Identification of peptide sequences as a measure of Anthrax vaccine stability during storage

Gail Whiting, Jun X Wheeler, and Sjoerd Rijpkema

Abbreviations: ACN, acetonitrile; AVA, anthrax vaccine adsorbed; AVP, anthrax vaccine, alum precipitated sterile filtrate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; 2D-DIGE, in-gel difference 2-dimensional gel electrophoresis; DTT, dithiothreitol; EF, edema factor; ET, edema toxin; kD, kilo Dalton; kVh, kilo Volt hours; LC, liquid chromatography; LF, lethal factor; LT, lethal toxin; Mab, monoclonal antibody; MS, mass spectrometry; MW, molecular weight; PA, protective antigen; RD, repeat domain; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; SFI, septum formation inhibitor; SHD, standard human dose; SOD, superoxide dismutase; SPE, sequential solid phase extraction; TFA, trifluoroacetic acid; THP succinyltransferase, 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase.

The UK anthrax vaccine is an alum precipitate of a sterile filtrate of Bacillus anthracis Sterne culture (AVP). An increase in shelf life of AVP from 3 to 5 years prompted us to investigate the in vivo potency and the antigen content of 12 batches with a shelf life of 6.4 to 9.9 years and one bulk with a shelf life of 23.8 years. All batches, except for a 9.4-year-old batch, passed the potency test. Mass spectrometry (MS) and in-gel difference 2-dimensional gel electrophoresis (DIGE) were used to examine antigens of the pellet and supernatant of AVP. The pellet contained proteins with a MW in excess of 15 kDa. DIGE of desorbed proteins from the pellet revealed that with aging, 19 spots showed a significant change in size or intensity, a sign of protein degradation. MS identified 21 proteins including protective antigen (PA), enolase, lethal factor (LF), nucleoside diphosphate kinase, edema factor, and S-layer proteins. Fifteen proteins were detected for the first time including metabolic enzymes, iron binding proteins, and manganese dependent superoxide dismutase (MnSOD). The supernatant contained 131 peptide sequences. Peptides representing septum formation inhibitor protein and repeat domain protein were most abundant. Five proteins were shared with the pellet: 2,3,4,5-tetrahydropyridine-6-dicarboxylate N-succinyltransferase, 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase.

Introduction

The UK anthrax vaccine (AVP) is manufactured by Public Health England and has been in production since the 1950s. AVP was first described in 1965, and is an aluminum potassium sulfate (alum) -precipitated sterile culture filtrate of Bacillus anthracis Sterne strain. The main mediator of protection is the binding subunit of the anthrax toxin: protective antigen (PA). Besides PA, AVP contains many other antigens including enolase, lethal factor (LF), and edema factor (EF). PA is also the active agent in the Anthrax Vaccine Adsorbed (AVA) produced by Emergent Biosolutions™, which is derived from the culture supernatant of non-virulent B. anthracis V770-NP1-R and uses aluminum hydroxide as adjuvant. By comparison this vaccine contains lower levels of LF and negligible levels of EF. Both EF and LF are enzymatic subunits that bind to multimeric PA to form the active toxins edema toxin (ET) and lethal toxin (LT) respectively. These toxins attack host cells and tissues in a variety of ways. Thus toxin neutralizing antibodies either directed to PA, LF, or EF can change the balance between pathogen and host to mitigate the outcome of anthrax and limit infection.

Due to their complex nature, the safety record of AVP and AVA has attracted the attention of the health care community. Following anthrax vaccination, the incidence of systemic side effects such as arthralgia, myalgia, and flu like syndrome was found to be significantly higher compared with immunization with vaccines based on purified antigens such as Tetanus Toxoid or Hepatitis A. However, studies among vaccinated British and American service personnel showed that side effects were mostly mild, localized at the site of injection and did not lead to an increase in medical consultations. Controversially, both anthrax vaccines were implicated in “Gulf War Syndrome” which
was described in veterans of the first Gulf War. However a causal link with the anthrax vaccine remained unproven, either in experimental studies using animal models or in epidemiological studies among Gulf War veterans. Thus AVP and AVA are considered safe and efficacious for human use.

In a previous study, we analyzed the antigen content of AVP by 2-dimensional gel electrophoresis. We showed that the number of spots tended to decrease with age of the batch, indicating degradation of vaccine antigens. Mass spectrometry (MS) identified the presence of anthrax toxin subunits and several antigens, not associated with the toxin, such as enolase, nucleoside diphosphate kinase, heat shock proteins, and S-layer proteins. Whether antibodies to these components contribute to the protection and/or adverse effects in vaccinated individuals remains to be proven.

Recently, the shelf life of AVP was increased from 3 to 5 y. To increase our insight into the stability of the vaccine during storage, we examined the potency and antigen content of 15 final lots (batches) and one bulk by MS.

All batches met specifications at the time of release and the shelf life of the batches examined in this study ranged from 1 to 10 y. The vaccine bulk was not released for human use and was 23.8 y old when analyzed.

The potency of batches that had exceeded their shelf life was retested in the guinea pig challenge model, to ascertain the potency of the batch at the time of analysis.

For analysis of the antigen content, batches of AVP were separated in a supernatant and a pellet fraction. The assumption was that the pellet contained proteins precipitated by the adjuvant and the supernatant contained dissociated or non-adsorbed protein fragments and oligopeptides. Supernatants were analyzed by reverse phase liquid chromatography (LC) followed by tandem peptide sequencing MS (LC-MS/MS). The proteins derived from desorbed pellets of individual batches were separated by in-gel difference 2-dimensional gel electrophoresis (2D-DIGE) and spots which showed a change in size or intensity, as the vaccine aged, were excised and submitted to MS/MS for analysis.

Here we confirm and extend our previous analysis of protein components of AVP. Due to improved sensitivity of LC-MS/MS and the combination of MS/MS and 2D-DIGE, novel proteins and peptides were identified in the supernatant and the pellet respectively. We show that antigens precipitated by the adjuvant notably enolase, EF, LF, and PA, are subject to degradation during prolonged storage and that peptides are abundant in the supernatant. The number of peptide sequences in the supernatant is related to the age of the batch and PA and LF peptides were only detected in the supernatant of batches, which had exceeded their shelf life. These findings are discussed in relation to vaccine potency to determine their relevance as markers for efficacy and stability of AVP.

