STRUCTURAL CHARACTERIZATIONS OF PROTEIN ANTIGENS FOR THE NEXT-GENERATION VACCINES AGAINST ANTHRAX AND PLAGUE

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ABSTRACT

Two recombinant protein antigens called rPA and F1-V are recommended by the U. S. Army as active pharmaceutical ingredients (API) for the next-generation vaccines to protect the warfighter against aerosolized anthrax and plague. Separate candidate vaccines containing purified antigen bound to aluminum hydroxide adjuvant in phosphate-buffered saline (PBS) have been tested at the research level, and each has satisfied requirements for advancement to product development. These criteria included: demonstrations of direct and surrogate efficacy in respective animal disease models; preliminary assessment of product safety by testing acute toxicity and API stability; demonstration of an in vitro correlate of protection for development as a potency test; and proposed baseline methods from which to develop a robust manufacturing process and assays for testing product purity, quality, strength, and stability. USAMRIID scientists provided the discovery and concept research in this regard for both candidate vaccines and have been performing ongoing supportive basic research to further the eventual translation of each into a viable product. In looking forward, one of many U. S. Food and Drug Administration (FDA) requirements for eventual human use of these vaccines is documentation of progressive studies that increase understanding of the chemical and biophysical properties of the API. Each of these two protein antigens exhibited unexpected physical features early in research that required further definition. This paper summarizes our characterizations of observed heterogeneity within these pure vaccine proteins: in primary structure for the rPA antigen; and in quaternary structure for the F1-V antigen. This work also illustrates the value of extending technology base research beyond the stages of discovery and proof of concept so as to better support the creation of medical biological products that will protect the warfighter against biological threats.

1. INTRODUCTION

Military policy to vaccinate the American soldier for possible biological threat dates back to General George Washington's order of mandatory variolation against smallpox during the Revolutionary War (Davis and Johnson-Winegar, 2000, Gibson, 1937). Awareness of the need for improved medical protection increased steadily through the past century to the heightened concern of today. Several events and discoveries underscore the current operational requirement for effective medical countermeasures against biological threat agents, and warrant specific protection against the bacteria that cause inhalation anthrax and pneumonic plague, *Bacillus anthracis* and *Yersinia pestis*, respectively. Most notably, the former Soviet Union had a large, clandestine offensive bio-warfare (BW) program (Hoffman, 1998, Alibek and Handelman, 1999), perhaps encouraged from the alleged successful use of tularemia against German panzer troops in the Battle of Stalingrad (Alibek and Handelman, 1999). From 1974 to 1981, the USSR is reported to have actively used chemical-biological weapons during conflicts in South East Asia and Afghanistan, and as recently as 1988, it reportedly earmarked anthrax, plague, and other BW agents for placement in SS-18 missiles and targeting against major U. S. cities (Alibek and Handelman, 1999, Davis and Johnson-Winegar, 2000). In 1995, Iraq acknowledged to the United Nations Special Commission that it had weaponized *B. anthracis* (Zilinskas, 1997), and at least 13 countries currently have probable or known BW programs or possess bio-threat agents, as determined through open literature sources (Carus, 1998, Monterey Institute for International Studies, 2002). Finally, actual risk of harm to the general public by bioterrorism was witnessed in 2001 as the mailing of at least five letters containing anthrax spores caused 22 confirmed or suspected cases of human anthrax and five deaths (Inglesby, 2002). Of even greater potential consequence is the specter of "designer" biological threat agents engineered for antibiotic resistance, latent activation, or other insidious properties (CIA, 2003). This danger is worsened by the global occurrences and distribution of new deadly source strains from naturally emerging and re-emerging diseases (Morens et al., 2004). The horizontal transfer of antibiotic resistance genes between *Y. pestis* and other bacteria in the flea midgut (Hinnebusch, 2002), for example, may help to explain the appearance of *Y. pestis* strains with unexpectedly wide-ranging antibiotic resistance isolated from recent human cases of urban and bubonic plague in Madagascar (Chanteau et al., 2000).

