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Lethal Factor Antibodies Contribute to Lethal Toxin Neutralization in Recipients of Anthrax Vaccine Precipitated¹

Eric K. Dumas^{a,b}, Lori Garman^{a,b}, Hannah Cuthbertson^c, Sue Charlton^c, Bassam Hallis^c, Renata J.M. Engler^d, Shyamal Choudhari^{e,1}, William D. Picking^e, Judith A. James^{a,b,f}, and A. Darise Farris^{a,b,*}

^aArthritis and Clinical Immunology Program, Oklahoma Medical Research Foundation (OMRF), 825 NE 13th St., Oklahoma City, OK 73104, USA

^bDepartment of Microbiology and Immunology, University of Oklahoma Health Sciences Center (OUHSC), 940 Stanton L. Young Blvd, Oklahoma City, OK 73104, USA

^cPublic Health England, National Infection Service, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom

^dWalter Reed National Military Medical Center, 8901 Wisconsin Ave, Bethesda, MD 20814, USA

^eDepartment of Pharmaceutical Chemistry, University of Kansas, 320B Multidisciplinary Research Building, 2030 Becker Dr., Lawrence, KS 66047, USA

^fDepartments of Medicine and Pathology, OUHSC, 1000 Stanton L. Young Blvd, Oklahoma City, OK 73104, USA

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 ED₅₀ values (AVP: 1464.0 ± 214.7, AVA: 544.9 ± 83.2, p<0.0001) and had

¹Abbreviations: AVA, anthrax vaccine adsorbed; AVP, anthrax vaccine precipitated; LF, lethal factor; LT, lethal toxin; PA, Protective Antigen

*Corresponding author at: Oklahoma Medical Research Foundation, Arthritis and Clinical Immunology Program, 825 NE 13th St., Oklahoma City, OK 73104, USA. Tel: +1 405 271 7389; fax: +1 405 271 4110. darise-farris@omrf.org.

¹Present address: Formulation Department, Cook Pharmica, LLC, 1300 S. Patterson Dr., Bloomington, IN 47403, USA

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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.

Keywords

Bacillus anthracis; anthrax; Anthrax Vaccine Adsorbed; Anthrax Vaccine Precipitated; Lethal Factor; Protective Antigen; Lethal Toxin; neutralization

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 Zn²⁺-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 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

terminally linked 6-His-containing proteins. LF1 was soluble in PBS and purified by standard Ni²⁺ affinity chromatography. LF2a/3, 2b and 4, were solubilized in 6M urea, and purified by Ni²⁺ affinity chromatography with a 20 mM imidazole wash step, and dialyzed into PBS containing 6M urea and 0.05% lauryldimethylamine oxide, followed by stepwise dialysis until the urea concentration was reduced to zero.

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 ED₅₀ values were calculated by four-parameter logistic regression [25].

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 3M 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.

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.

Results

AVP elicits LF antibodies while both AVP and AVA elicit PA antibodies in most vaccinated individuals

Serum samples from AVP recipients (n=39) matched 1:2 with plasma samples from AVA recipients (n=78) for number of vaccinations, time post-vaccination and age where known (Table 1), were tested for PA and LF antibodies. The overall median number of vaccinations in both groups was 4 (range 2–11). Samples from AVP recipients (37/39, 94.9%) were significantly more likely than matched AVA recipients (1/78, 1.3%) to contain anti-LF IgG at end titer ≥ 100 ($p < 0.0001$, odds ratio (OR) 1425), in keeping with the known presence of quantifiable LF in the AVP preparation. Moreover, the average LF IgG titer differed substantially between the AVP (1042 ± 243.3) and AVA groups (4.8 ± 1.3 , $p < 0.0001$; Fig. 1A). However, both groups produced similar concentrations of PA IgG after vaccination (AVP: 412.5 ± 69.6 $\mu\text{g/ml}$, AVA: 613.1 ± 112.7 $\mu\text{g/ml}$, $p = 0.320$, Fig. 1B). Samples were also examined for their LT neutralization capacity using an *in vitro* neutralization assay. AVP recipient samples had significantly higher average LT neutralization ED_{50} values than samples from matched AVA recipients (AVP: 1464 ± 214.7 , AVA: 544.9 ± 83.2 , $p < 0.0001$, Fig. 1C). Thus, AVP vaccination elicits higher levels of toxin neutralization, more prevalent and higher LF IgG responses, but similar levels of PA IgG compared to AVA vaccination.