Table 1. Characteristics of AVP batches and fractions thereof used in this study

| Batch ID | Age in years | Outcome of potency test | No. of SHD used | Supernatants analyzed by LC/MS-MS | Pellets analyzed by 2D-DIGE and LC/MS-MS |
|----------|--------------|-------------------------|-----------------|----------------------------------|------------------------------------------|
|          |              |                         |                 | C4 eluate | C8 eluate | C18 eluate | Combined eluates | Protein content in µg | No. of SHD used | Protein content in µg | Analyzed in 2D-DIGE | 2D-DIGE spots analyzed by LC/MS-MS |
| 1        | 23.6         | –                       | 12              | 63.9     | 17.8     | 11.0      | 92.7            | 7.7                | –                | –                 | –                | –                      |
| 2        | 9.9          | 0.9 (0.4–1.1)P          | 18              | 100.5    | 43.8     | 25.7      | 169.9          | 9.4                | 3                | 50.4             | 16.8             | Yes               |
| 3        | 9.4          | 0.5 (0.3–0.8)F          | 18              | 71.2     | 253.0    | 26.5      | 350.7          | 19.5               | 3                | 48.9             | 16.3             | Yes               |
| 4        | 8.8          | 1.1 (0.6–2.0)P          | 9               | 75.8     | 20.6     | 9.2       | 105.6          | 11.7               | –                | –                | –                | –                      |
| 5        | 8.7          | –                       | 19              | 67.0     | 79.5     | 26.5      | 173.0          | 9.1                | 3                | 66.0             | 22.0             | Yes               |
| 6        | 8.7          | 1.1 (0.7–1.7)P          | 8               | 55.3     | 19.5     | 8.9       | 83.7           | 10.5               | –                | –                | –                | –                      |
| 7        | 8.0          | 0.9 (0.4–1.7)P          | 9               | 73.9     | 19.7     | 8.0       | 101.6          | 11.3               | 3                | 45.0             | 15.0             | Yes               |
| 8        | 7.8          | 1.0 (0.4–2.3)P          | 8               | 202.8    | 18.5     | 9.5       | 230.8          | 28.9               | –                | –                | –                | –                      |
| 9        | 7.8          | 1.0 (0.5–2.0)P          | 9               | 56.5     | 20.9     | 11.2      | 88.7           | 9.9                | –                | –                | –                | –                      |
| 10       | 7.8          | –                       | 19              | 84.1     | 61.6     | 27.3      | 173.1          | 9.1                | 3                | 49.2             | 16.4             | Yes               |
| 11       | 7.3          | 1.0 (0.6–1.8)P          | 9               | 108.2    | 74.4     | 28.3      | 210.9          | 11.1               | 3                | 42.6             | 14.2             | Yes               |
| 12       | 7.2          | 0.8 (0.5–1.5)P          | 9               | 57.4     | 19.9     | 12.4      | 89.8           | 10.0               | 3                | 423              | 14.1             | Yes               |
| 13       | 6.4          | 0.8 (0.4–1.5)P          | 9               | 27.0     | 56.9     | 15.2      | 99.1           | 11.0               | 3                | 53.4             | 17.8             | –                 |
| 14       | 1.1          | 1.6 (0.9–2.9)P          | –               | –        | –        | –         | –              | –                  | 5                | 75.0             | 15.0             | Yes               |
| 15       | 1.0          | 1.1 (0.6–2.2)P          | 13              | 354.7    | 27.8     | 20.5      | 403.0          | 31.0               | 5                | 79.5             | 15.9             | Yes               |
| 16       | 1.0          | 1.5 (0.8–3.2)P          | 5               | 105.9    | 7.4      | 4.4       | 117.7          | 23.5               | 5                | 82.5             | 16.5             | Yes               |

1Age calculated at the time of testing. 2Potency of the batch relative to reference freeze-dried AVP standard NIBSC 99/790, (95% confidence interval) P = pass, F = Fail. 3Bulk 1 was used as a reference standard for the rabbit edema test and was not released as a final lot. 4Poor 2D-DIGE result excluded due to lack of material. 5Tested at release to market. 62D-DIGE spot pattern analyzed only. –, not done.
Results

Potency of vaccine batches

All AVP batches, except for batch 1, were intended for human use and met the release specifications for AVP as described in the European Pharmacopoeia monograph 2188. The potency of 10 batches which had exceeded their shelf life was retested. The results are given in Table 1. Only batch 3 was considered to have failed the potency test.

Analysis of vaccine supernatant by LC-MS/MS

Supernatant of multiple SHDs of 14 batches and one bulk were used to prepare sequential solid phase extraction (SPE) eluates for analysis by LC-MS/MS. The protein content of SPE eluates from batches which had exceeded their shelf life and bulk 1 was variable (Table 1). No correlation between protein content and age of the material was observed for this group. However, the average protein content of SPE eluates from batches which had exceeded their shelf life (n = 13, 12.2 µg ± 5.7) was significantly lower (P = 0.017) compared with the average protein content (n = 2, 27.3 µg ± 5.3) of SPE eluates from batches that were within shelf life. Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that proteins or protein fragments were absent from the supernatant with the exception of the supernatants of bulk 1, which contained a 20 kD fragment (results not shown).

Following trypsin digestion, LC-MS/MS identified 212 peptides and 207 of these matched the B. anthracis database and are thus members of the B. anthracis proteome. Of these, 131 peptide sequences were unique, 91 peptides were a product of trypsin digestion and 40 peptides were the result of other protease activity or spontaneous degradation (marked *, see Table 2; Supplementary Data 1). The 212 peptides identified represented 98 proteins, including 14 hypothetical proteins (Supplementary Data 1). Table 2 summarizes the sequences of the peptides that were present in the supernatant of 3 batches or more and that corresponds to 12 proteins, including 2 hypothetical proteins.

The number of peptides identified in the supernatant increased with vaccine age and this relationship was especially pronounced when the bulk (batch 1) was included in the analysis (Fig. 1A and B). The supernatant of batch 1 contained 44 peptides derived from 35 proteins, the largest number of proteins identified for any of the supernatants. The same trend was observed for PA peptides (Fig. 1A and B). The most frequently identified amino acid sequences were “MEEKQQNVTIK” representing the septum formation inhibitor (SFI) protein in 12 batches and ‘RLFLSTEGDADLIGDQALFG’ representing the repeat domain (RD) protein found in 11 batches (Table 2; Supplementary Data 1). The age of the batch did not correlate with the presence of peptides derived from the RD protein or the SFI protein.

Nineteen different PA peptides were found in the supernatant of 11 batches with an age of 7 y or more. PA peptide sequences GPTVPDRDNDGIPDS* and DNLQLPELK occurred most frequently as part of 15 longer amino acid sequences found in 9 batches and 10 sequences found in 7 batches respectively. The majority of the peptides (74%) in the vaccine supernatant were derived from 4 amino acid sequences of Domain 1. Most of these contained the core sequences mentioned above (Table 3). Three peptide sequences (16%) were positioned in Domains 2–4 (Table 3; Supplementary Data 1). Peptides representing the enzyme 2,3,4,5-tetrahydropyridine-2, 6-dicarboxylate N-succinyltransferase (THP succinyltransferase) were present in the supernatant of 7 batches with a shelf life in excess of 7 y.

The presence of LF peptides was limited to 2 batches with an age of more than 9 y and bulk 1 (see Table 2).

EF peptides were not detected in the supernatant for any of the batches tested. Peptide sequences representing 12 proteins were found in 2 batches, 74 peptide sequences occurred in one batch only. Five peptide sequences could not be identified by the search of the B. anthracis proteome (Supplementary Data 1). The latter group may represent carry over proteins from media components or contaminants.

