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Lethal factor antibodies contribute to lethal toxin neutralization in recipients of anthrax vaccine precipitated

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ABSTRACT

A major difference between two currently licensed anthrax vaccines is presence (United Kingdom Anthrax Vaccine Precipitated, AVP) or absence (United States Anthrax Vaccine Adsorbed, AVA) of quantifiable amounts of the Lethal Toxin (LT) component Lethal Factor (LF). The primary immunogen in both vaccine formulations is Protective Antigen (PA), and LT-neutralizing antibodies directed to PA are an accepted correlate of vaccine efficacy; however, vaccination studies in animal models have demonstrated that LF antibodies can be protective. In this report we compared humoral immune responses in cohorts of AVP (n = 39) and AVA recipients (n = 78) matched 1:2 for number of vaccinations and time post-vaccination, and evaluated whether the LF response contributes to LT neutralization in human recipients of AVP. PA response rates (≥95%) and PA IgG concentrations were similar in both groups; however, AVP recipients exhibited higher LT neutralization ED_{50} values (AVP: 1464.0 ± 214.7, AVA: 544.9 ± 83.2, $p < 0.0001$) and had higher rates of LF IgG positivity (95%) compared to matched AVA vaccinees (1%). Multiple regression analysis revealed that LF IgG makes an independent and additive contribution to the LT neutralization response in the AVP group. Affinity purified LF antibodies from two independent AVP recipients neutralized LT and bound to LF Domain 1, confirming contribution of LF antibodies to LT neutralization. This study documents the benefit of including an LF component to PA-based anthrax vaccines.

1. Introduction

Bacillus anthracis is a Gram positive, spore-forming rod that is the causative agent of anthrax. *B. anthracis* has two major, plasmid-encoded virulence factors, a poly-D-glutamic acid capsule and a secreted tripartite toxin [1]. The tripartite toxin is made up of Protective Antigen (PA), Lethal Factor (LF) and Edema Factor (EF) [2]. PA is an 83kD protein that acts as the common binding component for LF and EF. PA binds to one of the two major anthrax toxin receptors and forms a pore, allowing LF and EF access to the cytosol, where they exert their activities [3]. LF is a 90kD Zn^{2+}-dependent metalloprotease that cleaves MAPKKs, while EF is an 89kD calmodulin-dependent adenylate cyclase that converts ATP to cAMP [4,5]. PA and LF together make Lethal Toxin (LT), while PA and EF make Edema Toxin (ET) [2,6]. These toxins act to impair the host immunity [7] and have further systemic effects [8].

B. anthracis has been used as a weapon of bioterrorism. Since the intentional release of *B. anthracis* spores through the United States (US) postal system in 2001 [9], interest in understanding the immune response to anthrax vaccination has renewed. In the US, the currently licensed vaccine is Anthrax Vaccine Adsorbed (AVA), which is produced from a cell-free filtrate of a toxigenic, acapsulate bovine isolate (V770-NP1-R), containing an unquantified amount of

Abbreviations: AVA, Anthrax Vaccine Adsorbed; AVP, Anthrax Vaccine Precipitated; LF, Lethal Factor; LT, Lethal Toxin; PA, Protective Antigen.

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PA and small or negligible amounts of LF and EF [10,11]. In the United Kingdom (UK), the licensed vaccine is Anthrax Vaccine Precipitated (AVP), which is produced from a cell-free filtrate of a toxigenic, acapsulate strain (Sterne 34F2) and contains roughly 7.9 μg/mL PA and 1.9 μg/mL LF [12]. The current AVA vaccination course consists of 5 intramuscular doses, administered at 0, 1, 6, 12 and 18 months, with subsequent annual boosters [10]. AVP vaccination consists of 4 intramuscular doses, administered at 0, 3, 6 and 32 weeks, with an annual booster [13]. Other distinctions between the vaccines include adsorption to aluminum hydroxide gel (AVA, 0.6 mg Al/dose) versus precipitation with aluminum potassium sulfate (AVP, 0.4 mg Al/dose) and use of different preservatives [14,15]. These vaccines are thought to protect by eliciting LT-neutralizing PA antibodies [16]. Consequently, recombinant PA alone has been developed as a next generation vaccine and has demonstrated safety and immunogenicity in humans [17–20]. Studies in animal models have demonstrated that LF without PA can provide protection. Immunization with LF alone protected mice from LT challenge [21], and immunization of mice with spores producing bacilli making LF but not PA provided equivalent protection against Sterne spore challenge as compared to immunization with spores producing only PA [22]. These results suggest that the added presence of LF in the AVP vaccine may contribute to toxin neutralization in human vaccinees.