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Because of increased resistance and other concerns associated with the intentional wide spread use of antibiotics, a new national strategy of protection that focuses on antibiotic therapy requires careful expert review before being adopted into policy (Nevas, 2002, Inglesby, 2002). As a case in point, an expert panel recommended the use of antibiotics for postexposure *prophylaxis* in a mass casualty setting, while at the same time noting that the FDA has not yet approved any antibiotic therapy specifically for the *treatment* of symptomatic inhalation anthrax (Inglesby, et al. 2002, and subsequent published author comments). Ciprofloxacin will not kill dormant spores remaining in the lung and will not treat a late-stage illness in which bacteria are already expressing toxins, beta-lactamase, and other virulence factors (Inglesby, et al. 2002, and subsequent published author comments; H. Heine, personal communication). Therefore, the advancement and improvement of existing and new vaccines should be steadfastly pursued along with programs to discover new technologies for prophylaxis, treatment, and detection (Cieslak et al., 2000). This paper illustrates a portion the detailed investigation that supports the creation of two significant vaccines for protecting the warfighter against anthrax and plague.

1a. Anthrax Vaccine

One hundred and twenty-three years ago (May 1881), Louis Pasteur's preparations became the first bacterial vaccine adopted for veterinary use. In public demonstrations, he had shown that inoculations with oxidized/aged preparations of *B. anthracis* protected sheep and cattle against lethal doses of anthrax spores (reviewed in Turnbull, 1991). This preparation and subsequent vaccines based on live non-encapsulated avirulent strains brought tremendous agricultural cost savings and public health benefit to France and the rest of the world since then. For safety reasons, the live attenuated veterinary vaccine was considered unsuitable for human use. Successive studies since 1904 in search of an acellular anthrax vaccine showed protective antigen protein (PA) as a common causative factor that could elicit protective immunity after being derived from purulent cutaneous anthrax lesions, bacteremic blood plasma from infected animals, and *in vitro* culture supernatants. The first U. S. vaccine for human use was developed in 1954 (Wright et al., 1954). It was an alum-precipitated, cell-free filtrate from an aerobic culture that contained PA and other undefined components. The current U. S. anthrax vaccine, called AVA for anthrax vaccine adsorbed, is based on similar technology, being an inactivated cell-free filtrate from a non-encapsulated strain of *B. anthracis* and adsorbed to aluminum hydroxide. AVA was licensed in 1970 (Michigan Department of Public Health, 1978), and is produced by Bioport Corporation (Lansing, MI), which at that time had a single U. S. manufacturing facility for producing the vaccine. Limited production capacity, and a temporary hold on production to redress FDA citations of GMP non-compliance, generated concern for force protection readiness after mandatory anthrax vaccination was ordered for all active- and reserved-duty personnel in 1997. In response to concerns of safety, including the publicized Desert Storm Syndrome (Asa, et al., 2000), medical reviews indicated that only one percent of AVA injections given to 1583 people in the past 25 years were associated with one or more systemic events (e.g., headache, itching, etc.)(Pittman et al., 2001). Finally, after a thorough review commissioned by the CDC, the Institute of Medicine concluded that AVA was acceptably safe (IOM, 2002). Nevertheless, a Defense Technology Objective accelerated research for a replacement vaccine that would be effective in two or fewer doses, as opposed to AVA which requires six doses over 1 ½ years as currently licensed. The new vaccine candidate, which contains only recombinant PA (rPA) as its API and is formulated with aluminum hydroxide, was shown to be equally effective in eliciting protective immunity for several animal models of anthrax (Ivins, et al., 1998, Little et al., 1997). The rPA vaccine will soon enter phase 2 clinical trials under separate sponsorship of the NIAID and the U. S. Army's Prime Systems Contactor, DVC. While demonstrations of safety and effectiveness for the new rPA vaccine are paramount, its eventual licensure and distribution also entails adherence to FDA guidelines for detailed studies to gain structural understanding, manufacturing controls, and many other requirements. By conservative estimates, the current lifespan and cost for discovery and development of a modern biologic drug begins at 10 years and $500 million, and at only 10 % of the total cost, the research and development phase leverages a great effect on final success. For this reason, each early contribution supporting the successful evalua-