Analysis of vaccine pellets by 2D-DIGE

The protein content from pellets of 11 batches was determined following dissolution of the pellet (Table 1). The average protein content of the pellet from one SHD did not differ significantly for batches 2, 3, 5, 7, and 10–13, which were over 6 y of age (n = 8, 16.6 ± 2.6 µg) compared with batches 14–16 that were within shelf life (n = 3, 15.8 ± 0.8 µg). The desalting required for 2D-DIGE analysis may have reduced the protein yield and therefore our results may underestimate the total amount of protein absorbed to the alum adjuvant, these results can be used to compare the amount of protein in the pellet of each batch.

2D-DIGE analysis of the protein content of batches 14–16 revealed an average of 1103 ± 99 spots per gel and 595 of the larger well delineated spots were matched across gel images generated from all gels. The vast majority of these spots (91.9%) did not change significantly in abundance and only 8.1% of the spots showed a significant change (P ≤ 0.05) in protein abundance between the batches.

2D-DIGE analysis of 7 batches with a shelf life of 7 y or older showed an average of 919 ± 81 protein spots per gel. Batch 13 was excluded from this analysis due to low protein content of the pellet (Table 1). A total of 504 well-delineated spots were matched across 33 gel images generated from 11 gels. Of these, 248 spots (49%) showed a significant change (P ≤ 0.05) in protein abundance. Many of these changes were related to the age of the batch (Table 4). The most pronounced changes in spot size and intensity were found when patterns from batch 2 (9.9 y) were compared with batch 12 (7.2 y).

Identification of protein species in excised spots

Twenty-four spots which showed a significant change in protein abundance and one spot which showed a decrease in size were excised (Fig. 2 and Table 4). Indicating that proteins present in these spots are susceptible to degradation during prolonged storage of the vaccine over time. Spots 14, 17, 19, 20, 21, and 22 only showed a change for batch 5 (Table 4). Proteins were extracted for MS/MS analysis and peptide sequences were identified in 19 of the 25 spots, representing 21 proteins (Supplementary Data 2). The remainder of the spots did not contain sufficient protein. PA peptides that were detected in the pellet are given in Table 3. The identity of all extracted proteins is given in Table 5. Of the 379 peptide sequences, 159 peptides were unique, and all were
the result of trypsin digestion (Supplementary Data 2). The analysis confirms that PA is the major protein in the pellet fraction. PA was detected in 15 spots (79%) and represented by 146 peptide sequences (39% of total) of which 36 peptide sequences are unique (Table 3; Supplementary Data 2). The location of the PA peptides detected in the pellet fraction differed when compared with PA peptides of the supernatant. In the pellet fraction, 12 peptides belonged to Domain 1 (33%), followed by 11 peptides (31%) from Domain 4, eight peptides (22%) from Domain 2 and five peptides from 3 (14%; see Table 3; Supplementary Data 1 and 2). Thus compared with the supernatant, domain 1 peptides of PA were under represented in the pellet fraction.

Other abundant proteins were LF, present in 9 spots (47%; 54 peptide sequences of which 21 are unique), enolase present in 4 spots (69 peptide sequences of which 21 are unique) and S-layer protein present in spots 16, 18, 20, and 25 (10 peptide sequences of which 6 are unique; see Table 5; Supplementary Data 2). The presence of these proteins and nucleoside diphosphate kinase and the edema factor is in line with findings of our previous study.5 Full size LF and PA tended to co-migrate (see Table 5, spots 1, 3, 6, 7, 16, and 25). PA was also present as lower MW fragments (see Table 5, spots 11, 12, 19, and 20).

This study also identified 14 proteins for the first time in AVP—6-phosphofructokinase, DNA-binding response regulator, formate acetyltransferase, glucose-6-phosphate isomerase, 2 iron binding proteins, lipoprotein Bmp family, manganese dependent superoxide dismutase (MnSOD), phosphoesterase, purine nucleoside phosphorylase DooD-type, serine hydroxymethyltransferase, sporulation-control protein Spo0M, THP succinyltransferase, and transaldolase (Supplementary Data 2). Peptide sequences of 5 precipitated proteins were identified in the supernatant of AVP—enolase, LF, MnSOD, PA, and THP succinyltransferase. Peptide sequences of SFI and RD proteins which were abundant in the supernatants were not detected in the pellet fraction. We attribute the detection of a wider range of antigens to improved extraction procedures and an increased sensitivity of the DIGE technique and the MS/MS.

Discussion

AVP is a complex mixture of PA, LF, EF, and numerous other antigens. Proteins are precipitated by the adjuvant and reside in the pellet, whereas peptides are found in the supernatant. We analyzed the antigen content of the pellet and the potency of AVP batches which had exceeded their shelf life. All but one of the AVP batches passed the in vivo potency test, including batches which exceeded their shelf life by a considerable margin (Table 1). Batches that were within shelf life were not examined. Despite this omission, the evidence that AVP is efficacious up to and beyond its shelf life is compelling.

Analysis of the pellet fraction indicated that protein decay is common among batches which had expired. However, the lack information on the decay of specific antigens during shelf life, limits our understanding of the significance of this process for vaccine stability and the effect on the antigens identified in Table 5. We did examine the peptide composition of the vaccine supernatant in 3 1-y-old batches and this provided a useful reference for the results of batches, which exceeded their shelf life (Table 2).

The presence of PA, EF, enolase, LF, and S-layer proteins in the vaccine pellet confirmed findings of our previous study.5 Two members of the S-layer protein family were identified, sap located on the chromosome and one S-layer protein located on pXO1.23,24 Both enolase and S-layer proteins have been detected in spent medium of B. anthracis cultures, which explains their abundance in the pellet of AV, and both proteins are also secreted by B. anthracis under culture conditions that simulate the host environment.25,26 Thus besides the toxin subunits, enolase and S-layer proteins may elicit antibodies following vaccination. Enolase binds human plasminogen and is considered a virulence factor that could be involved in tissue lysis.27 S-layer proteins are immunogenic and S-layer vaccines protected mice against anthrax challenge.28-31

Also present in AVP are—enzymes required for vegetative growth such as transaldolase and THP succinyltransferase, membrane proteins such as iron transport proteins, lipoprotein Bmp, and MnSOD (Table 5). Like enolase and S-layer proteins, these antigens reflect the use of culture supernatant as the source of AVP. THP succinyltransferase is required for the biosynthesis of Diaminopimelic acid, a substrate for Sortase C and essential for anchoring of surface proteins to the cell envelope of B. anthracis.25,33 The iron transport-associated protein (Table 5, spot 24) is part of a family of iron-regulated surface determinant proteins containing NEAr Transporter domains.34 These proteins are secreted during culture and were shown to be immunogenic in convalescing guinea pigs and rabbits and in humans following immunisation with AVA.34,35 Antibodies to transaldolase and enolase were found in recipients of AVA.35 Thus several of the proteins identified in the pellet of AVP are immunogenic and shared with AVA. MnSOD, is present in the exosporium of B. anthracis and is considered a virulence factor which protects the bacterium against reactive oxygen species in the host and during culture.56

The proteins mentioned above were all detected in spots that with one exception showed a reduction in size or intensity for older AVP batches, indicating loss of these proteins from the pellet with increased age of AVP. Indeed, a considerable proportion of spots decreased in size among batches that were over 6 y of age compared with spot patterns of 1-y-old batches. In addition the total number of spots per individual batch was lower in batches that had exceeded their shelf life. Previously we noted a reduction in the number of spots for older batches of AVP and an increase in the inter-batch variation with increasing age of the samples.5 Thus both studies point to a decay of protein antigens during prolonged storage of AVP.

