Anthrax Vaccine Precipitated Induces Edema Toxin-Neutralizing, Edema Factor-Specific Antibodies in Human Recipients

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ABSTRACT Edema toxin (ET), composed of edema factor (EF) and protective antigen (PA), is a virulence factor of *Bacillus anthracis* that alters host immune cell function and contributes to anthrax disease. Anthrax vaccine precipitated (AVP) contains low but detectable levels of EF and can elicit EF-specific antibodies in human recipients of AVP. Active and passive vaccination of mice with EF can contribute to protection from challenge with *Bacillus anthracis* spores or ET. This study compared humoral responses to ET in recipients of AVP (*n* = 33) versus anthrax vaccine adsorbed (AVA; *n* = 66), matched for number of vaccinations and time postvaccination, and further determined whether EF antibodies elicited by AVP contribute to ET neutralization. AVP induced higher incidence (77.8%) and titer (229.8 *H*11006 58.6) of EF antibodies than AVA (4.2% and 7.8 *H*11006 8.3, respectively), reflecting the reported low but detectable presence of EF in AVP. In contrast, PA IgG levels and ET neutralization measured using a luciferase-based cyclic AMP reporter assay were robust and did not differ between the two vaccine groups. Multiple regression analysis failed to detect an independent contribution of EF antibodies to ET neutralization in AVP recipients; however, EF antibodies purified from AVP sera neutralized ET. Serum samples from at least half of EF IgG-positive AVP recipients bound to nine decapeptides located in EF domains II and III. Although PA antibodies are primarily responsible for ET neutralization in recipients of AVP, increased amounts of an EF component should be investigated for the capacity to enhance next-generation, PA-based vaccines.

KEYWORDS *Bacillus anthracis*, antibody, edema toxin, vaccine

*Bacillus anthracis* is a Gram-positive, spore-forming bacterium that is the causative agent of anthrax infection. While anthrax is a disease of antiquity (1), it has been in the spotlight in recent years due to its use as a weapon of bioterrorism (2). *B. anthracis* has two major virulence factors, a poly-D-glutamic acid capsule and a secreted tripartite toxin (3). The capsule has an antiphagocytic role, allowing *B. anthracis* to evade engulfment by macrophages (4). The toxin is made up of three components, protective antigen (PA), lethal factor (LF), and edema factor (EF) (5). These proteins combine to form 2 different AB toxins, lethal toxin (LT), which is a combination of the active component LF with the binding component PA, and edema toxin (ET), having EF as its active component with PA as its binding component (5, 6). LT is a zinc-dependent...
metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPKKs) (7) and exerts a multitude of effects on the immune system during *B. anthracis* infection (8, 9).

ET is a calmodulin-dependent adenylate cyclase that converts ATP into cyclic AMP (cAMP) (10). ET also exerts several effects on the immune system during *B. anthracis* infection, including inhibition of macrophage chemotaxis (11), rescue of macrophages from TLR4-induced apoptosis (12), inhibition of neutrophil priming and motility (13–15), impairment of dendritic cell (DC) cytokine secretion, enhancement of DC maturation and chemotaxis (16–18), and suppression of T cell activation and chemotaxis, as well as skewing CD4+ T cell differentiation to the Th2 subset (11, 19–21). Interestingly, EF has an adjuvant effect when administered intranasally with PA (22). While the exact mechanism of this adjuvant effect is unknown, it requires preservation of EF’s adenylate cyclase activity as well as delivery of EF to the cytosol (23) and is associated with DC maturation (18, 23).

