induration, and grade 4 was given to a reaction with an erythema of > 40 mm with accompanying induration [3]. Immediately after the skin test reading, all guinea-pigs were bled by cardiac puncture and the sera were separated by centrifugation.

#### Antibody determination by ELISA

The wells of MaxiSorp microtitre plates (Nunc, Roskilde, Denmark) were coated with 100 μL of the corresponding antigen (5 mg/L in 50 mM bicarbonate buffer pH 9.5) for 16 h at 4 °C. The plates were washed three times with PBS containing Tween-20 0.05% v/v (PBST), and were blocked with 200 μL bovine serum albumin 3% w/v in PBS (PBS-BSA) for 2 h at room temperature. After three additional washes, 100 μL of serum, diluted 1 : 100 in PBS-BSA, was added and incubated for 2 h at room temperature. After washing, 100 μL of affinity-purified alkaline phosphatase-conjugated, goat anti-guinea-pig IgG antibodies (Jackson Immuno-Research Laboratory, West-Growe, PA, USA) diluted 1:10 000 in PBS-BSA were added and plates were incubated for 1 h at room temperature. After five washes with PBS-BSA, the plates were developed with 1000 mg/L p-nitrophenylphosphate (Sigma, St Louis, MO, USA) and the absorbance was determined at 405 nm.

#### T-cell response to the antigens

Single cell suspensions were prepared from the spleens of five immunised and five control animals by meshing on a 100-μm nylon filter, followed by a series of washings with RPMI-1640 medium supplemented with syngeneic serum 1% v/v. Red blood cells were lysed with 0.15 M $NH_4Cl$, 1 mM $KHCO_3$, 0.1 mM $Na_2EDTA$ pH 7.3. Aliquots of $3 \times 10^5$ cells in RPMI containing penicillin 100 000 U/L, streptomycin 10 mg/L, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, $5 \times 10^{-5}$ M 2-mercaptoethanol, and syngeneic serum 1% v/v, were plated into each well of 96-well flat-bottomed plates (Nunc) in the presence of the following stimuli: concavalin A (Con-A 1.5 mg/L; Sigma), EAP, anthraxin and ATE (5, 2.5, and 1.25 mg/L, respectively). Each stimulation was performed in triplicate. Plates were incubated for 72 h at 37°C in $CO_2$ 5% v/v. For the last 12 h, cultures were pulsed with 1 μCi of [$^3$H]-thymidine. The cells were harvested on to glass-fibre filters (Brandel, Gaithersburg, MD, USA) and the [$^3$H]-thymidine incorporation into DNA was measured by a β-counter (Packard 1600 TR; Hewlett Packard, Palo Alto, CA, USA). Results, in counts per minute (cpm) were expressed as an index of stimulation (IS) where IS = (cpm with antigen – cpm without antigen) ÷ cpm without antigen.

## RESULTS

Anthraxin and ATE preparations (20 μg/guinea-pig) elicited similar results in immunised animals, whilst the average reaction grade was lower for EAP (Table 1). The skin test results of non-immunised animals were negative for all three antigens.

Levels of IgG to all three antigens were raised significantly in the sera of immunised animals. The highest levels were observed with EAP (Table 2). The results for five tested and five non-tested animals in the control group were combined, as there were no differences in antibody levels to the antigens ($p > 0.05$).

**Table 1.** Graded reactions after skin tests with *Bacillus anthracis* antigens in guinea-pigs immunised with the Sterne veterinary vaccine

| Anthrax antigens | Number of tested guinea-pigs | Total points positive[a] | Mean value |
|---|---|---|---|
| Anthraxin | 9 | 16 | 1.8 |
| ATE | 9 | 18 | 2.0 |
| EAP | 6[b] | 8 | 1.3 |

[a]Skin reactions were graded on a scale of 0–5.
[b]The antigen produced was sufficient to test six animals only.
ATE, themoextractable antigen; EAP, extractable antigenic protein.

**Table 2.** IgG antibody levels in sera of immunised and control guinea-pigs measured by ELISA using different *Bacillus anthracis* antigens

| Antigen | Immunised ($n = 10$) | Control ($n = 10$) | p value |
|---|---|---|---|
| EAP | 1.441 ± 0.122 | 0.370 ± 0.07 | $p < 0.001$ |
| Anthraxin | 0.569 ± 0.154 | 0.265 ± 0.03 | $p < 0.01$ |
| ATE | 1.124 ± 0.121 | 0.351 ± 0.06 | $p < 0.001$ |

ATE, themoextractable antigen; EAP, extractable antigenic protein.

T-cell immune response in immunised animals revealed a significant proliferation of splenocytes after stimulation with EAP (Table 3).

## DISCUSSION

Anthraxin, a cell-wall extract from the vegetative, non-capsulated Sterne strain of *B. anthracis*, consists mainly of a complex of peptidoglycans and polysaccharides [9]. Immunological analysis of animals immunised with the Sterne veterinary vaccine revealed that the antibodies elicited recognise a cell-associated extractable antigen, characterised as a major S-layer protein of the vegetative Sterne strain [10]. Therefore, this antigen, EAP, was included in the present study.