Peptides derived from a total of 98 proteins were detected in the supernatant and only 5 proteins were detected in the pellet: PA, enolase, LF, MnSOD, and THP succinyltransferase (Tables 2 and 5). The supernatants of 1-y-old batches contained 4 or 6 peptide sequences and as the age of the batch increased, the number
Table 2. Most frequently identified peptide sequences in vaccine supernatant and the B. anthracis protein they represent

| B. anthracis protein/ NCBI accession number | Present in supernatant of batch | Peptide sequence (position) |
|------------------------------------------|---------------------------------|----------------------------|
| Septum formation inhibitor /NP_846895    | 3, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 16 | MEEKKQVNYVTIK (1–12) |
| Repeat domain protein/ EJY191466         | 1, 3, 5, 6, 8, 9, 11, 12, 13, 15, 16 | RLFLSSTEGDAIDLGGQALFG* (230–250) |
| Protective antigen/AAA22637              | 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12 | GPTVPDRDNDGIPDS* (172–186) |
| 2,3,4,5-Tetrahydropyridine-2, 6-dicarboxylate N-succinyltransferase /NP_846430 | 2, 3, 4, 5, 6, 9, 12 | AGVIEPPPSAK* (155–165) |
| Amino acid permease/YP_028011            | 6, 7, 8, 9, 10, 11, 16 | STELGEQKLKNTK (16–28) |
| Hypothetical protein /NP_843324          | 6, 8, 9, 10, 11, 13, 15 | ELLSENIR (127–134) |
| HAD family hydrolase, subfamily IIIB /NP_844477 | 1, 4, 5, 10, 12 | SQFNAGINTI* (51–61) |
| Hypothetical protein/ZP_00391958         | 3, 4, 6, 10, 13 | IEEPENDEKNKE (293–305) |
| Enolase /NP_847538                      | 1, 3, 6, 9 | IELGTBPNGKLGL* (95–107) |
| Alcohol dehydrogenase/NP_844655         | 3, 6, 9 | IKVDIKPPOG* (156–166) |
| Inosine-uridine preferring nucleoside hydrolase family protein/NP_845876 | 10, 12, 13 | YGNVTQEKTSAAYLQLLAG* (36–56) |
| Lethal factor/CAC39332                   | 1, 2, 3 | IQVDSNPLSEK (365–376) |

*Indicates peptide not cleaved by trypsin.

of peptide sequences steadily increased to 44 for the 23.8 y old bulk. Peptides from the pellet fraction all resulted from trypsin digestion, which is part of the sample preparation. Whereas a large proportion of the peptides in the supernatant resulted from other protease activity, for example during fermentation or during temperature controlled storage of the vaccine. Although data are lacking for 2 to 5 y old batches, there is a correlation between age and the number of peptide sequences and this correlation was also found for PA peptides. However, it remains unclear whether protein decay during storage is a gradual process or whether it accelerates as batches age beyond their shelf life.

Despite the presence of peptides from SFI and RD proteins in the supernatant of 75% of the batches, SFI and RD proteins were not detected in the pellet. The function of these proteins is unknown. The RD protein was recently identified in the genome of B. anthracis isolated from cattle, but a function was not allocated. One protein, likely to be related to RD, was shown to be associated with the spore capsule in Bacillus strains. The septum plays a role in the onset of sporulation. This implies that SFI protein may therefore have a role during the vegetative growth of the Sterne strain although currently its function is unknown.

Most of the PA peptides in the supernatant belonged to domain 1 and are represented by sequences “GPTVPDRDNDGIPDS” and “DNPLQPLELK” (Table 3). In the pellet fraction, PA peptides from domains 2–4 dominated. This observation is of particular interest for vaccine efficacy and stability. Domain 1 is the N-terminal domain of PA, which contains the furin cleavage site RKKR. Furin is a host protease that removes a 20 kD N-terminal fragment from domain 1. Upon activation by furin cleavage, PA assembles into a heptamer ring-shaped structure with a large hydrophobic surface, which is then able to bind EF and LF. Domain 2 and domain 3 are required for the polypeptide translocation and oligomerization respectively. Domain 4 is involved in the binding of the PA complex to the host cell. Peptide GPTVPDRDNDGIPDS was not the result of trypsin digestion suggesting that specific Bacillus proteases may have cleaved this peptide during fermentation or that this peptide fragment may have dissociated from the adjuvant spontaneously during storage. Accelerated degradation of an experimental rPA vaccine, which contained Alum hydroxide as adjuvant, showed that domain 1 epitopes became more accessible for binding to monoclonal antibodies (Mabs) compared with epitopes in domains 2 to 4. These changes were explained as a result of the unfolding of parts of PA, which exposes normally hidden epitopes which are situated at domain interfaces and the disruption of conformational epitopes at the surface of PA. Thus domain 1 of PA is likely to be more unstable than other parts of the molecule and therefore may be susceptible to desorption. This hypothesis supports our finding that domain 1 is prone to dissociation and degradation during storage. A study in mice showed that adsorption of rPA to alum adjuvant did not induce higher titers of toxin neutralizing antibodies. However, unfolding of the PA molecule did affect the induction of toxin neutralizing antibodies in vaccinated mice. Toxin neutralizing antibodies interfere with the formation of PA heptamers, the formation of the toxin complex and/or prevent binding of anthrax toxin to the host receptor. In humans, anthrax vaccination induces an anti-PA IgG response which is mainly directed toward domain 1, but only a minority of these antibodies are able to neutralize LT, which contrasts with the higher proportion of toxin neutralizing antibodies generated that bind to PA. Human or humanised Mabs that bind to domain 1, specifically the furin cleavage site, and 2 mouse Mabs which bind close to the furin cleavage site are protective in vitro and in a mouse model.

Epitope VKNKRTFLPSWISNIHEKE of domain 1 is recognized by toxin neutralizing murine Mab 19D9 and present in adsorbed PA (Table 3; Supplementary Data). Steric hindrance reduces enzyme activity and slows or prevents cleavage...
of PA\textsubscript{43}, which in turn would limit formation of active LT in vivo. However, the efficacy of anti-domain 1 antibodies could be affected by the propensity of domain 1 to be freely available in the serum of infected individuals. Free subunits of domain 1 are then able to bind to anti-domain 1 antibodies, rendering these antibodies incapable of neutralizing the holo-toxin.\textsuperscript{46,48} In view of these observations, epitopes of domain 1 may be considered to be of less importance for the induction of toxin neutralizing antibodies than epitopes of domains 3 and 4. Indeed most therapeutic Mabs, including the recently licensed Raxibacumab\textsuperscript{TM}, are directed against domains 3 or 4.\textsuperscript{44,51}

Despite the abundance of LF in the pellet, LF peptides were only detected in the supernatant of bulk 1 and 9 to 10 y old production batches. The presence of both PA and LF peptides and the failure of batch 3 to pass the potency test may be associated. However, we cannot rule out that this effect is due to a batch specific variation and therefore further work is required to support this observation.