Herein, we compared humoral responses to the AVP and AVA vaccines and tested the hypothesis that human LF antibodies elicited by anthrax vaccination can contribute to LT neutralization. We observed that AVP vaccination induced higher LT neutralization, higher prevalence and titer of LF antibodies, but similar levels of PA antibodies compared to AVA vaccinees matched for number of vaccinations and time post-vaccination. PA and LF IgG independently contributed to *in vitro* LT neutralization in these samples. Moreover, LF antibodies purified from the plasma of AVP vaccinees neutralized LT and recognized LF Domain 1. These data show that AVP vaccination elicits LF-specific antibodies that contribute to LT neutralization, demonstrating the benefit of including an LF component in PA-containing human vaccine formulations.

2. Methods

2.1. Collection of human blood samples

Individuals were enrolled with informed consent and had been immunized with licensed AVP (n = 39) or AVA (n = 78). Existing plasma samples from non-vaccinated individuals (n = 100) were used as controls to establish thresholds of positivity in ELISA assays. Institutional Review Board approval was obtained from the Oklahoma Medical Research Foundation, University of Oklahoma Health Sciences Center, and Walter Reed National Military Medical Center. Consent was obtained from staff at Public Health England, United Kingdom. Sera or plasma isolated from AVP or AVA recipients were stored at ≤ -20 °C until further use.

2.2. PA, LF and LF domain ELISAs

ELISAs were performed as described [23]. Briefly, 96-well plates were coated with 1 μg/well of recombinant PA, LF (List Biologicals, Campbell, CA), or LF domains (see below). Plates were blocked, followed by a 2 h incubation with serum or plasma at room temperature (RT). Alkaline phosphatase-labeled anti-human IgG was used for detection, and optical density ($OD_{410/490}$) was measured. LF endpoint titer was defined as the last 10-fold dilution giving an OD greater than the average OD plus 2SD above values of 100 unvaccinated controls. PA IgG concentration was calculated using a standard curve of reference serum AVR801 (Center for Disease Control and Prevention, Atlanta, GA) containing antibodies to PA, serially diluting twofold at a starting concentration of 109.4 μg/mL [24].

2.3. Production of LF domains

LF domain expression was accomplished by cloning cDNAs encoding LF 1 (aa 1-262), LF2a/3 (aa263-386), LF2b (aa 387-550) and LF4 (aa551-776) into pET expression vectors. LF1 was cloned into pET28a using the HindIII reverse site without a stop codon reverse primer to produce 6xhis tags at both the N- and C-termini, which was required for effective purification. LF2a/3, LF2b and LF4 were cloned into pET15b to produce standard N-terminally linked 6-His-containing proteins. LF1 was soluble in PBS and purified by standard Ni^{2+} affinity chromatography. LF2a/3, 2b and 4, were solubilized in 6 M urea, and purified by Ni^{2+} affinity chromatography with a 20 mM imidazole wash step, and dialyzed into PBS containing 6 M urea and 0.05% lauryldimethylamine oxide, followed by stepwise dialysis until the urea concentration was reduced to zero.

2.4. J774a.1 LT neutralization assay

This assay was performed as described [12], with minor modifications. Briefly, plasma serial dilutions were pre-incubated with 25 ng PA and 5 ng LF for 30 min at 37 °C then added to J774a.1 cells (plated the previous day at 90,000 cells per well in 96-well plates) for 3 h. Next, 50 μL of 1.5 mg/mL MTT was added, and the plates were incubated for 1 h. The OD was measured, and ED_{50} values were calculated by four-parameter logistic regression [25].

2.5. Purification of LF specific IgG

Recombinant LF and PA were produced as previously described [26,27] and individually bound to cyanogen bromide-preactivated Sepharose 4B following the manufacturer's protocol (GE Healthcare, Piscataway, NJ). To purify LF-specific antibodies, serum samples were passed over the LF column 3 times. On each passage, samples were eluted with 3 M NaSCN, buffer exchanged into PBS and concentrated to starting plasma or serum volume (1 mL) using EMD Millipore Amicon™ Ultracel 30,000 NMWL Centrifugal Filter Units (EMD Millipore, Billerica, MA). These antibodies were then passed over a PA column once, and the unbound antibodies (affinity purified LF antibodies) were concentrated to 1 mL.

2.6. Statistical analyses

AVP-vaccinated individuals (n = 39) were matched 1:2 with AVA-vaccinated individuals (n = 78) by number of vaccinations, time post-vaccination within 0.3 years, and age where known (Table 1). Between-group comparisons of medians were assessed by Mann-Whitney *U* test. In all comparisons, mean ± SEM is reported. Between-group proportions were compared by Fisher's exact test, and associations were reported as odds ratios (OR). Univariate correlations of log-transformed data were analyzed by linear regression.

Multiple linear regression of log-transformed data was used to assess the contribution of PA and LF antibodies to *in vitro* LT neutralization, as well as influence of vaccination history and demographic factors on antibody responses and LT neutralization. Initial models included interaction term(s). Non-significant interaction term(s) were dropped from the final models. Partial correlation of determination was used to quantify the contribution of terms to the models. Multi-collinearity was assessed by variance inflation factor. Multiple regression analyses were performed in R 3.2.3; all other statistical analyses were performed using GraphPad Prism 6.0.