Two human anthrax vaccines are currently given in the western world, anthrax vaccine adsorbed (AVA) and anthrax vaccine precipitated (AVP) (24–26). AVA is the only anthrax vaccine currently approved for use in the United States, while AVP is licensed in the United Kingdom. The efficacy of both vaccines depends upon elicitation of an antibody response to the secreted anthrax toxin; however, there are several key differences between the formulations. AVA is composed of a cell-free filtrate of the acapsular, toxigenic *B. anthracis* strain V770-NP1-R that is adsorbed onto aluminum hydroxide, and it contains an unquantified amount of PA and only trace amounts of LF and EF (25, 27). Vaccination consists of five intramuscular injections at 0, 1, 6, 12, and 18 months, followed by annual boosters (25). The AVP vaccine is composed of a cell-free filtrate of the acapsular, toxigenic *B. anthracis* Sterne 34F2 strain that is precipitated with aluminum potassium sulfate (Alum) (26). Unlike the AVA vaccine, AVP contains all three toxin components, with roughly 7.9 μg/ml PA, 1.9 μg/ml LF, and detectable amounts of EF (26). Vaccination with AVP consists of 4 intramuscular doses, administered at 0, 3, 6, and 32 weeks, with annual boosters (28).

While both vaccines elicit PA-specific antibody responses that mediate protection, we recently observed that the presence of LF in the AVP formulation elicits LF-specific antibodies that additively contribute to LT neutralization (29). However, the potential contribution of EF antibodies to ET neutralization in AVP recipients remains unclear. Several animal studies have shown that EF-specific antibodies can neutralize ET and protect animals from ET challenge (30–32) as well as contribute to protection from spore challenge (33–36). Turnbull et al. showed that AVP recipients make antibodies to all three components of the tripartite anthrax toxin, although EF antibodies appeared later and were of lower titer than responses to PA and LF (37). A recent study confirmed the production of EF antibodies by recipients of AVP (38). However, these studies evaluated few subjects, and an EF seroconversion rate in response to AVP was not established. In a phase 4 clinical study evaluating the impact of annual AVP boosters, all 120 enrolled individuals had measurable EF responses (Defense Science and Technology Laboratory and PHE, unpublished data). To our knowledge, no study has determined whether EF antibodies elicited by AVP can neutralize ET.

Here, we compared humoral responses to ET in recipients of AVP and recipients of AVA matched for number of vaccinations and time postvaccination to test the hypothesis that human EF antibodies elicited by anthrax vaccination can contribute to ET neutralization. We observed that, unlike AVA, AVP elicited measurable EF antibodies in more than 75% of recipients, the antibodies bound common sequential epitopes in EF domains II and III, and the EF-specific antibodies neutralized ET. Despite these observations, the EF humoral response did not detectably contribute to ET neutralization independently of PA antibodies in AVP recipients. Moreover, ET neutralization was indistinguishable between the AVA and AVP groups, indicating that PA antibodies mediate ET neutralization as a result of AVP vaccination. We conclude that addition of an EF component in quantities exceeding that found in AVP should be investigated for the capacity to enhance next-generation PA-based vaccines.
RESULTS

Only AVP elicits EF antibodies, while both AVP and AVA elicit PA antibodies in vaccinated individuals. Serum samples from AVP recipients (n = 33), matched 1:2 with plasma samples from AVA recipients (n = 66) for number of vaccinations, time postvaccination, and age, where known (Table 1), were tested by enzyme-linked immunosorbent assay (ELISA) for antibodies directed against EF and PA. Samples from AVP recipients (26/33; 78.8%) were significantly more likely than those from matched AVA recipients (2/66; 3.1%) to contain EF IgG at an end titer of \( \geq 100 \) (\( P < 0.0001 \); odds ratio [OR], 118.9), which is to be expected, since the AVP vaccine contains EF (26, 39). In addition, the average EF IgG titer also differed between the AVP (244.3 ± 63.3) and AVA (6.6 ± 2.1) groups (\( P < 0.0001 \)) (Fig. 1A). However, both groups produced similar serum concentrations of PA-specific IgG (AVP, 459.0 ± 79.4 μg/ml; AVA, 646.7 ± 128.0 μg/ml) (\( P = 0.46 \)) (Fig. 1B).