The absence of EF peptides in the supernatant and the limited presence of EF among precipitated proteins is in line with previous observations that fermentation conditions for production of AVP result in high levels of PA followed by LF and that levels of EF are very low.\textsuperscript{2,4,5}

In conclusion, MS analysis of the supernatant of adsorbed vaccines like AVP, can provide valuable information about vaccine stability and antigen content. With continuing advances in this area, such methods should become more accessible to national control laboratories. However, as the current study shows the selection of relevant markers for efficacy and stability is not straightforward for a complex vaccine such as AVP, in vivo assays remain a crucial component of the batch release process to ascertain the potency and safety of AVP.

**Materials and Methods**

**Anthrax vaccine batches**

Fifteen final lots of AVP and one bulk used in this study are given in Table 1. All were donated by Public Health England Porton (previously Health Protection Agency). In this study, the oldest sample, the bulk, was assigned number 1 and the final lots (batches) were assigned numbers 2 to 16. The bulk was not released for use in humans. All batches met specifications and were released. Batches 2–13 and bulk 1 had a shelf life that exceeded 5 y at the time of testing. Batches 14–16 were all in date at the time of testing (Table 1).

**Guinea pig potency test**

The potency of the AVP batches was measured as the relative potency compared with freeze-dried AVP reference standard NIBSC 99/790. The potency test used in this study was a modification of the challenge assay in guinea pigs described in Monograph 2188 of the European Pharmacopeia.\textsuperscript{22} The modifications related to the dilutions of the vaccine used to immunise guinea pigs and the pass criteria. Groups of animals received 2 immunizations of AVP diluted 1/10, 1/30, 1/90, or 1/270 SHD. To reflect the achievable precision of the assay, the pass criteria applied to batches, which had exceeded their shelf life were: the relative potency exceeds 1.0 or the 95% confidence interval includes 1.0 and the lower 95% confidence limit is not less than 33% of the relative potency.
Protein detection
The amount of protein in the vaccine supernatant or pellet was determined by BCA (Thermo Scientific Pierce) or Bradford assay (Bio-Rad).

Direct peptide sequencing of vaccine supernatant by LC-MS/MS
Excipients were removed from vaccine supernatants by SPE prior to LC-MS/MS analysis. SPE used 3 types of reverse phase matrices represented by cartridges C4, C8, and C18 (CHROMABOND® HR-Xpert polymers, Macherey Nagel). Cartridge C4 retains the hydrophobic peptides, C18 retains smaller less hydrophobic peptides and C8 is an intermediary in both respects. Cartridges were pre-treated with 3 mL of 50% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA) and equilibrated with 3 mL of 0.1% TFA. The supernatants of several SHDs were pooled (Table 1) and passed through a cartridge 3 times followed by a wash step (3 mL of 0.1% TFA). Following passage through a C4 cartridge, the non-binding material was passed through a C8 cartridge and subsequently through a C18 cartridge. Bound peptides were eluted with 1.5 mL of 50% ACN and 0.1% TFA. Eluates from C4, C8, and C18 cartridges were dried and reconstituted in 100 µL of 50 mM ammonium bicarbonate pH 8.5.

The amount of protein in a 50 µL aliquot of the eluate from each sample was measured by BCA assay. Trypsin digestion was performed on the remaining sample unless the sample contained more than 20 µg of protein in which case a volume equivalent to 20 µg of protein was used. The sample was incubated in the presence of 1% Rapigest (Waters; sodium 3-[2-methyl-2-undecyl-1,3-dioxolan-4-y1]methoxy]-1-propanesulfonate) at 100 °C for 5 min to solubilise proteins and peptides. Trypsin was added once the sample was at RT (ratio of 20:1 w/w enzyme/protein).

Table 3. Protective antigen peptides detected in the vaccine supernatant and pellet

| PA Domain | Oligo peptide | Position | Identified in |
|-----------|---------------|----------|--------------|
| 1–2       | HPLVAAYPIVHVDMENISSLKNEDQSTQNTDSTQR | 253–287 | Pellet       |
| 2         | TWAETmGLNTADTAR | 345–359 | Pellet       |
| 2         | YVNVTAPIYVNLPTTSVLGK | 366–388 | Pellet       |
| 2         | AKENQSLQLAPNNYPSK | 396–414 | Pellet       |
| 2         | NLAPIALNAQDDFSSTPmYNQFLEK | 415–444 | Pellet       |
| 2         | LDTQVVYGIATYNFENG | 450–468 | Pellet       |
| 2–3       | VRVDTGSNWSV ELPVLQIETTAR | 469–490 | Supernatant/Pellet |
| 3         | DNLV | 497–503 | Pellet       |
| 3         | RIAAVNPSDPLETTKPDmTLK | 504–525 | Pellet       |
| 3         | IAFGFNPNGNLQYGQK | 529–545 | Pellet       |
| 3         | NQLAEELNATYVLDK | 564–580 | Pellet       |
| 3         | LNAKLNSILIR | 583–592 | Pellet       |
| 4         | FHYDRNNAVGADESVVK | 596–613 | Supernatant/Pellet |
| 4         | EVINSSTEGLLNNIDK | 617–633 | Pellet       |
| 4         | IVIEIDTEGLKE* | 642–654 | Supernatant   |
| 4         | YDmLNISSLR | 660–669 | Pellet       |
| 4         | QDGKTIFDFFK | 670–680 | Pellet       |
| 4         | YNQKLPYISNPNVK | 681–695 | Pellet       |
| 4         | VNVYAWTK | 696–703 | Pellet       |
| 4         | ENTIINPSEGDTSTNGIK | 704–722 | Pellet       |

1Protective antigen protein sequence AA22637. 2The longest oligo peptide sequence is given. Sequences in bold were detected individually (Supplementary Data 2). m, oxidized form of methionine, possibly a site of post translational modification. *Indicates peptide not cleaved by trypsin.
and incubated at 37 °C overnight. Hydrochloric acid was added to terminate digestion and ensure breakdown of Rapigest.

The digests were analyzed using LC-MS/MS equipped with a nano-electrospray ion source and 2 mass detectors, linear trap and orbitrap, coupled with an Ultimate 3000 nano-LC system, comprising a solvent degasser, a loading pump, a nano-pump, and a thermostatted autosampler (Thermo Fisher). After automated injection, the extracted peptides were trapped in a cartridge (PepMap reversed phase C18; 5 µm [100 Å], 300 µm internal diameter x 5 mm length) and eluted on to a C18 reversed phase nano-column (3 µm [100 Å], 75 µm internal diameter x 15 cm length), and followed by a 60 min separation under a column flow rate of 0.3 µL/min using linear gradient from 5–70% acetonitrile and 0.1% formic acid. After a first survey MS scan (from m/z 400–2000) in the linear trap, the 5 most intense ions were fragmented in the linear ion trap at collision induced energy of 35%. The total cycle time was approximately 30 ms. Data was collected in data dependent MS/MS mode with dynamic exclusion set to 2 counts.