EAP proved to be the most active antigen. Firstly, EAP produced positive skin test reactions in guinea-pigs vaccinated with a live anthrax vaccine. Consequently, EAP can be used to evaluate cellular immunity *in vivo*, similar to the conventional anthraxin test. Secondly, EAP was used as an ELISA antigen for the determination of antibodies in the sera of animals vaccinated with live anthrax vaccine. Thirdly, use of EAP identified a lymphocyte proliferation response from immunised animal cells, allowing it to be used in an in-vitro test evaluating cell-mediated immunity to anthrax.

The two other antigens demonstrated comparable results in skin tests and satisfactory serological

**Table 3.** Splenocyte proliferation after stimulation of guinea-pigs with different *Bacillus anthracis* antigens

| Antigen concentration (mg/mL) | Index of stimulation (mean ± sd)[a] | | | | | |
|---|---|---|---|---|---|---|
| | EAP | | ATE | | Anthraxin | |
| | Immunised | Control | Immunised | Control | Immunised | Control |
| 1.25 | 4.32 ± 1.23 | 0.87 ± 0.27 | 1.77 ± 0.29 | 1.13 ± 0.18 | 1.69 ± 0.14 | 0.9 ± 0.075 |
| 2.5 | 6.21 ± 0.98 | 1.28 ± 0.36 | 1.75 ± 0.33 | 1.24 ± 0.14 | 1.82 ± 0.12 | 1.1 ± 0.09 |
| 0.5 | 6.13 ± 0.9 | 1.32 ± 0.7 | 1.84 ± 0.56 | 1.37 ± 0.11 | 1.78 ± 0.1 | 0.93 ± 0.06 |

[a]Five animals were tested in each group.
ATE, themoextractable antigen; EAP, extractable antigenic protein.

activity in ELISA, but were not active in the lymphocyte proliferation assay.

Cellular and humoral reactions following exposure to these three envelope antigens would arise only after contact with the vegetative form of *B. anthracis*, or following vaccination with the whole bacterium. These reactions would not be expected to be positive in individuals immunised against anthrax with the protective antigen vaccine unless these individuals had also been exposed to the anthrax microorganism. In addition, these reactions would be expected to be positive in non-immunised individuals who had been exposed to *B. anthracis*. Thus, these antigens could be used to differentiate between individuals who have not been immunised but have been exposed, and those individuals who have been immunised and either exposed or not exposed to the organism. Such differentiation could prevent unnecessary antibiotic therapy and reserve antibiotic prophylaxis for anthrax-exposed individuals.

In conclusion, data obtained in this preliminary study indicated that extractable antigens from the cell wall of the vegetative *B. anthracis* organism can be used to evaluate cell-mediated immunity with in-vivo skin tests, and to test for specific humoral and T-cell-mediated immunity *in vitro*.

## REFERENCES


1. Lew DP. *Bacillus anthracis* (Anthrax). In: Mandell, GI, Bennet, JE, Dolin, R, eds. *Principles and practice of infectious diseases*. Philadelphia; Churchill Livingstone, 2000, **2**: 2215–2220.
2. Friedlander AM, Welkos SL, Pitt MLM *et al*. Postexposure prophylaxis against experimental inhalation anthrax. *J Infect Dis* 1993; **167**: 1239–1242.
3. Shlyakhov E, Rubinstein E. Evaluation of the anthraxin skin test for diagnosis acute and past human anthrax. *Eur J Clin Microbiol Infect Dis* 1996; **5**: 242–245.
4. Shlyakhov E, Rubinstein E. Human live anthrax vaccine in the former USSR. *Vaccine* 1994; **12**: 727–730.
5. Pfisterer RM. Retrospective verification of the diagnosis of anthrax by means of the intracutaneous skin tests with the Russian allergen – 'Anthraxin' in a recent epidemic in Switzerland. Proceedings of the International Workshop on Anthrax. *Salisbury Med Bull* 1990; **68** (suppl): 80.
6. Stepanov AV, Marinin LI, Pomerantsev AP, Staritsin NA. Development of novel vaccines against anthrax in man. *J Biotechnol* 1996; **44**: 155–160.
7. World Health Organisation. *Guidelines for the surveillance and control of anthrax in humans and animals*, 3rd edn. Geneva: 1998, WHO.
8. Shlyakhov E. Anthraxin: confrontation with live and acellular anthrax vaccine. In. *Proceedings of the third international conference on anthrax*. Plymouth, UK 1998, 41.
9. Shlyakhov EN. *Allergy in anthrax*. Kartia Moldoveniasca. Kishinev, 1968, 188.
10. Ezzell JW, Abshire TG. Immunological analysis of cell-asociated antigens of *Bacillus anthracis*. *Infect Immun* 1988; **56**: 349–356.