PA IgG concentration and LF IgG titer are independently and additively associated with *in vitro* LT neutralization

In univariate analyses, LF IgG titer correlated with *in vitro* LT neutralization only in the AVP cohort ($R^2 = 0.577$, $p < 0.0001$; Fig. 2A), while PA IgG concentration was associated with *in vitro* LT neutralization in both AVA ($R^2 = 0.598$, $p < 0.0001$) and AVP ($R^2 = 0.798$, $p < 0.0001$; Fig. 2B) cohorts. To determine the relative contribution of PA and LF antibodies to LT neutralization in the AVP group, multiple regression analyses were performed. The initial model detected no evidence of statistical interaction (synergy) between PA IgG concentration and LF IgG titer ($p = 0.180$). A final regression model was constructed without interaction terms (Table 2). This model was unaffected by collinearity (variance inflation factor = 1.9), indicating that LF IgG titer predicts *in vitro* LT neutralization significantly and independently of PA IgG concentration.

Gender modulates humoral responses to AVP and AVA

To explore the influence of age and gender on humoral responses to anthrax vaccination, a cohort of 26 AVP samples for which these variables were fully known and their matched AVA samples (n=52) were evaluated by multiple regression analysis. Though matching ensured no differences in age between subgroups (AVP mean $\pm$ SD: 33.2 ± 12.1 , AVA: 31.3 ± 8.9), the AVA group contained more male vaccinees (AVP: 50% male, AVA: 82.7% male, OR 4.78, $p = 0.006$). Potential interaction between PA and LF was eliminated from an initial model using an approach of backwards stepwise regression (not shown). In the AVP group, PA IgG concentration positively associated with LT neutralization ED_{50} (coefficient 0.897, 95% CI=0.597–1.197) in both males and females, whereas LF IgG titer positively associated with LT neutralization ED_{50} only in females (coefficient 0.297, 95% CI=0.063–0.531, $p = 0.016$; Suppl. Table 1). In the AVA population, LF titer had no association with ED_{50} , as

expected. However, PA IgG concentration positively associated with ED₅₀ only in males (coefficient 1.011, 95% CI= 0.769–1.252, $p<0.0001$). Increased ED₅₀ values occurred in males of the AVA cohort ($p=0.009$, Mann-Whitney U test, Suppl. Figure 1). Thus, gender effects on humoral immunity to AVP and AVA vaccination are detected.

Purified LF specific antibodies neutralize LT *in vitro*

To confirm the LT-neutralizing capacity of LF antibodies in AVP recipients, LF antibodies were purified from AVP serum samples by affinity purification, and their LT neutralization capacity determined. Samples were first subjected to positive selection on an LF affinity column followed by negative selection on a PA affinity column. This approach resulted in pure samples of LF-specific antibodies without contaminating PA antibodies (Fig. 3A). Purified LF antibodies from two of the four samples with LF IgG titers of at least 1:320 neutralized LT in a dose-dependent manner (Fig 3B and C). The remaining two samples with LF IgG titers of only 1:40 failed to neutralize LT, possibly as a result of low antibody concentration. These data directly show that human LF IgG raised by AVP vaccination neutralizes LT.

Purified LF antibodies primarily bind LF Domain 1

The purified human LT-neutralizing LF antibodies were examined to determine whether their neutralizing specificity could be assigned to a particular domain. Although the starting whole serum samples of all four subjects exhibited IgG binding to multiple LF domains (Fig. 4A, B), the purified LF antibodies with neutralizing activity from AVP subjects 1 and 4 showed specificity for only the PA-binding Domain 1 of LF (Fig. 4C). Thus, although the affinity purification strategy resulted in unintended selective loss of LF antibodies specific for Domains 2a/3, 2b and 4, the results nevertheless show that purified LF antibodies with LT neutralization activity bind to LF Domain 1.

Discussion

Currently licensed anthrax vaccines suffer from the necessity for multiple initial injections and yearly boosters [13, 28]. Moreover, while 95% of AVA recipients make an anti-PA IgG response, the quality of this response is highly variable. For example, 7.5% of AVA recipients with anti-PA IgG titers 1:1000 had LT neutralization activity that was indistinguishable from unvaccinated controls [29]. Initiatives to develop less reactogenic and more effective anthrax vaccines resulted in development of recombinant PA [17–20] and consideration of additional vaccine targets including LF [30].

Studies suggest that anthrax vaccination protects by eliciting LT-neutralizing PA antibodies [16]. Although no direct human correlate of protection exists, studies in *Rhesus macaques* showed that LT neutralization ED₅₀ values of 338 and 367 were associated with 80 and 88% probability of survival, respectively, in animals that had received two intramuscular injections [31, 32]. While PA antibodies are viewed as the primary source of protection, several animal studies have shown that inclusion of LF and/or EF in a PA-based vaccine results in increased levels of protection [21, 22, 33]. Turnbull demonstrated that more guinea pigs immunized with AVP were protected from challenge with the fully virulent Ames strain

compared to guinea pigs immunized with AVA [34]. However, no studies in humans have established whether LF or EF contribute to LT neutralization or protection following vaccination.

The quantifiable presence of LF in the AVP formulation [12] and evidence of its relative paucity in AVA [23, 34] led us to compare humoral responses of the two vaccines in order to determine whether LF antibodies contribute to LT neutralization in AVP vaccinees. As expected, LF IgG (titer ≥ 100) was detected primarily in AVP recipients and rarely in AVA vaccinees, with response rates of 95% and 1%, respectively. This is the largest AVP cohort for which an LF IgG response rate based on ELISA titers has been published, although a study of 15 month post-vaccination samples from 41 Swedish volunteers who received the AVP formulation according to an atypical schedule of three doses at three week intervals showed a wide range of responses [35]. The LF response rate to AVA is in general agreement with our prior larger study of a real world military AVA cohort demonstrating an LF IgG response rate of 6.9% (n=1000) [23].

While many studies have examined human and animal responses to AVP and AVA separately [23, 29, 35–37], few studies have compared PA and LF antibody responses in human recipients of AVP and AVA [34, 38, 39]. These prior studies evaluated small numbers of vaccinees (ranging from 4–6 individuals per group [38] to 11–14 individuals per group [39]), did not match the groups for vaccination history, and did not establish whether LF contributes to LT neutralization in human vaccination. The size and design of our study permitted the use of multiple linear regression to investigate the contribution of the LF IgG response to LT neutralization in AVP recipients. Initial regression modeling revealed no statistical interaction between PA IgG concentration and LF IgG titer, indicating that contributions of these two factors to LT neutralization are additive rather than synergistic. This is in contrast to apparent synergistic activity between passively transferred PA and LF-specific monoclonal antibodies for protecting rats from LT challenge [40] or rabbits from *B. anthracis* challenge [41]. Importantly, the final multiple regression model indicated that LF IgG titer predicts *in vitro* LT neutralization significantly and independently of PA IgG concentration in AVP recipients. Equivalent PA responses in both vaccine groups support the concept that enhanced LT neutralization in the AVP group is attributable to the AVP-induced LF response; however, the contribution of adjuvant or other formulation differences to this result cannot be excluded.

The present study uncovered gender effects in response to both AVP and AVA. Male AVA vaccinees showed positive association between PA IgG concentration and LT neutralization and had higher ED₅₀ values. In our study evaluating 1,465 AVA vaccinees [42], we observed that male AVA recipients had higher PA IgG concentrations than females, consistent with results herein. In contrast, PA IgG concentration positively associated with LT neutralization ED₅₀ values in both male and female AVP recipients. Gender also affected LF IgG titers in AVP vaccinees; LF IgG titer positively associated with ED₅₀ selectively in females, regardless of similar ED₅₀ values in both AVP gender groups. A larger study will be required to fully reveal effects of gender on the humoral immune response to AVP.

Finally, we showed that purified LF antibodies with final LF titers 1:320 from two independent AVP vaccinees neutralized LT in a dose-dependent manner. Domain-specific ELISA assays demonstrated that these LT neutralizing antibodies bound to LF Domain 1. Along with previous work in which immunization with domain 1 of LF was able to protect mice from a lethal spore challenge [43], this finding supports the hypothesis that the inclusion of LF domain 1 in a PA-based anthrax vaccine, could increase the protective response [43]. The possibility that LT-neutralizing LF antibodies elicited by AVP are also directed to other LF domains is not excluded, however, since the LF affinity column used herein preferentially purified Domain 1-specific LF antibodies.

We conclude that while PA-specific antibodies are the primary contributor to LT neutralization following vaccination with AVP, LF-specific antibodies also contribute to LT neutralization in an independent and additive manner. Inclusion of an LF component in recombinant PA vaccine formulations can be expected to increase the breadth and magnitude of the LT neutralization response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- AVP and matched AVA recipients make similar IgG responses to PA
- AVP recipients have higher ED₅₀ LT neutralization responses
- LF IgG makes additive contribution to LT neutralization in AVP recipients
- LF antibodies affinity purified from AVP recipients bind Domain 1 and neutralize LT
- Gender influences humoral immunity to AVP and AVA

Fig. 1. AVP vaccinees produce robust IgG responses to Lethal Factor and have higher levels of Lethal Toxin neutralization compared to matched AVA vaccinees

A) End-point titers of serum IgG to recombinant Lethal Factor (LF) from AVP (n=39) compared to matched AVA (n=78) vaccinees. Two AVA data points are below the axis and not shown. **B)** Levels of serum IgG to recombinant Protective Antigen (PA) in the same samples shown in A) and C). **C)** 50% effective dilution (ED₅₀) values for serum neutralization of Lethal Toxin determined in a J774A.1 macrophage-based Lethal Toxin neutralization assay. Samples are the same as those shown in A) and B). Red lines show mean ± SEM for all panels. p-values determined by unmatched t-tests.

Fig. 2. Correlation of Lethal Factor (LF) and Protective Antigen (PA) IgG levels with Lethal Toxin (LT) neutralization values in AVA and AVP vaccines

A) Linear regression showing correlation between serum LF titer and LT neutralization

ED₅₀ values in AVP but not matched AVA samples. **B)** Linear regression showing correlation between serum PA IgG levels and LT neutralization ED₅₀ values in both AVP and matched AVA sample groups.

Fig. 3. Purified Lethal Factor (LF) antibodies from AVP serum samples neutralize Lethal Toxin (LT)

A) End-point IgG titers of reactivity to Protective Antigen (PA) or LF in samples of purified LF antibodies from four independent AVP vaccinees before (pre) and after (post) purification. Post-purification samples were brought to the starting pre-purification volumes. **B)** Example of results showing the capacity of affinity purified LF antibodies from AVP Sample 4 to neutralize LT-mediated killing of the J774a macrophage cell line (blue bars). A neutralizing PA- specific human monoclonal antibody is included as a positive control (gray bars). Black and red bars are from the control conditions indicated. **C)** Post-purification LT ED₅₀, LF titer and PA titer of all four affinity purified LF antibody samples from four

independent AVP vaccinees. Two samples with LF titers of at least 1:320 neutralized LT *in vitro*.

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Fig. 4. Purified Lethal Factor (LF) antibodies with Lethal Toxin (LT)-neutralizing activity bind to LF domain 4

A) LF domain preparations separated by SDS polyacrylamide gel electrophoresis and stained with Coomassie blue. **B)** Average binding of serum IgG from 39 AVP and 39 unvaccinated individuals to each LF domain by ELISA. Error bars are SEM. **C)** Reactivity to the LF domains before and after LF purification for AVP samples 1 and 4 by ELISA. Optical density thresholds for positivity to each domain as determined by the mean + 2 SD above the mean of values from 54 unvaccinated controls for LF1, LF2a/3, LF 2b and LF4 were 0.228, 0.150, 0.534 and 0.299, respectively.

Table 1

Vaccination history of AVP and AVA recipients

	AVP (n = 39)	Matched AVA (n = 78)
Age^a:		
Average (SD)	33.2 (12.1)	30.6 (8.9)
IQR	24–42.5	23–37
Range	19–64	19–61
Number of Vaccinations:		
Average (SEM)	5.4 (0.39)	5.4 (0.28)
Median	4	4
Range	2–11	2–11
Years post-Vaccination:		
Average (SEM)	0.097 (0.031)	0.151 (0.021)
Median	0.04	0.10
Range	0.02–1.0	0.01–0.98

^a Age was known for 26 of 39 AVP samples and was matched 1:2 in 52 AVA samples. Race was known for only 10 of 39 AVP samples and thus was not considered as a covariate.

Table 2

Multiple linear regression of LT neutralization

	R²	Coefficient (CI)^a	p value
AVP			
Log ₁₀ (PA IgG µg/mL)	0.612	0.805 (0.588, 1.021)	<0.0001
Log ₁₀ (LF Titer)	0.188	0.251 (0.075, 0.427)	0.007
Matched AVA			
Log ₁₀ (PA IgG µg/mL)	0.586	0.761 (0.614, 0.908)	<0.0001
Log ₁₀ (LF Titer)	0.011	0.121 (−0.139, 0.381)	0.356

^aCI=confidence interval