Mass spectra were processed and peptides were identified by a search of the Proteome Discoverer v 1.2 with built-in Sequest (Thermo Electron) against B. anthracis FASTA database using either trypsin as the enzyme or no enzyme for specific or non-specific searches to identify both trypsinized peptides as well as peptides generated by Bacillus proteases or by natural decay during storage at 2–8 °C. For the search, initial mass tolerances for protein identification by MS were set to 10 ppm and the peptide mass must be in rank 1 to be considered as a positive identification. Subsequently, the identity of the protein and the location of the oligopeptide sequences (Supplementary Data 1) were confirmed by searching against the non-redundant B. anthracis proteins in the Protein Blast database of the US. National Centre for Biotechnology Information (NCBI).54

Table 4. 2D-DIGE spots that showed a change in protein level

| Spot No. | Observed change | P value |
|----------|-----------------|---------|
| 1        | Decrease with age of batch | 0.0012  |
| 2        | Decrease with age of batch | 0.0029  |
| 3        | Decrease with age of batch | 0.0039  |
| 4        | Increase with age of batch | 0.00012 |
| 5        | Increase with age of batch | 0.0011  |
| 6        | Decrease with age of batch | 0.0089  |
| 7        | Decrease with age of batch | 0.0015  |
| 8        | Decrease with age of batch | 0.00036 |
| 9        | Decrease with age of batch | 0.033   |
| 10       | Decrease with age of batch | 0.011   |
| 11       | Decrease with age of batch | 0.073¹ |
| 12       | Decrease with age of batch | 0.02    |
| 13       | Decrease with age of batch | 0.0021  |
| 14       | Decrease only in batch 5 | 0.00002 |
| 15       | Decrease with age of batch | 0.00027 |
| 16       | Decrease with age of batch | 0.00031 |
| 17       | Decrease only in batch 5 | 0.0045  |
| 18       | Increase with age of batch | 0.00015 |
| 19       | Decrease only in batch 5 | 0.0029  |
| 20       | Decrease only in batch 5 | 0.018   |
| 21       | Decrease only in batch 5 | 0.0014  |
| 22       | Decrease only in batch 5 | 0.000018 |
| 23       | Increase with age of batch | 0.0045  |
| 24       | Decrease with age of batch | 0.002   |
| 25       | Decrease with age of batch | 0.00084 |

¹Change is not significant due to large size of spot.

Preparation of anthrax vaccine pellet for 2D-DIGE analysis

Antigens from pellets of selected batches (Table 1) were prepared as follows. For batches 14–16, a homogenous suspension of 2.5 mL (5 SHDs) was concentrated using Amicon Ultra 3k 0.5 mL centrifugal filter units (Millipore) to yield 0.5 mL concentrated vaccine. Anthrax antigens were desorbed from the alun by the addition of 15 µL 10M NaOH to 0.5 mL concentrated vaccine and the sample was vortexed until the suspension cleared. The solution was immediately neutralized by the addition of 15 µL of 3M sodium citrate and centrifuged briefly to remove insoluble material. Vaccine antigens were precipitated using 2D clean up kit (GE Healthcare) and stored at ~20 °C until required.

Due to limited availability of batches 2, 3, 5, 7, 10, 11, 12, and 13, antigens were prepared from 1.5 mL (3 SHDs) vaccine. The samples were centrifuged for 5 min at 13000 g. The pellet was resuspended in 100 µL sterile distilled water; proteins were desorbed and precipitated as described above.

Labeling of anthrax vaccine proteins with fluorescent dye

Proteins from desorbed pellets of AVP were resuspended in 50 µL of sample buffer (30 mM Tris, 7 M Urea, 2 M Thiourea, 4% (w/v) 3-[3-cholamidopropyl] Dimethylammonio]-1-propanesulphonate, pH 8.5 [CHAPS]). The pH was determined by spotting a small volume (~1 µL) onto a pH indicator strip. If necessary the pH was adjusted to 8.5. The protein concentration was determined and the samples were stored in sample buffer at -70 °C until required. Two CyDye DIGE Fluor minimal dyes (NHS-Cy3 and Cy5) were used to label proteins derived from batches 14, 15, and 16. The internal standard, consisting of equal amounts of protein from each vaccine batch included in the experiment, was prepared by pooling the vaccine protein preparations. The internal standard was labeled with 400 pmol Cy5 per 50 µg of protein. Proteins, from the desorbed pellet of 3 batches of AVP were labeled with 400 pmol Cy5 per 50 µg protein.

Three CyDye DIGE Fluor minimal dyes (NHS-Cy2, Cy3, and Cy5) were used to label proteins derived from batches 2, 3, 5, 7, 10, 11, and 12. The internal standard, consisting of equal amounts of protein from each batch included in the experiment, was prepared by pooling the vaccine protein preparations. The internal standard was labeled with 400 pmol Cy2 per 50 µg of protein. Proteins, from desorbed pellets of a batch of AVP were labeled with 400 pmol Cy3 or Cy5 per 50 µg protein. Protein labeling was performed according to the manufacturer’s
instructions (GE Healthcare). Briefly, samples were mixed with the appropriate concentration of dye and incubated on ice in the dark for 30 min. The reaction was terminated by the addition of lysine (10 pmol per 400 pmol dye) followed by a further 10 min incubation on ice in the dark.

**Isoelectric focusing and SDS-PAGE**

Isoelectric focusing (IEF) and SDS-PAGE were performed according to the manufacturer’s recommendations. For IEF of batches 14–16, 2 different protein samples were run on a single 2D gel, the Cy3-labeled pooled internal control and a Cy5-labeled AVP test sample. For IEF of batches 2, 3, 5, 7, 10, 11, and 12, three different protein samples were run on a single 2D gel, the Cy2-labeled pooled internal control and a Cy3 and a Cy5-labeled AVP test sample. The differently labeled protein samples were combined in a single microfuge tube and mixed. The labeled protein samples were diluted in rehydration buffer (7 M Urea, 2 M Thiourea, 2% [w/v] CHAPS, 2% [w/v] IPG Buffer pH 3–11, 18 mM Dithiothreitol [DTT], trace of Bromophenol blue) to the required final volume of 600 µL. Samples were loaded onto Immobiline DryStrips (pH 3–11 NL, 24 cm, GE Healthcare) for 16 h using the in-gel rehydration technique. Isoelectric focusing was performed for 100 kVh using the Ettan IPGphor 3 isoelectric focusing system (GE Healthcare). Prior to SDS-PAGE separation, the IPG strips were equilibrated for 10 min in equilibration buffer (6 M urea, 75 mM Tris, pH 8.8, 30% v/v glycerol, 2% w/v SDS) containing 130 mM DTT and for a further 10 min in equilibration buffer containing 150 mM Iodoacetamide. The strips were placed on top of a 12% polyacrylamide gel (24 cm x 24 cm x 1 mm) cast between low fluorescence glass plates using Next Gen 2DEoptimizer gel caster and then sealed with 0.5% agarose in SDS electrophoresis running buffer(25 mM Tris, 192 mM Glycine, 0.2% [w/v] SDS). Gels were run on the ETTAN 6 vertical gel system (GE Healthcare).