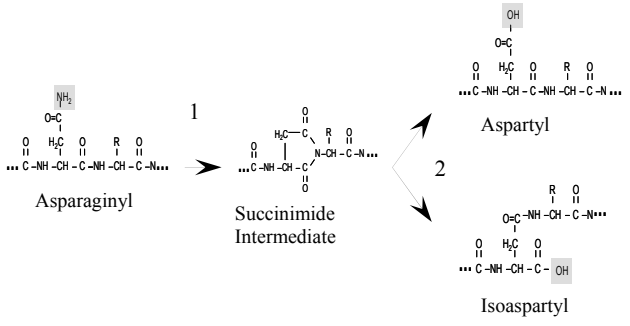

Fig. 1 Protein asparaginyl deamidation. The chemical reaction for spontaneous degradation of protein by deamidation of asparaginyl sites occurs through two steps: 1, loss of ammonium ion from asparaginyl (left grey box) leads to an unstable cyclic succinimide intermediate; 2, which hydrolyzes (right grey boxes) giving aspartyl and isoaspartyl in 1:3 ratio. The loss of ammonium (-17) and addition of water (+18) produces a net change of 1 mass unit, and decreases the functional group isoelectric point. A similar but much less frequent deamidation reaction occurs at glutaminyl sites (Cleland et al., 1993).

tion and manufacture of rPA vaccine is of immeasurable value. The issue of product heterogeneity arose early in development (pre-IND meeting, 2002), and was recently resolved at USAMRIID by a combination of tests as summarized in this paper (Powell et al., and Ribot et al., unpublished work).

1b. Plague Vaccine

There is no currently licensed U. S. vaccine for plague. The formerly licensed Plague Vaccine USP protected experimental animals against parenteral challenge, but was later shown to be ineffective against pneumonic plague caused by typical F1 capsule positive (F1+) strains of *Y. pestis* (Meyer, 1970, Pitt, 1994), as well as against natural isolates of non-encapsulated (F1-) *Y. pestis,* since the vaccine's primary immunogen was fraction 1 antigen, F1 (Heath, et al., 1998). A survey of other known *Yersinia* virulence factors as potential additional or substitute components showed that a combination of both F1 and the classic virulence antigen, V, provided effective protection against lethal challenge by encapsulated and non-encapsulated *Y. pestis* in animal models (Benner et al., 1999). However, we later showed that a novel fusion protein joining both F1 and V subunits (F1-V) provided slightly greater protection than the two-component F1 + V vaccine (Heath et al., 1998). An improved F1-V fusion protein antigen was chosen for further study and early development at USAMRIID (Plague Vaccine IPT Meeting, 8 Dec 2000). A competitive candidate vaccine product comprising separate F1 + V subunits is similarly under research and development in the U. K. The data summarized here provides evidence that a single fusion protein would be more robust than a two-component vaccine.