AVA and AVP elicit similar ET-neutralizing antibody responses. ET is a calmodulin-dependent adenylate cyclase that increases the levels of intracellular cAMP in intoxicated cells. While there have been several studies examining ET neutralization with commercially available immunoassay-based cAMP detection reagents, none of these assays generated reproducible data with human serum samples in our hands, possibly due to interference from serum or plasma constituents. To measure ET neutralization reproducibly, we employed a cAMP-inducible luciferase reporter cell line (40). This assay resulted in 95.4% (103/108) and 100% (108/108) of repeated experiments having neutralization values within 12% and 26%, respectively, of the first experiment. Using this assay, we observed that samples from both AVP and AVA groups neutralized ET,

| Parameter                      | Value by recipient type | Matched AVA (n = 66) |
|--------------------------------|-------------------------|----------------------|
|                                | AVP (n = 33)            |                      |
| Age\(^a\) (yr)                 | Avg (SD)                | 31.7 (12.5)          |
|                                | Interquartile range     | 23–35                |
|                                | Range                   | 19–64                |
| No. of vaccinations            | Avg (SEM)               | 5.3 (0.4)            |
|                                | Median                  | 4                    |
|                                | Range                   | 2–10                 |
| Yr postvaccination             | Avg (SEM)               | 0.08 (0.03)          |
|                                | Median                  | 0.04                 |
|                                | Range                   | 0.02–1.00            |

\(^a\)Donor age was known for 22 of 33 AVP samples and was matched 1:2 in 44 AVA samples.

FIG 1 AVP vaccinees produce IgG responses to edema factor. (A) Endpoint titers of serum IgG to recombinant edema factor (EF) from AVP (n = 33) compared to matched AVA (n = 66) vaccinees. (B) Levels of serum IgG to recombinant protective antigen (PA) in the samples shown in panel A. Two AVA data points are below the axis and not shown. Red lines show means ± SEM for all panels. \( P \) values were determined by unpaired two-tailed Mann-Whitney U tests.
with no difference in percent neutralization capacity between the groups (AVP, 75.0 ± 4.5; AVA, 75.4 ± 3.3; P = 0.98) (Fig. 2A).

Univariate analysis of log10-transformed data revealed that PA IgG concentrations correlated with ET neutralization in both AVP (R² = 0.56; P < 0.0001) and AVA (R² = 0.37; P < 0.0001) groups (Fig. 2B). As expected, EF titer correlated with ET neutralization only in the AVP cohort (R² = 0.26; P = 0.003) (Fig. 2C). Multiple regression models were next employed to reveal whether EF IgG titer makes a contribution to ET neutralization independently of PA IgG concentration within the AVP group. The initial model detected no evidence of statistical interaction (i.e., synergy) between EF titer and PA IgG concentration (coefficient of partial determination [partial R²] = 0.009; P = 0.61; data not shown). The final model without interaction terms revealed association between ET neutralization and PA IgG concentration (partial R² = 0.39; P = 0.0002) but not EF titer (partial R² = 0.04; P = 0.26) (Table 2). This result, together with the observation of equivalent ET neutralization in EF antibody-positive AVP recipients versus the largely EF antibody-negative AVA group, indicates that the EF IgG response does not contribute additively to ET neutralization following AVP vaccination (Table 2).

Gender influences ET neutralization in AVP and AVA recipients. We next explored the effect of gender and age on ET neutralization. For this analysis, we employed log10-transformed data from a subset of 28 AVP recipients (13 male and 15 female) and their 56 matched AVA controls (49 male and 7 female) for which gender was fully known, although there were missing values for age in 6 AVP samples. Univariate linear regression showed significant correlation of EF IgG responses with ET neutralization only in female AVP recipients. In contrast, AVP recipients of both genders and AVA males showed correlation between PA IgG responses and ET neutralization (see Fig. S1 in the supplemental material). Overall, there was no significant difference in ET

### TABLE 2 Multiple linear regression assessing relative contribution of PA and EF IgG responses to ET neutralization

| Recipient type | Partial R² | Coefficient (CI) | P value |
|----------------|------------|------------------|---------|
| **AVP**        |            |                  |         |
| Log₁₀(PA IgG concentration) | 0.39       | 37.4 (19.8, 55.0) | 0.0002  |
| Log₁₀(EF titer)      | 0.04       | 6.2 (−4.9, 17.4)  | 0.26    |
| **Matched AVA** |            |                  |         |
| Log₁₀(PA IgG concentration) | 0.60       | 23.3 (18.6, 28.1) | <0.0001 |
| Log₁₀(EF titer)      | 0.03       | −5.3 (−13.3, 2.7) | 0.19    |

*Results from separate AVP and AVA models are shown.

*Partial R², coefficient of partial determination.

*CI, 95% confidence interval.
neutralization responses in AVP males (means ± standard errors of the means [SEM], 1.87 ± 0.04) versus females (1.75 ± 0.09) (P = 0.56), while male AVA recipients (1.887 ± 0.04) had slightly higher ET neutralization values than their female counterparts (1.665 ± 0.11) (P = 0.02) (Fig. S1).

Multiple regression modeling using ET neutralization as the outcome and backward model selection from a saturated model (including as variables PA IgG concentration, EF titer, gender, age, and interactions between any two; excluding 6 samples with missing age values) failed to detect any effects of age on ET neutralization responses (not shown). Therefore, age and corresponding interaction terms were dropped from subsequent models that used data from all 28 AVP and 56 AVA recipients. EF IgG responses did not show significant effects on ET neutralization in AVP recipients, but a marginal interaction between PA response and gender was detected (P = 0.07), with changes in PA IgG concentration resulting in larger changes in ET neutralization in females than males (Table S1). In AVA recipients, PA IgG concentration, EF titer, and gender all had significant effects on ET neutralization (Table S2). PA concentration and EF titer showed significant interaction, with changes in PA IgG concentration resulting in larger changes in ET neutralization as EF titer increases, although only two AVA samples (one male and one female) had EF titers of ≥100. Higher ET neutralization values in male compared to female AVA recipients were confirmed to occur after controlling for PA IgG concentration and EF IgG titer. However, no interaction between gender and PA or EF IgG responses was detected.

**EF-specific antibodies purified from sera of AVP recipients neutralize ET in the absence of PA antibodies.** Lack of EF antibody contribution to ET neutralization in AVP recipients could be due to low EF antibody titers elicited by the low EF content (relative to PA and LF) (26) of AVP or alternatively or additionally could be a consequence of nonneutralizing EF specificities. Therefore, ET neutralization capacity of purified, EF-specific antibodies was examined. EF antibodies were purified from AVP serum samples by affinity column chromatography, and their ET neutralization capacity was determined. Serum samples from four independent AVP recipients with EF titers of ≥320 were independently passed over an EF affinity column to positively select EF-specific antibodies, followed by passage over a PA affinity column to negatively select contaminating PA antibodies. This method resulted in pure samples of EF antibodies lacking detectable PA reactivity (Fig. 3A). The cAMP reporter-based ET neutralization assay revealed that purified EF antibodies from three of the four AVP recipients neutralized ET activity in a dose-dependent fashion (Fig. 3B). Interestingly, however, titers of EF reactivity did not correspond to ET neutralization titers in these ET-neutralizing samples, as the sample with the highest EF titer (1,280) demonstrated the lowest positive ET neutralization titer (20) (Fig. 3C). Moreover, the non-ET-neutralizing sample had an EF titer equivalent to that of a neutralizing EF sample (Fig. 3C). Together, these data directly demonstrate that human EF antibodies elicited by AVP vaccination can neutralize ET and further suggest that some AVP-induced EF antibodies are nonneutralizing.

**Nine common sequential epitopes of EF are bound by antibodies from AVP recipients.** To determine the antibody specificity of EF antibody-positive serum samples from AVP recipients to sequential epitopes of EF, solid-phase humoral epitope mapping was performed. The samples were analyzed using a modified ELISA to detect IgG binding to EF decapeptides, overlapping by eight amino acids (aa), spanning the entire EF protein. Nine decapeptides were bound by over 50% of the EF IgG-positive samples that were assayed (Fig. 4A and B). Of the nine decapeptides recognized, four were located in the catalytic core of EF (domain II), one was in the linker region that connects the catalytic core to the helical domain, and the remaining four were located in the C-terminal helical domain (domain III) (41). No common sequential epitopes localized to the N-terminal domain (domain I).

The lack of correlation between ET neutralization by purified EF antibodies and EF antibody titer in whole serum samples suggested heterogeneity in the fine specificity of the response, with some samples likely recognizing predominantly nonneutralizing
epitopes. To investigate this, EF decapeptide binding by whole serum samples from three AVP subjects with neutralizing EF antibodies (AVP 1, 2, and 4; Fig. 3C) was compared to that of the AVP recipient containing EF antibodies with no or very low neutralizing activity (AVP 3; Fig. 3C). Whole-serum IgG from AVP recipients with high-neutralizing EF antibodies (AVP 1 and AVP 2) demonstrated very similar peptide binding patterns (Fig. 5), sharing positive binding to 45 decapeptides, 38 of which were not bound by the AVP 3 serum sample, which contained EF antibodies with no or very low neutralizing activity (Table S3). Six of these decapeptides corresponded to common epitopes 1, 2, 4, 5, 6, and 7, shown in Fig. 4 (Table S3). Only two decapeptides were bound by all three AVP samples with ET-neutralizing EF antibodies but not by the AVP sample with EF antibodies with no or very low neutralizing activity, EF aa 613 to 622.

**FIG 3** Purified edema factor antibodies from AVP serum samples neutralize edema toxin. (A) Endpoint IgG titers of reactivity to protective antigen (PA) or edema factor (EF) in samples of EF antibodies purified from four independent AVP vaccinees before (Pre) and after (Post) purification. Postpurification samples were returned to the starting prepurification volumes. (B) Example of results showing the capacity of affinity-purified EF antibodies from AVP sample 4 to neutralize EF-induced cyclic AMP production in a mouse macrophage cyclic AMP reporter cell line. A neutralizing PA-specific human monoclonal antibody is included as a positive control (gray bars). Forskolin was used at a concentration of 50 μM. RLU, relative light units. (C) Postpurification ET endpoint neutralization titer for capacity to inhibit 20% of ET-induced cAMP production, EF titer, and PA titer of all four affinity-purified EF antibody samples from four independent AVP vaccinees. Three samples with EF titers of at least 1:20 neutralized ET in vitro.
and neither of these was part of a common epitope. Serum from the AVP 3 recipient with EF antibodies containing no or very low neutralizing activity bound to multiple decapeptides within the region corresponding to EF amino acids 249 to 354 (decapeptides 125 to 173), while sera from AVP recipients with neutralizing EF antibodies bound almost no decapeptides within this region (Fig. 5 and Table S3), suggesting that these are nonneutralizing EF epitopes. No common EF epitopes localized to this region.

Serum from AVP 3 with EF antibodies with no or very low neutralizing activity bound to EF common epitopes 8 and 9, shown in Fig. 4, suggesting that these are nonneutralizing epitopes. In summary, these data indicate that the humoral response to EF among AVP recipients is heterogeneous and identify EF epitopes that associate with ET-neutralizing EF antibody responses.

**DISCUSSION**

The U.S. postal attacks in 2001 brought anthrax back into the spotlight as a potential agent of bioterror. The current measures for dealing with anthrax infection are antibiotic treatment and passive immunotherapy once infection occurs, along with vaccination as a preventive measure. However, work from our group has previously shown that the toxin-neutralizing quality of PA antibodies from AVA recipients is highly variable (42). This has led to research seeking to develop more effective anthrax vaccines, including recombinant PA (43–46), alternative adjuvants (47, 48), and additional vaccine targets (49–51).

The current anthrax vaccines are believed to provide protection by eliciting anthrax toxin-neutralizing PA antibodies (52). While PA-specific antibodies appear to be the primary source of protection, many animal studies have shown that inclusion of additional antigens, including EF, into PA-based vaccines results in increased levels of
In the present study, we sought to determine the contribution of human EF-specific antibodies to ET neutralization. We sought to study these antibodies by examining serum samples from recipients of the AVP vaccine, which contains detectable EF (26). EF-specific IgG at titers of $\geq 100$ was observed in 79% of the AVP recipient samples compared to 4% in the AVA group; however, there was no statistical difference in ET neutralization between the two groups. There were two possible explanations for this result. On the one hand, EF antibodies generated from AVP vaccination might not be neutralizing; on the other hand, the very low quantity of EF in the AVP formulation may simply lead to EF-neutralizing antibodies that are too low in titer to make a measurable contribution to ET neutralization in the presence of a much higher concentration of PA antibodies. Indeed, we observed that PA titers were approximately 20-fold higher than EF titers in AVP recipients (not shown).

To determine if EF antibodies alone could neutralize ET, EF-specific antibodies were purified from the sera of four AVP recipients using EF and PA affinity columns. The EF column was used first to positively select EF IgG from the samples; however, PA-specific

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**FIG 5** EF decapeptide binding by AVP serum samples with known EF antibody-mediated ET neutralization status. EF decapeptide binding of whole serum samples from the same subjects evaluated in Fig. 4 expressed as the number of SD above the mean from six unvaccinated controls. AVP 1, AVP 2, and AVP 4 sera contained EF antibodies with ET neutralizing activity. AVP 3 serum contained EF antibodies with no or very low ET neutralizing activity.
IgG was still detectable, which has been noted by others (54). To obtain pure samples of EF-specific IgG, the samples were then passed over a PA-depleting column that effectively eliminated the PA IgG contamination. EF-specific antibodies from three of four samples evaluated neutralized ET, supporting the concept that EF antibodies fail to detectably contribute to ET neutralization in AVP serum samples due to their low concentration as a consequence of the low quantity of EF in the AVP formulation. The actual concentration of EF in AVP is unclear but is lower than that of LF (26). In contrast to the present results, we recently showed that LF antibodies in AVP vaccinees contribute to LT neutralization (29). As LF is present in AVP at a concentration of approximately 1.9 µg/ml (26), we suggest that this should be the minimum amount of EF or its derivative that should accompany recombinant PA in any future studies investigating the capacity of EF to enhance recombinant PA vaccines.

Age had no impact on ET neutralization responses in recipients of AVP or AVA. Gender has been known to play a role in responses to both viral and bacterial vaccines, with women generally having higher antibody responses to vaccination than men (55). The marginal effect of gender on ET neutralization in response to AVP reflected interaction with PA IgG concentration, where changes in PA antibody concentration resulted in detectably larger changes in ET neutralization in females than males. In contrast to our recent report noting a selective association between LF titers and LT neutralization in female recipients of AVP (29), no interaction between gender and EF IgG response in the outcome of ET neutralization in AVP recipients was detected. In contrast, gender significantly impacted the AVA response, with ET neutralization being higher in males than females. This is consistent with prior observations showing higher LT neutralization (29) and higher PA IgG responses (56) in male AVA recipients using samples from a real-world military cohort. A large, well-controlled clinical trial evaluating different routes and schedules of AVA vaccination showed no gender effects on PA IgG concentration after subcutaneous administration and higher PA IgG concentrations in females at early but not late time points after intramuscular vaccination (57). Our gender analysis included only 7 samples from female AVA recipients and thus was not only underpowered for detecting true gender effects in response to AVA but also too small to control for potentially confounding variables, such as time postvaccination and number of vaccinations, that may explain the gender effect we observed.

Analysis of purified EF antibodies showed that ET neutralization failed to closely correlate with EF antibody titer. One possible explanation for this observation is heterogeneity in the fine specificity of the EF antibody response to AVP, wherein proportions of ET-neutralizing versus nonneutralizing EF antibody responses vary from subject to subject. Indeed, we previously observed that as many as 12% of AVA vaccinees producing robust PA antibody responses failed to neutralize LT (58 and unpublished observations). Analysis of AVP serum samples by sequential humoral epitope mapping using solid-phase ELISAs provided results supporting this concept. Evaluation of sera from 11 AVP recipients with EF titers of at least 100 revealed nine common sequential epitopes of EF, only one of which had been described previously in EF-immunized mice (32). Careful evaluation of EF decapeptide binding patterns by sera from the four AVP subjects from whom ET neutralization activity of purified EF antibodies was determined showed that serum antibodies from an AVP recipient with EF antibodies with no or very low neutralizing activity recognized multiple epitopes in a region of EF (aa 249 to 354, including parts of the N-terminal and catalytic domains) that were largely unrecognized by three other samples with ET-neutralizing EF antibodies. In addition, samples with the highest EF antibody-mediated neutralization activity harbored EF antibodies binding to six of the common epitopes, none of which were bound by antibodies from the AVP subject lacking ET-neutralizing EF antibodies. Furthermore, two EF epitopes were bound by all three neutralizing EF antibody samples but not by EF antibodies with no or very low neutralizing activity. Thus, AVP gave rise to both neutralizing and nonneutralizing EF antibodies with differing fine specificities. The source of variation in these responses in unclear. We previously
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demonstrated in genetically identical mice that substantial epitope variation in the response to immunization with PA is stochastic (59).

Curiously, none of the common sequential epitopes described here were located in the N-terminal, PA binding domain of EF, and four of the epitopes were located in the C-terminal helical domain. This is in contrast to previous work from our group evaluating EF responses in mice that showed common sequential epitopes residing preferentially in the N-terminal and catalytic domains, with no binding in the C-terminal helical domain (32). Nevertheless, IgG from AVP samples bound to a recombinant N-terminal fragment (aa 1 to 290) of EF, indicating that these samples recognize a conformational epitope(s) in this domain (data not shown). It is important to note that in our previous work we examined mice immunized with EF alone, while the AVP vaccine contains all three components. PA binding with EF could result in different structures being presented to the immune system, potentially contributing to sequential epitope differences between the two studies. Although details of physical association between EF and PA in AVP are unknown, the AVP formulation itself lacks toxin activity. Evaluation of ET neutralization activity and EF epitopes elicited by vaccination with soluble PA plus an inactive mutant of EF (53) would be informative, and we suggest in particular the EF-S414N mutant, which has reduced adenyly cyclase activity but still displays a known adjuvant effect (23). Only a small number of reports have examined the effects of EF antibodies, and even fewer have attempted to determine the specificity of the antibody response. Most of the few existing reports have characterized neutralizing mouse monoclonal antibodies (MAb) directed to EF and have demonstrated that neutralizing EF antibodies can be directed to all three EF domains (30, 35, 60). Although no prior reports evaluated the specificity of the human EF antibody response, one study isolated a neutralizing, EF-specific Fab clone from a chimpanzee library that bound to a region in the catalytic domain within aa 316 to 456 (31). ET neutralization-associated common epitopes 1, 2, and 4, described in the present study, map to this same fragment.

In summary, while PA antibodies are the main contributors to ET neutralization in AVP recipients, EF antibodies with ET neutralization activity are elicited by AVP in most recipients despite the very low EF content in the AVP preparation. We conclude that addition of an EF component in concentrations exceeding that found in AVP should be investigated for the capacity to enhance future PA-based vaccines.

MATERIALS AND METHODS

Collection of human blood samples. All individuals were enrolled with informed consent and had been immunized with licensed AVP (n = 33) or AVA (n = 66). Existing plasma samples from nonvaccinated individuals (n = 100) were used as controls to establish thresholds of positivity in enzyme-linked immunosorbent assays (ELISAs). 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 volunteers at Public Health England, United Kingdom. Sera and plasma were isolated from AVP and AVA recipients and stored at −20°C until further use.

PA and EF ELISAs. ELISAs were performed as described previously (58). Briefly, 96-well plates were coated at 4°C overnight with 1 µg/well of recombinant PA or EF (List Biologicals, Campbell, CA). Plates were blocked, followed by a 2-h incubation with serum or plasma at room temperature (RT). Samples of serum or plasma taken from the same subjects gave equivalent results and thus were used interchangeably. Plates were then incubated with anti-human IgG for 2 h at RT, followed by the addition of substrate and measurement of optical density at 410 and 490 nm (OD410/490). For EF ELISAs, the endpoint was determined as the reciprocal of the last 10-fold dilution with an OD greater than the average plus two standard deviations (SD) for 100 unvaccinated controls. For PA ELISAs using serum or plasma, PA IgG concentration was determined using a standard curve of reference serum AVR801 (Centers for Disease Control and Prevention, Atlanta, GA) containing antibodies to PA, serially diluting 2-fold at a starting concentration of 109.4 µg/ml (61). For PA and EF ELISAs of purified antibodies, the endpoint of serial 2-fold dilutions of samples concentrated back to the original serum or plasma volumes was determined as the last dilution with an OD greater than the average plus two standard deviations for 100 unvaccinated controls.

Solid-phase peptide construction and epitope mapping. Decamer peptides overlapping by 8 amino acids and spanning the entire length of the EF protein (GenBank accession number AAA79215) were covalently synthesized onto polyethylene solid-phase supports in a 96-well format as previously described (62). Peptides were incubated with serum samples at dilutions of 1:200 for 2 h at RT. After
washing, peroxidase-labeled goat anti-human IgG (KPL, Gaithersburg, MD) was added and incubated overnight at 4°C. The following day, bound antibodies were detected on washed peptides by the addition of SureBlue Reserve TMB substrate (KPL, Gaithersburg, MD). A common epitope was defined as one or more overlapping solid-phase peptides with an OD$_{450}$ greater than or equal to the average OD$_{450}$ plus two standard deviations for a group of six unvaccinated control samples for each peptide, OD greater than 0.2, and being recognized by more than 50% of all EF-positive vaccine recipient serum samples evaluated.

**ET neutralization assay.** Cyclic AMP production and ET neutralization were assessed using a RAW 264.7 CAMP response element (CRE) luciferase reporter cell line (40). Cells (100,000/well) were cultured in 96-well plates for 18 h at 37°C. The cells then were treated with final concentrations of 0.25 μg/ml PA and 0.25 μg/ml EF (equivalent to 0.03 μg of each/well) either without (ET only) or with sample (serum, MAb, or purified human EF antibodies) and incubated at 37°C for 4 h. Cells then were washed twice with phosphate-buffered saline (PBS), lysed by the addition of 50 μl passive lysis buffer (Promega, Madison, WI), and stored at ~80°C. Luciferase expression levels were quantified using a luciferase assay system (Promega, Madison, WI), followed by measurement of luminescence with an LMax II 384 luminometer (Molecular Devices, Sunnyvale, CA). Luciferase expression in untreated wells was subtracted from that for all treatment wells to account for endogenous cAMP. Neutralization percentages were calculated as ET only/ET + sample × 100.

**Purification of EF-specific IgG.** Production of recombinant EF and PA for affinity columns was performed as previously described (63). The purified proteins were bound to cyanogen bromide-pretreated Sepharose 4B medium separately by following the manufacturer's protocol (GE Healthcare, Piscataway, NJ). To obtain purified EF-specific antibodies, 1 ml of serum was passed over the EF column 3 times, eluted with 3 M NaSCN, buffer exchanged into PBS, and concentrated back to the original 1-ml volume after each passage. These antibodies then were passed over the PA column once, and the flowthrough was concentrated back to 1 ml.

**Statistical analyses.** The 33 AVP-vaccinated individuals were matched to 66 AVA-vaccinated individuals by time postvaccination, number of vaccinations, and age, where known (2 AVA to 1 AVP). For assessment of effects of gender and age, a subgroup of samples from 28 AVP and 56 matched AVA recipients, for which gender was fully known and age data were missing for 6 AVP recipients, was analyzed. Between-group comparisons were assessed by unpaired 2-tailed Mann-Whitney U test. In all comparisons, means ± SEM are reported. Between-group proportions were compared by Fisher's exact test, and associations were reported as odds ratios (OR). Univariate correlations were analyzed by linear regression. Multiple linear regression was used to assess the relative contribution of PA and EF antibodies to ET neutralization and to evaluate the effects of age and gender on ET neutralization. For the latter purpose, appropriate models were selected from saturated models by backward model selection using Bayesian information criterion. Coefficients of partial determination (partial $R^2$), which measure the contribution of one explanatory variable when all others are already included in the selected model, are reported. Multicollinearity was assessed by variance inflation factor. Multiple regression analysis was performed in R 3.2.3. All other statistical analyses were performed using GraphPad Prism 6.0.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/CVI.00165-17.

**SUPPLEMENTAL FILE 1,** PDF file, 0.1 MB.

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