**Image acquisition, analysis, and spot picking**

2D-DIGE gels were scanned within the low fluorescence glass plates. Imaging was performed at 10 µm resolution using a Typhoon 9410 variable mode imager (GE Healthcare). Cy2, Cy3, and Cy5 scans were made of each gel as required. The

---

*Figure 2.* Position of the excised spots on the 2D-DIGE gel stained with Novex colloidal blue. The spot number is related to numbers presented in Tables 4 and 5.
appropriate excitation and emission wavelengths were used for each dye—Cy2 with a blue 488 nm laser and a 520 nm emission filter; Cy3 with a green 532 nm laser and a 580 emission filter; and Cy5 with a red 633 nm laser and a 670 nm emission filter. A prescan was performed to determine the optimal photomultiplier tube voltage required for the scanning of each image.

2D-DIGE analysis of 1-y-old batches 14, 15, and 16 comprised 4 replicates of each batch. Twelve gels were analyzed with each gel containing a Cy3-labeled internal standard and a Cy5-labeled test sample. 2D-DIGE analysis of batches 2, 3, 5, 7, 10, 11, and 12, which were over 7 y of age comprised 11 gels. Each gel contained a Cy2-labeled internal standard and a Cy3 and Cy5-labeled vaccine sample. At least 3 replicates of each vaccine batch were included in the analysis. Image analysis was performed using the biological variance analysis module of the DeCyder differential analysis software (GE Healthcare, v7.2). Quantitative comparison of spots across multiple gels was enabled by the incorporation of an internal standard in the experimental design. Spot patterns from the different gels were matched using the internal standard present on each gel to allow comparison and statistical analysis.

| Spot No. | B. anthracis protein | NCBI accession number | Mr/pl | No. of peptides (coverage) |
|----------|----------------------|-----------------------|-------|----------------------------|
| 1        | Protective antigen   | AAA22637              | 82.8/5.90 | 18 (34%)                 |
| 1        | Lethal factor        | CAC93932              | 93.7/5.95 | 18 (24.1%)               |
| 3        | Protective antigen fragment | AAA22637 | 82.6/5.72 | 10 (15.8%)             |
| 3        | Lethal factor endopeptidase | CAC93932 | 93.7/5.95 | 6 (6.9%)                 |
| 4        | Enolase              | NP_847538             | 46.4/4.77 | 25 (62.4%)               |
| 4        | Protective antigen fragment | AAA22637 | 82.6/5.72 | 2 (4.5%)                 |
| 5        | Enolase              | NP_847538             | 46.4/4.77 | 23 (58.9%)               |
| 5        | Protective antigen fragment | AAA22637 | 82.6/5.72 | 2 (3.8%)                 |
| 6        | Enolase              | NP_847538             | 46.4/4.77 | 14 (47.1%)               |
| 6        | Protective antigen   | AAA22637              | 82.8/5.90 | 20 (34.0%)               |
| 6        | Enolase              | NP_847538             | 46.4/4.77 | 10 (35.5%)               |
| 6        | Lethal factor        | CAC93932              | 93.7/5.95 | 8 (12.5%)                |
| 6        | Glucose-6-phosphate isomerase | NP_847316 | 50.3/5.14 | 4 (16.4%)               |
| 6        | Serine hydroxymethyltransferase | NP_847716 | 45.1/5.91 | 2 (5.3%)                 |
| 7        | Enolase              | NP_847538             | 46.4/4.77 | 10 (35.5%)               |
| 7        | Protective antigen   | AAA22637              | 82.8/5.90 | 20 (31.1%)               |
| 7        | Lethal factor        | CAC93932              | 93.7/5.95 | 3 (3.7%)                 |
| 7        | Glucose-6-phosphate isomerase | NP_847316 | 50.3/5.14 | 2 (4.4%)                 |
| 8        | Protective antigen fragment | AAA22637 | 62.8/5.83 | 12 (21.9%)               |
| 9        | Protective antigen fragment | AAA22637 | 62.8/5.83 | 21 (45.1%)               |
| 11       | Protective antigen fragment | AAA22637 | 26.6/7.46 | 2 (18.9%)                |
| 11       | Lipoprotein, Bmp family | NP_846172 | 38.3/8.48 | 2 (6.5%)                 |
| 11       | 6-Phosphofructokinase | NP_847047             | 34.3/6.29 | 2 (8.5%)                 |
| 12       | Protective antigen fragment | AAA22637 | 26.6/7.46 | 3 (23.2%)                |
| 12       | Iron compound ABC transporter, iron compound-binding protein | NP_847506 | 35.9/6.71 | 6 (21%)                  |
| 12       | Lipoprotein, Bmp family | NP_846172 | 38.3/8.48 | 2 (6.5%)                 |
| 13       | Protective antigen fragment | AAA22637 | 82.6/5.72 | 11 (16.5%)               |
| 13       | Iron compound ABC transporter, iron compound-binding protein | NP_847506 | 35.9/6.71 | 3 (11.7%)                |
| 13       | Lethal factor        | CAC93932              | 93.7/5.95 | 2 (2.7%)                 |
| 13       | 6-Phosphofructokinase | NP_847047             | 34.3/6.29 | 3 (9.7%)                 |
| 13       | Lipoprotein, Bmp family | NP_846172 | 38.3/8.48 | 2 (6.5%)                 |
| 13       | Edema factor fragment | AAA79215              | 29.4/5.19 | 2 (5.2%)                 |
| 14       | Lethal factor        | CAC93932              | 93.7/5.95 | 5 (12.5%)                |
of spot-volume ratios. Spot detection, background subtraction, and normalization were performed, however manual editing was required to merge and separate spots and identify landmark spots before automatic matching could occur. For spot picking, selected gels were stained overnight with Novex colloidal blue stain and then destained briefly in deionised water (Fig. 2). Protein spots of interest were excised from gels.