2. METHODS

rPA protein (82,674 Da) was from bulk product produced under good manufacturing practices (GMP) by contract to USAMRIID (Biopharmaceutical Development Program, Frederick, MD), or from archives of research grade protein produced at USAMRIID and stored at – 70 degrees. F1-V protein (53,192 Da) was produced at USAMRIID (Powell et al., unpublished work) or supplied as bulk product by contract (BioSciences, Baltimore, MD) then exchanged into PBS at USAMRIID. F1 protein (15,694) was produced essentially as previously described (Andrews et al., 1996), and V protein (37,240 Da) was purified after engineering the expression vector to yield a product with a natural N-terminus (Powell et al., unpublished work). Non-denaturing polyacrylamide gel electrophoresis (native PAGE) employed the PhastSystem as prescribed by the manufacturer (Amersham Biosciences, Piscataway, NJ). A protein isoaspartyl methyl transferase assay (Promega Corp., Madison, WI) employing HPLC detection (Agilent Technologies Inc., Wilmington, DE) was used to measure protein isoaspartate content as described (Shurter and Aswad, 1991). Liquid chromatography coupled to electrospray ionization ion trap mass spectrometry (Agilent Technologies, Inc.) with Mascot Daemon search engine software (Matrix Sciences Limited, London, UK) was used to detect and measure protein deamidation, as performed on trypsin-digested whole protein or protein isoforms isolated by native PhastSystem gel or two-dimensional gel electrophoresis (2-DE), with computer-assisted image analysis (BioRad, Emeryville, CA). Synthetic peptides mimicking the native and principal deamidated state of tryptic peptide 529-IAFGFNEP-*N*GNLQYQGK-545 were prepared by contract. Liquid chromatography coupled to multi-angle light scattering spectroscopy, differential refractometry, and photon correlation spectroscopy (Wyatt Technologies Corp., Santa Barbara, CA) were used to define solution phase quaternary structures for F1, V, and F1-V proteins.

3. RESULTS

3a. Asparagine Deamidation Causes rPA Isoforms

During early research and development, purified rPA

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000 EVKQENRLLN ESESSSQGLL GYYFSDLNFQ APMVVTSSTT
041 GDLSIPSSEL ENIPSENQYF QSAIWSGFIK VKKSDEYTFA
081 TSADNHVTMW VDDQEVINKA SNSNKIRLEK GRLYQIKIQY
121 QRENPTEKGL DFKLYWTDSQ NKKEVISSDN LQLPELKQKS
161 SNSRKKRSTS AGPTVPDRDN DGIPDSLEVE GYTVDVKNKR
201 TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT
241 GRIDKNVSPE ARHPLVAAYP IVHVDMENII LSKNEDQSTQ
281 NTDSQTRTIS KNTSTSRTHT SEVHGNAEVH ASFFDIGGSV
321 SAGFSNSNSS TVAIDHSLSL AGERTWAETM GLNTADTARL
361 NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ
401 LSQILAPNNY YPSKNLAPIA LNAQDDFSST PITMNYNQFL
441 ELEKTKQLRL DTDQVYGNIA TYNFENGRVR VDTGSNWSEV
481 LPQIQETTAR IIFNGKDLNL VERRIAAVNP SDPLETTKPD
521 MTLKEALKIA FGFNEPNGNL QYQGKDITEF DFNFDQQTSQ
561 NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKRFHYDR
601 NNIAVGADES VVKEAHREVI NSSTEGLLLN IDKDIRKILS
621 GYIVEIEDTE GLKEVINDRY DMLNISSLRQ DGKTFIDFKK
681 YNDKLPLYIS NPNYKVNVYA VTKENTIINP SENGDTSTNG
721 IKKILIFSKK GYEIG
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Fig. 2 Summary of LC/MS/MS analysis for deamidation within the rPA protein. The entire amino acid sequence of rPA is shown by standard single letter code, and is identical to natural protective antigen excluding the first 29 amino acid signal sequence (accession P13423). Bold text indicates amino acid sequence experimentally observed. Underline text indicates tryptic fragments containing confirmed deamidation, and grey text indicates sites of verified asparagine deamidation including: N399, N408, N537, N539, N601, N602, N713 and N719.

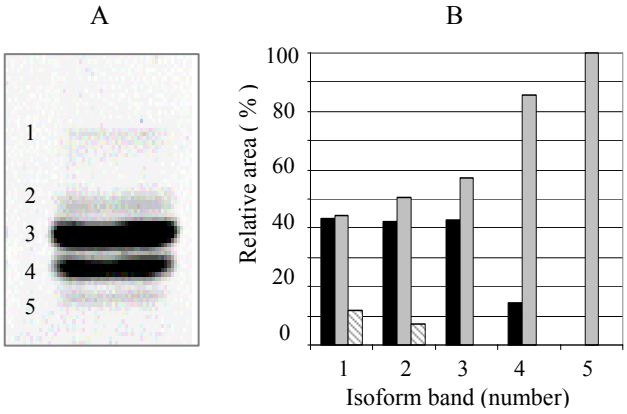

Fig. 3 Analysis of rPA isoforms by native PAGE and ion trap mass spectrometry. A, Coomassie blue-stained native PhastSystem gel of purified GMP grade rPA protein. Isoform bands of increasing negative charge are numbered sequentially. B, Relative amounts (comparative ion peak areas, ordinate) of peptide 529-IAFGFNEP*N*G*N*LQYQGK-545 having native amino acid sequence (dark bar), deamidation at Asn537 (grey bar), or deamidation at both Asn537 and Asn539 (hatched bar) are shown for each isolated isoform band from 1 to 5 (abscissa).

protein was observed to contain at least four isoforms that all shared the expected molecular weight of 83,000 by SDS-PAGE but differed incrementally in apparent charge by capillary electrophoresis and native PAGE (Ribot et al., 2003 and unpublished observations). In contrast, GMP grade rPA protein revealed only two major isoforms and several minor isoforms (see Fig. 3A and Fig. 4, top panel). Because asparagine deamidation is a common mode of protein deterioration that also adds negative charge to the affected protein (Fig. 1, Cleland et al., 1993), and because isoaspartate is a sentinel marker of this event, we first tested whole rPA protein for isoaspartate content. GMP grade rPA contained 0.16 mole fraction of isoaspartate, which would be in agreement with full deamidation of up to 14 of its 67 total asparagine residues. Mass spectrometry was then employed to identify modified amino acids and measure their extent of departure from native structure. Analysis of trypsinized protein by liquid chromatography coupled to tandem ion trap mass spectrometry (LC/MS/MS) covered 71 % of the entire rPA sequence (Fig. 2, bold text) and revealed several deamidated amino acids. Manual inspection of all automatically assigned modifications confirmed the presence of eight deamidated asparagines on four tryptic peptides (Fig. 2, italic text and underlines), with no other amino acid modification of significant amount. The extent of deamidation did not exceed 52 % in any one of these eight asparagines (data not shown). Because these measured values represented population averages of all isoforms present in the bulk material, we next inspected individual isoforms isolated by native PhastSystem gel (Fig. 3A) and by 2-DE (Fig. 4, top panel). The data revealed that the four predominantly affected asparagines (N408, N537, N601 and N713) generally increased in percent deamidation with increasing net negative charge of the isoform, as illustrated for Asn537 of peptide 529-IAFGF-NEPNGNLQYQGK-545 (Fig. 3B and Fig 4, bottom panel). Similar trends were also observed using 2-DE separations with immobilized pH gradients of wider (3 - 10) and narrower (4.7 - 5.9) ranges for first dimension (data not shown). Furthermore, the amount of asparagine deamidation at each spot was greater in protein that was artificially aged through successive freeze/thaw cycles as compared to freshly thawed protein (data not shown). Faint isoform spots located at the extreme pH ends of 2-DE separation did not fall into this trend (e.g., Fig 4, spot 2; other data not shown). In all of these results, no clear correlation was seen that linked percent deamidation to the relative abundance of any isoform band or spot. Of practical benefit is our observation that Asn537 contained

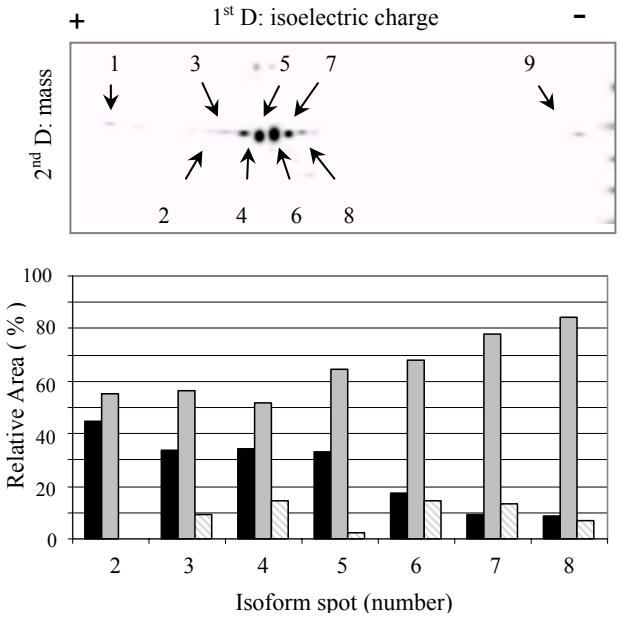

Fig. 4 Analysis of rPA isoforms by 2-DE and ion trap mass spectrometry. Top panel, Master image averaged from duplicate coomassie blue-stained two-dimensional polyacrylamide gels (1st D: pH 4-7, 2nd D: 4 - 12 % PAGE in Bis-Tris buffer) of purified GMP grade rPA protein. Isoform spots of increasing net negative charge are numbered sequentially from 1 to 9. For clarity and vertical alignment between isoform spots and their corresponding percent deamidation in to the bar graph below, the 2-DE image is shown in reverse orientation, with instrument cathode on the right and labels referring to the proteins being separated (conventional orientation places instrument cathode and standard mass markers on left). Bottom panel, Relative amounts (peak area, ordinate) of peptide 529-IAFGFNEP-*N*G*N*LQYQGK-545 having native amino acid sequence (dark bar), deamidation at Asn537 (grey bar), or deamidation at both Asn537 and Asn539 (hatched bar) are shown for each isolated isoform spot (abscissa). Data above background was not retrieved for the faint isoform spots 1 and 9.

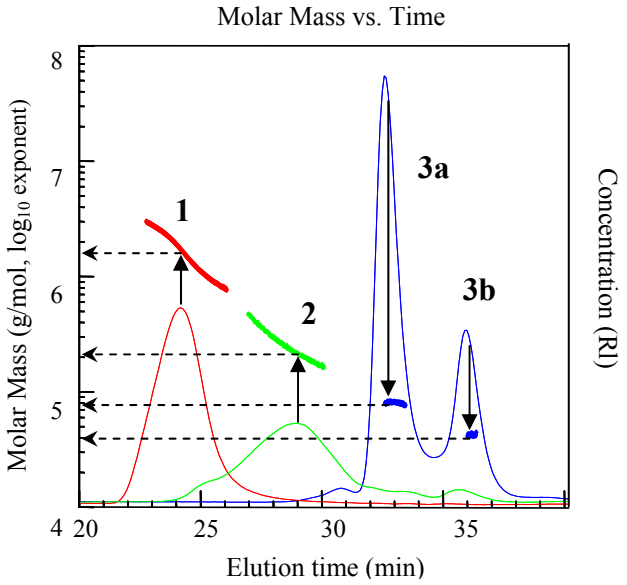

Fig. 5 Analysis of molar mass distributions for F1, F1-V and V proteins by SEC-MALS in PBS at 37 ° C. Chromatograms for three separate experiments are overlaid to show concentration and molar mass vs. elution time for F1 protein (red), F1-V fusion protein (green) and V protein (blue). Thin lines trace concentration as measured by differential refractometry (RI, right hand ordinate); thick lines trace absolute molar mass (left hand ordinate) across the indicated elution range. Solid arrows connect the point of highest concentration to the point of from which mass is extrapolated by hand (dashed arrow) or by instrument (Table I) for F1 (1), F1-V (2) and V (3a and 3b).

the highest percent deamidation within all isoforms measured and by all separations tested. The values of percent deamidation (i.e., peak areas of specific parent ions) obtained for these experiments were compared against standard curves of synthetic peptides designed to mimic the native and d-Asn537 states, and found to be well within linear range (data not shown).

3b. F1 Subcomponent Causes F1-V Aggregation

While purifying and testing the F1-V plague vaccine antigen, we observed spontaneous aggregation of the fusion protein under common handling conditions, and developed a size-exclusion chromatography multi-angle laser light-scattering (SEC-MALS) assay to measure soluble quaternary structure (Cushman et al., 2000, Powell et al., 2002). Soluble structures were compared between separate lots of purified F1-V fusion protein and individual F1 and V subcomponent proteins in PBS buffer at pH 7.4 and 37 ° C to simulate physiologic conditions (Fig. 5, Table I). Pure F1-V fusion protein comprised a polydisperse population of molecules ranging from the expected monomeric mass (53 kDa) to large soluble aggregates (Fig. 5, Fig. 6, Table I), irrespective of the protein's method of purification or original solubility (not shown). However, most of F1-V fusion protein (greatest concentration peak) had an absolute molar mass of 212,000 at both 37 ° C and room temperature, in agreement with a tetrameric form (Fig. 5 curve 2, Fig. 6B). In contrast, pure F1 protein (15 kDa monomer mass) existed as an even larger polydisperse aggregate with the bulk population at 1.5 million at 37 ° C (Fig. 5 curve 1, Fig. 6A), and shifted to 3 million in absolute molar mass at room temperature (Fig. 6A). Thus, under either condition, F1 aggregated to a mass far greater than its monomeric mass and ten times greater than F1-V. Pure V protein existed in monomeric, dimeric, and tetrameric form with no detectable higher aggregation (Fig. 5 curves 3a & 3b, Fig 6C). This distribution was shifted to predominantly monomeric form by reducing agents (Fig. 6C), which is to be expected as V contains a single cysteine. Increased salinity, like decreasing temperature, also exacerbated the aggregation of F1 (data not shown). In contrast, reducing agents did not noticeably shift the molar mass distribution of F1-V (Fig 6B). The data show that the soluble molecular structure of F1-V is more stable than are either of the subcomponent proteins under these

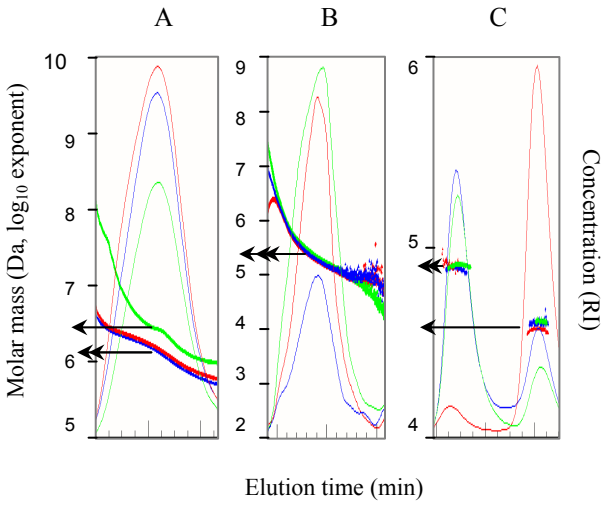

Fig. 6 Comparison of mass distributions of F1, F1-V, and V proteins by SEC-MALS under three different physical conditions. Chromatograms for three separate experiments per protein are overlaid to show concentration and molar mass vs. elution time for F1 protein (A), F1-V fusion protein (B) and V protein (C), as tested at 37 ° C (blue lines), 25 ° C (green lines) and reducing conditions at 37 ° C (red lines). Thin lines trace concentration as measured by differential refractometry (RI, right hand ordinate); thick lines trace absolute molar mass (left hand ordinate) across the indicated elution range as described (Wyatt Technologies, Inc., www.wyatt.com). Arrows connect sample populations of highest concentration to their measured absolute molar mass for each of the three conditions. Note that while the bulk population of F1-V (panel B) remains at 212,000 under all three conditions, the bulk population of F1 protein (panel A) shifts from 1,500,000 to 3,000,000 by lowering the temperature 12 degrees to room temperature. Greater aggregation size is observed by further lowering to 18 degrees (data not shown). V protein shifts from dimer to monomer under reducing condition (panel C).

Table I. Summary of Measured Absolute Molar Masses and Mass Distributions for Pure Protein Antigens F1, V, and F1-V.

Protein Name	Protein Function	Expected Mass (kDa)	Observed Mass (kDa) [calculated multiples of monomeric mass]	
			Over Observed Concentration Range	At Peak Concentration
Fraction I protein, F1	Proteinaceous capsule, interferes with phagocytosis	15.694	450 – 3,000 [n = 30 - 200]	1,500 [n = 100]
Virulence antigen, V	Secreted effector protein, interferes with cytokine mediated cell regulation	37.240	37 – 74 [n = 1, n = 2]	74 [n = 2]
F1-V Fusion protein	Active pharmaceutical ingredient of candidate vaccine against plague	53.192	53 – 2,100 [n = 1 – 40]	212 [n = 4]

Protein name, function, expected mass, and observed absolute mass distributions are given for SEC-MALS measurements performed at 37 degrees in PBS. Expected masses are inferred from nucleic acid sequence of the gene. Observed absolute molar masses are presented in kilo Daltons (kDa), across the detected range of protein elution and at the predominant concentration peak, with inferred multiples of monomeric units having equivalent mass shown in brackets ([ ]).

conditions, and that aggregates of F1-V fusion protein are much smaller than those of the isolated F1 protein.

4. CONCLUSIONS

The results presented here indicate that: 1) asparagine deamidation is the dominant cause of micro-charge heterogeneity observed in the rPA protein antigen for the anthrax vaccine; and 2) while a natural property of the F1 subcomponent causes molecular aggregation in saline solution, the F1-V fusion protein antigen designed for the next plague vaccine greatly reduces the final size of aggregation and also provides structural consistency.

With respect to rPA, we found that more negatively charged isoforms generally contained more deamidation. Since we did not find specific correlation between the abundance of a given isoform and percent deamidation at any observed asparagine, these results support the notion that each observed isoform band or spot represents a separate cumulative molecular charge resulting from a unique sum of various deamidations. As such, even an isolated isoform would actually comprise a heterogeneous population of proteins that each contains a given number of asparagine deamidations but at unspecified positions. This proposal is in agreement with the fact that fractional deamidation does not occur within a single position on a single molecule. As combined with related research, which demonstrated that separate vaccine preparations containing each of the two primary rPA isoforms equally protected rabbits in an experimental model of anthrax disease (Ribot et al., 2003), these experiments have added critical documentation to support upcoming FDA review of rPA vaccine as an investigational new drug (IND). Furthermore, this work reveals a means for measuring rPA deamidation that could be developed into an additional assay for testing manufacture consistency and product stability.

With respect to the plague vaccine F1-V antigen, our findings on the soluble molecular structure of *Y. pestis*-based protein antigens reveal that the observed aggregation of F1-V fusion protein is attributable to a natural tendency for self-association imparted by the F1 protein. Importantly, the bulk (i.e., primary concentration peak) of the F1-V fusion protein formed an aggregate mass at least ten-times smaller than that for the individual F1 protein under common handling conditions (Fig. 5 and Fig. 6). This disparity is worsened by temperatures even lower than 25 ^{o} C, such as holding on ice. This evidence places additional burden on manufacturing controls for a possible two-component F1 + V vaccine, and indicates that a single F1-V fusion protein would be a more robust GMP product in this regard. The SEC-MALS assay developed by this work can serve as a useful measure of protein structural quality and purity, and, indeed, we have transferred details of this technology to the army product developer, DVC. These results reveal new details about the structural properties of two vaccine antigens and join with many other required documents to assist in the successful development of vaccines as future medical biological products to protect the warfighter against a growing potential biological threat.

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