Identification of peptides in 2-D DIGE spots

Peptides were extracted from 2D DIGE spots as described by Wheeler et al. In brief, excised 2-D gel spots were washed with ammonium bicarbonate, then 50% ACN in ammonium bicarbonate and finally in 100% ACN. Each step lasted for 30 min. This procedure was repeated once. Fifty µL of ammonium bicarbonate containing 0.1 µg Trypsin was added to the dehydrated gel spot and incubated o/n at RT. Peptides were extracted sequentially in 4 steps: adding 50 µL of 1% TFA, transferring the supernatant, then adding 100 µL of 50% ACN in 0.2% TFA twice and transferring supernatant twice and finally 100 µL of 100% ACN was added. The supernatants were pooled and dried in a centrifugal evaporator. The pellet was reconstituted in 100 µL 0.1% formic acid and subjected to LC-MS/MS analysis. Mass spectra were processed and extracted peptides were identified by a search of the Proteome Discoverer v 1.2 with built-in Sequest (Thermo Electron) against B. anthracis FASTA database using trypsin as the enzyme. Peptide sequences (Supplementary Data 2) were confirmed by searching against the non-redundant B. anthracis proteins in the Protein Blast database of NCBI.

Statistical analysis

One way ANOVA was used to analyze the protein content of vaccine fractions and to determine the significance of variations in spot intensity for different batches following 2D-DIGE analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Pam Ford and Howard Tranter (Public Health England, Porton Down, United Kingdom) for donation of AVP batches and AVP bulk 1 and for sharing the in vivo potency data, the Department of Health for encouragement and financial support.

Table 5. Proteins identified by MS/MS in 19 spots excised from 2D-DIGE gel (continued)

| Spot No. | B. anthracis protein | NCBI accession number | Mr/pI | No. of peptides (coverage) |
|----------|----------------------|-----------------------|-------|---------------------------|
| 18       | S-layer protein       | AAA22637              | 26.6/7.46 | 2 (6.4%)     |
| 19       | Protective antigen fragment | AAA22637 | 26.6/7.46 | 3 (10.3%) |
| 20       | S-layer protein       | AAA22637              | 26.6/7.46 | 3 (10.3%)     |
| 21       | Protective antigen fragment | AAA22637 | 26.6/7.46 | 3 (10.3%)     |
| 22       | Lethal factor fragment | AAA22637              | 26.6/7.46 | 3 (10.3%)     |
| 23       | Nucleoside diphosphate kinase | AAA22637 | 26.6/7.46 | 3 (10.3%)     |
| 24       | Iron transport-associated protein | AAA22637 | 26.6/7.46 | 3 (10.3%)     |
| 25       | Protective antigen fragment | AAA22637 | 26.6/7.46 | 3 (10.3%)     |
| 26       | Lethal factor fragment | AAA22637              | 26.6/7.46 | 3 (10.3%)     |
| 27       | S-layer protein SAP   | AAA22637              | 26.6/7.46 | 3 (10.3%)     |

www.landesbioscience.com  Human Vaccines & Immunotherapeutics  1679

©2014 Landes Bioscience. Do not distribute.
37. Antwerpren M, Proença DN, Rückert C, Licht K, Kalinowski J, Hancezaruk M, Tiemann C, Grass G. Draft genome sequence of Bacillus anthracis BF-1, isolated from Bavarian cattle. J Bacteriol 2012; 194:6360-1; PMID:23105087; http://dx.doi.org/10.1128/JB.01676-12
38. Mukhopadhyay S, Akmal A, Stewart AC, Hsia RC, Read TD. Identification of Bacillus anthracis spore component antigens conserved across diverse Bacillus cereus sensu lato strains. Mol Cell Proteomics 2009; 8:1174-91; PMID:19208616; http://dx.doi.org/10.1074/mcp.M800403-MCP200
39. Marraffini LA, Schneewind O. Targeting proteins to the cell wall of sporulating Bacillus anthracis. Mol Microbiol 2006; 62:1402-17; PMID:17074072; http://dx.doi.org/10.1111/j.1365-2958.2006.05469.x
40. Petosa C, Collier RJ, Klimpel KR, Leppla SH, Liddington RC. Crystal structure of the anthrax toxin protective antigen. Nature 1997; 385:833-8; PMID:9039918; http://dx.doi.org/10.1038/385833a0
41. Wagner L, Verma A, Meade LD, Reiner K, Narum DL, Brady RA, Little SF, Burns DL. Structural and immunological analysis of anthrax recombinant protective antigen adsorbed to aluminum hydroxide adjuvant. Clin Vaccine Immunol 2012; 19:1465-73; PMID:22815152; http://dx.doi.org/10.1128/CVI.00174-12
42. Berthold I, Pombo ML, Wagner L, Arciniega JL. Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants. Vaccine 2005; 23:1993-9; PMID:15734073; http://dx.doi.org/10.1016/j.vaccine.2004.10.014
43. Chen Z, Moayeri M, Purcell R. Monoclonal antibody therapies against anthrax. Toxins (Basel) 2011; 3:1004-19; PMID:22069754; http://dx.doi.org/10.3390/toxins3081004
44. Reason DC, Ullal A, Liberato J, Sun J, Keine W, Zhou J. Domain specificity of the human antibody response to Bacillus anthracis protective antigen. Vaccine 2008; 26:4041-7; PMID:18565627; http://dx.doi.org/10.1016/j.vaccine.2008.05.023
45. Reason D, Liberato J, Sun J, Keine W, Zhou J. Frequency and domain specificity of toxin-neutralizing paratopes in the human antibody response to anthrax vaccine adsorbed. Infect Immun 2009; 77:2030-5; PMID:19223482; http://dx.doi.org/10.1128/IAI.01254-08
46. Rivera J, Nakouzi A, Abboud N, Revskaya E, Goldman D, Collier RJ, Dadachova E, Casadevall A. A monoclonal antibody to Bacillus anthracis protective antigen defines a neutralizing epitope in domain 1. Infect Immun 2006; 74:4149-56; PMID:16790789; http://dx.doi.org/10.1128/IAI.00150-06
47. Abouz N, De Jesus M, Nakouzi A, Cordero RJ, Pujato M, Fisher A, Rivera J, Casadevall A. Identification of linear epitopes in Bacillus anthracis protective antigen bound by neutralizing antibodies. J Biol Chem 2009; 284:25077-86; PMID:19617628; http://dx.doi.org/10.1074/jbc.M109.022061
48. Smith K, Crowe SR, Garman L, Guthridge CJ, Muhler JJ, McKee E, Zheng NY, Farris AD, Guthridge JM, Wilson PC, et al. Human monoclonal antibodies generated following vaccination with AVA provide neutralization by blocking furin cleavage but not by preventing oligomerization. Vaccine 2012; 30:4276-83; PMID:22425791; http://dx.doi.org/10.1016/j.vaccine.2012.03.002
49. Migone TS, Subramanian GM, Zhong J, Healey LM, Corey A, Devalaraja M, Lo L, Ullrich S, Zimmerman J, Chen A, et al. Raxibacumab for the treatment of inhalational anthrax. N Engl J Med 2009; 361:135-44; PMID:19587338; http://dx.doi.org/10.1056/NEJMoa0810603
50. Wheeler JX, Whiting G, Rijpkema S. Proteomic analysis of the response of the human neutrophil-like cell line NB-4 after exposure to anthrax lethal toxin. Proteomics Clin Appl 2007; 1:1266-79; PMID:21136624; http://dx.doi.org/10.1002/pca.200700074
51. Cited 2014 Feb 8. Available from: http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome