Mouse monoclonal antibodies to anthrax edema factor protect against infection

Running Title: Anti-EF antibodies protect against anthrax infection

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Abstract

*Bacillus anthracis* is the causative agent of anthrax, and the tripartite anthrax toxin is an essential element of its pathogenesis. Edema factor (EF), a potent adenylyl cyclase, is one of the toxin components. In this work, anti-EF monoclonal antibodies (MAb) were produced following immunization of mice, and four of the antibodies were fully characterized. MAb 3F2 has an affinity of 388 pM, was most effective for EF detection, and appears to be the first antibody reported to neutralize EF by binding to the catalytic Cβ domain. MAb 7F10 shows potent neutralization of edema toxin activity *in vitro* and *in vivo*; it targets the N-terminal protective antigen-binding domain. The four MAb react with three different domains of edema factor, and all were able to detect purified edema factor by western blot analysis. None of the four MAb cross-reacted with the lethal factor toxin component. Three of the four MAb protected mice in both a systemic edema toxin challenge model and a subcutaneous spore challenge edema model. A combination of three of the MAb significantly delayed the time to death in a third mouse model involving spore infection in the neck. This appears to be the first direct evidence that monoclonal antibody-mediated neutralization of EF alone is sufficient to delay anthrax disease progression.

Introduction

*Bacillus anthracis* is the bacterium that is the causative agent of anthrax, the zoonotic disease and bioterrorism threat. The virulence of this gram positive bacterium is mediated through its poly-D-glutamic acid capsule (11) and its tripartite toxin, composed of protective antigen (PA), lethal factor (LF), and edema factor (EF) (21,35). PA is the intermediary that
binds mammalian receptors capillary morphogenesis gene 2 (CMG2) and tumor endothelial marker 8 (TEM8) and conducts LF and EF, the effector proteins, into the host cell cytosol. LF is a zinc metalloprotease (15) that cleaves mitogen-activated protein kinase kinases (MEKs) (7), causing dysregulation of signal transduction, and EF is a calmodulin-dependent adenylyl cyclase that depletes cellular ATP while creating cAMP, a cellular second messenger (16). Edema toxin (ET), which is the combination of PA and EF, induces pathogenic effects in mice, causing lesions and death (10). PA is the dominant antigen for immunization, and as such, vaccination and therapeutic efforts have focused on it; however, lethal factor and edema factor should still be targeted as they are important effectors during anthrax infection.

The structure and function of EF have been elucidated through numerous studies using X-ray crystallography, NMR spectroscopy, surface plasmon resonance, enzyme kinetics, and fluorescence resonance energy transfer (FRET) (5,6,27,28,32). Three functional domains have been recognized (Fig. 1). The N-terminal 257 amino acids constitute the PA-binding domain, which is spatially separated from the remainder of EF. The next 332 amino acids comprise the catalytic domain, which can be further divided into the catalytic A and B domains (CA and CB) (31). The active site for adenylyl cyclase activity lies at the interface of the CA and CB domains (5). The C-terminal 178 amino acids of EF are referred to as the helical domain. Calmodulin binding takes place at the interface of the CA and helical domains, causing the interface of the CA and CB domains to reorient themselves into the active conformation (5).

A large fraction of the countermeasures to anthrax toxin that are currently in development are antibodies, and the majority of these target the receptor binding domain of PA (domain IV) (3), thereby blocking binding of PA to cellular receptors (35). It is prudent, from a biodefense perspective, to build redundancy into any countermeasures targeting these toxins in order to
prevent loss of therapeutic effect due to natural variation or deliberate manipulation of PA. As such, efforts have been undertaken to develop antibodies targeted toward LF and EF.

Previous efforts to raise antibodies to EF have met with varied levels of success. Little et al. produced a number of immunoglobulin G (IgG) antibodies of moderate affinity to EF, one of which (9F5) was able to inhibit binding of EF to PA and prevent physiological effects of EF on Chinese Hamster Ovary (CHO) cells (18). Winterroth and colleagues described six antibodies of moderate affinity, including one IgM (KD 17 nM) that could neutralize EF activity in CHO cells (34). This antibody had no significant protective effect against a Sterne strain infection in a mouse model, but extended the mean time to death when combined with a sub-protective dose of anti-PA antibody. Chen and colleagues developed chimpanzee antibodies that included one having very high affinity (50-120 pM) that competes with calmodulin for binding to the helical domain of EF (4). This antibody was very effective at preventing ET-mediated edema in a mouse footpad model, and provided significant protection in a systemic toxin challenge.

The studies presented here had three goals. We sought to isolate antibodies (1) having the ability to prevent EF-mediated edema and systemic infection, (2) that could potentially serve as reagents in future diagnostics, and (3) that would be useful reagents for further molecular studies of EF action. We present a panel of antibodies of nanomolar and subnanomolar affinity that bind to epitopes on three different domains of EF. Some of these antibodies neutralize EF activity in vitro and in vivo and have significant protective effects at sub-stoichiometric ratios to toxin. Additionally, these antibodies are useful as laboratory reagents and could be utilized after further development as a platform for diagnostic assays.
Materials and Methods

Proteins

PA was prepared from *B. anthracis* as previously described (33). EF was isolated from an *Escherichia coli* expression system and was purified as previously described (29). The chimpanzee anti-EF IgG EF13D was produced as previously described (4).

Calmodulin was expressed in *E. coli* as previously described. Briefly, BL21 (DE3) Gold cells harboring the plasmids pProEx-modified-rCaM and pUBS520 (the kind gift of Wei-Jin Tang, University of Chicago) (6), were grown in autoinducing medium ZYM-5052 (30) for 24 h at 30 °C while shaking. Centrifugation, cell lysis, and purification using phenyl-Sepharose was accomplished as detailed by Maune and colleagues (20).

EF truncation mutant plasmids were described previously (4). These plasmids use a pET31b backbone and harbor the various EF truncations shown in Fig. 1, with a C-terminal His6 tag (accession numbers JN540812-15). Plasmids were transformed into *E. coli* strain BL21 (DE3) Gold. Cells were grown overnight in terrific broth (12 g tryptone, 24 g yeast extract, and 4 ml glycerol per liter, buffered with 17 mM KH2PO4 and 72 mM K2HPO4) with 100 µg/ml ampicillin at 37 °C, then subcultured 1:10 into the same medium at 37 °C for 4 h, centrifuged, and resuspended in 1x IMAC buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 10 mM imidazole, 0.1% Tween-20). Cells were lysed by ultrasonication, cell debris was pelleted, and the supernatant was incubated with Ni-NTA agarose beads (Qiagen, Germantown, MD) on ice for 1 h with gentle shaking. After thorough washing, the proteins were eluted with 250 mM imidazole in 1x IMAC buffer. Proteins were dialyzed into HBS-T (10 mM Hepes, pH 7.4, 150 mM NaCl,
Mouse immunization, hybridoma fusion, antibody expression, purification, and subtyping

Two Balb/cJ mice that were initially 10 weeks old were immunized subcutaneously (SC) with EF (10 µg/100 µl in PBS, without adjuvant) five times, at 2-week intervals, followed by a sixth and final immunization 4 weeks after the fifth immunization. Mice were bled to assess immune response and spleens collected 3 days after the final boost. Splenocytes isolated from the EF-immunized mice were fused to SP2 cells (ATCC, Manassas, VA) by standard methods to produce hybridoma clones that were grown in 96-well plates in HAT medium (RPMI-1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 100 µg/ml penicillin/streptomycin, 10 µg/ml gentamycin, all from Invitrogen, Carlsbad, CA, further supplemented with 100 µM hypoxanthine, 0.4 µM aminopterin, and 800 µM thymidine, Sigma). Supernatants were collected after 7-14 days and stored at -80 °C until used for the initial ELISA screen. Positive clones were plated in HT medium (HAT medium lacking aminopterin, Sigma) in 24-well plates, subcloned, expanded, and frozen in 10% DMSO/90% FBS.

Scale-up of antibody expression was accomplished by culturing hybridoma cell lines in Cell Stack culture chambers with CellBind surface treatment (Corning). Hybridomas were cultured in RPMI-1640 with 10% FBS supplemented with 2 mM Glutamax (Invitrogen), 1 mM sodium pyruvate, and 10 µg/ml gentamycin. Medium was harvested, sterile filtered, and stored at 4 °C until purification. Purification was carried out using a Protein A HiTrap MabSelect SuRe column (GE Healthcare, Piscataway, NJ) under high salt conditions on an ÄKTA FPLC (GE Healthcare) as detailed by Harlow and Lane (12). The fractions containing antibody were pooled.
and dialyzed against a buffer of 10 mM Hepes, 300 mM NaCl, and 0.5 mM EDTA.

Concentration was assessed by measuring the $A_{280}$ on an Ultrospec 4000 UV/Visible spectrophotometer (GE Healthcare), where 1 mg/ml was equated to $A_{280} = 1.4$. Purity was assessed by non-reducing SDS-PAGE, and activity was confirmed by mixing of antibody with EF followed by resolution using native PAGE to observe a supershift attributed to complex formation. Antibodies were stored at -80 °C. Subtyping of antibodies was performed using the Rapid Isotyping Kit for Mouse (Thermo Scientific, Rockford IL).

**Initial ELISA screen**

Enzyme immunoassay (EIA) 96-well plates (Corning #3590, Corning, NY) were coated with EF (10 µg/ml, or 111 nM) in PBS (5.6 mM Na$_2$HPO$_4$, 1 mM KH$_2$PO$_4$, pH 7.4, 154 mM NaCl) overnight at 4 °C. After washing the plates twice with PBS, the hybridoma supernatants were mixed 1:1 with 10 mM Tris, pH 8.0, 100 mM NaCl containing either 2 mM EDTA or 0.5 mM EDTA, 2 mM CaCl$_2$, and 10 µg/ml (0.6 µM) calmodulin (alternate incubation buffers), and incubated for 1 h at room temperature. After washing five times with the incubation buffer, anti-mouse antibody conjugated to alkaline phosphatase (1:2000 dilution, Southern Biotech, Birmingham, AL) prepared in the previous buffer was added for 1 h. Plates were washed five times, and then detection was accomplished with the p-nitrophenyl phosphate liquid substrate system (Sigma). The reaction was stopped with 3 M NaOH upon saturation of signal of at least one clone and plates were read at 405 nm with a 96-well plate spectrophotometer. Previously verified mouse polyclonal antiserum to EF was used as a positive control, while the lack of primary antibody was the negative control. An absorbance of 0.1 or greater was considered a positive signal.
Surface plasmon resonance (SPR) measurements

Candidate MAb were then screened by SPR for binding to EF using a ProteOn XPR36 (Bio-Rad, Hercules, CA). EF was immobilized onto a GLC low binding capacity chip using standard amine coupling procedures. Briefly, a GLC chip was activated with 0.4 M ethyl(dimethylaminopropyl) carbodiimide (EDC)/0.1 M N-hydroxsulfosuccinimide (sulfo-NHS) at 30 µl/min flow rate for 300 s. EF was immobilized by passing a 10 µg/ml (111 nM) solution in 10 mM acetate buffer at pH 5.5 for 60 s at 25 µl/min flow rate. The surface was deactivated with an injection of 1 M ethanolamine at 30 µl/min for 300 s. This resulted in approximately 960 Resonance Units of immobilized EF. Human serum albumin (Sigma) was similarly immobilized as a negative control, and a surface that was not activated acted as an additional negative control. Undiluted supernatants from each clone were injected as the analyte at 25 µl/min for 60 s, and dissociation was monitored for 600 s. Regeneration was accomplished with a 30 s injection of 4 M MgCl₂ at 50 µl/min.

Kinetic parameters of the purified antibodies were determined using the ProteOn XPR36. Each antibody was immobilized on a GLC chip in 10 mM acetate buffer, pH 5, using standard amine coupling conditions. This resulted in approximately 961 – 1225 Resonance Units of immobilized antibody. A concentration series of EF from 75 nM to 18.75 nM was injected at 100 µl/min for 60 s, and dissociation was monitored for 10 min. Regeneration was accomplished by a 30 s injection of 100 mM HCl at 100 µl/min. Samples were run in triplicate. Data was analyzed using ProteOn Manager software with the Langmuir model.

Epitope mapping was accomplished using the same surface as for kinetic analysis. A 22 nM injection of EF at 100 µl/min for 60 s was immediately followed by a 15 µg/ml (100 nM)
IgG injection at 100 µl/min for 60 s. Because of the multiplex nature of the ProteOn, all antibodies were immobilized in their vertical lanes, and EF followed by different IgGs were injected in the horizontal direction. In this way, the entire matrix of antibody epitopes could be analyzed with respect to one another in a single experiment. Regeneration was performed as previously noted.

**Epitope mapping by EF truncation ELISA**

ELISAs were used to identify the domains of EF to which each antibody binds. EIA plates were coated with the purified anti-EF MAb at 2.1 µg/ml (14 nM) in PBS overnight at 4°C. The plates were washed with PBS-T (PBS with 0.05% Tween-20), and blocked with 2% milk (Bio-Rad) in PBS-T for 3 h. The plates were then washed with PBS-T and the EF truncation variants were allowed to incubate for 1 h at room temperature in 2% milk-PBS-T.

After washing in PBS-T, anti-Penta His HRP at a 1:1000 dilution (Qiagen) was allowed to bind for 1 h at room temperature in 2% milk-PBS-T. After washing, detection was accomplished with 3,3,5,5-tetramethylbenzidine substrate (TMB, R&D Systems, Minneapolis, MN), quenched with 1 M H₂SO₄, and absorbance was read at 405 nm.

**EF neutralization in tissue culture**

RAW264.7 macrophage cells were grown in DMEM with Glutamax (Invitrogen), supplemented with 10% FBS, 1 mM sodium pyruvate, 10 mM Hepes buffer, pH 7.3, and 10 µg/ml gentamicin. Cells were plated at 80-90% confluence 24 h prior to toxin treatment. PA (500 ng/ml, =6 nM), EF (2.25 µg/ml, =25 nM), and 500 µM 3-isobutyl 1-methylxanthine (IBMX, A.G. Scientific, San Diego, CA) combined in culture medium were incubated with various concentrations of IgG at room temperature for 30 min prior to addition to cells. Cell
culture media was aspirated from the plates, and the toxin/antibody mix was placed on cells for 1 h at 37 °C, then cells were lysed with Cisbio lysis buffer supplemented with 50 mM EDTA (to stop enzyme activity), and cAMP levels were assessed according to manufacturer’s protocol using the cAMP HiRange HTRF kit (Cisbio, Bedford, MA).

**Western blot analysis**

Purified proteins (50 ng each PA, LF, or EF) were run on Novex 4-20% Tris-Glycine gradient gels (Invitrogen) under denaturing conditions, and then transferred to nitrocellulose membranes, which were then blocked for 30 min at room temperature in 4% milk in PBS-T. Each separate membrane was then exposed to a different monoclonal antibody or the control mix of polyclonal antisera to assess specificity of the primary antibody. MAb to EF were incubated with the membranes at a concentration of 1.4 µg/ml (9.5 nM). PA, LF, and EF controls were detected with polyclonal rabbit antisera developed in our laboratory and used at 1:1000 dilutions. All antibodies were incubated with the blots in 4% milk-PBS-T overnight at 4 °C. After washing in PBS-T, secondary antibodies (goat anti-mouse IgG-IRDye700DX and goat anti-rabbit IgG-IRDye700DX, Rockland, Inc.) were applied at 1:5000 dilutions in 4% milk-PBS-T for 1 h. After washing in PBS-T, membranes were imaged with a Licor Odyssey (Lincoln, NE) using infrared fluorescence detection. SeeBlue Plus2 prestained standard was the molecular weight marker used (Invitrogen).

**EF catalytic activity capture assay**

EIA 96-well plates were coated with anti-EF antibodies at 2.1 µg/ml (14 nM) in HBS (10 mM Hapes, pH 7.4, 150 mM NaCl) overnight at 4 °C. Plates were then washed and blocked at room temperature for 3 h in 2% milk in PBS-T. A series of EF concentrations were allowed to
incubate for 1 h at room temperature in 2% milk-PBS-T. After washing in PBS-T, 100 µl of 20 mM MnCl₂, 80 µg/ml BSA (1.2 µM), 20 µg/ml calmodulin (1.2 µM), 1 mM ATP, and 10 mM Hepes, pH 7.3, was added and allowed to incubate for 1 h at 37 °C to allow EF synthesis of cAMP (reaction conditions were chosen based upon several previous studies) (14,17,27). Then 100 µl of Cisbio lysis buffer supplemented with 50 mM EDTA was added and cAMP levels were assessed using the cAMP HiRange kit (Cisbio).

Spore preparation

Spores were prepared from Ames 35, the avirulent, nonencapsulated, toxigenic B. anthracis strain (25) as previously described (13). Briefly, bacteria were grown at 37 °C overnight followed by 7 days at 28 °C on NBY sporulation agar (8 g nutrient broth, 3 g yeast extract, 15 g agar per liter water), and were then monitored by microscopy to confirm that sporulation was greater than 95%. Spore purification was accomplished through four cycles of centrifugation followed by washing in sterile water, and were then subjected to heat treatment at 70 °C for 0.5 h. Spore quantification was carried out using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA) and verified by dilution plating.

In vivo studies

Female BALB/cJ or C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were used at 8-12 weeks of age. For the footpad edema model, BALB/cJ mice (n = 6-9/group) were injected in one footpad (20 µl) with ET (0.5 µg EF + 0.5 µg PA) pre-mixed with either antibody (3.6 µg) or PBS. Footpad edema was monitored 24 h after injection by measuring footpads in the dorsal/plantar direction using digital calipers (Mitutoyo Corporation, Aurora, IL). In experiments assessing leg edema following spore infection, C57BL/6J mice (n = 4/group) were
injected intravenously (IV) with 71.4 µg of antibody prepared in 100 µl PBS. After a delay of 10 min, the mice were infected with 2 x 10^7 spores (20 µl) via SC injection in the right foreleg. The limited edema spread in this location allows consistent quantification of edema. Edema was assessed at 24 h after infection by measuring the limb in the sagittal dorsal/ventral direction using digital calipers. To test antibody efficacy against ET lethality, C57BL/6J mice (n = 5/group) received a single antibody injection (10.7-35.7 µg, IV) 1 h prior to administration of a lethal dose of ET (25 µg EF + 25 µg PA, IV) and survival was monitored for 200 h. To test antibody efficacy against spore infection, C57BL/6J mice were injected with a cocktail of all three antibodies (7F10, 3F2, and 4A6, 12.5 µg each, IV, n = 5) or with EF13D (25 µg, IV, n = 8) at 18 h and 1 h prior to administration of a lethal dose of spores (2 x 10^7, SC). Survival was compared to control mice which received PBS instead of antibody (n = 18). All mouse experiments were performed under protocols approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

**Results**

**Immunization, hybridoma screening, and selection**

Mice immunized SC with EF in the absence of adjuvant showed high ELISA titers (data not shown), and two spleens were used for hybridoma fusion. After fusion, 11 plates were seeded and individual clones were screened by ELISA for binding to EF in the absence or presence of calcium and calmodulin. Initially, screening was performed with the intent to assess differential binding of these MAb to EF in the absence or presence of bound calmodulin. However, most of the antibodies that were shown to bind to EF in this screen did so in both conditions. Therefore, further characterization of this kind was not conducted. The 25 clones
that exhibited the best binding by ELISA were then selected for subcloning and further
screening. Antibodies from a total of 82 subclones were then assessed for EF binding by SPR.
Of these, four antibodies were selected for further characterization because they had the lowest
apparent off-rates, which is usually the best indicator of high affinity.

Scale-up, purification, subtyping, and initial characterization

Scale-up and purification of the four selected clones yielded approximately 7-11 mg of
purified antibody from each. Native gel electrophoresis showed that all antibodies ran as single
bands, and native gel electrophoresis of antibody mixed with EF revealed a supershift indicative
of an EF/antibody complex (data not shown). Subtyping of each purified antibody revealed that
all were of the IgG1 subclass.

IgG Kinetics

Binding kinetics for each IgG/EF interaction were assessed by SPR using a ProteOn
XPR36. For this measurement, each IgG was immobilized on the surface so that the monovalent
EF/IgG interaction could be monitored without avidity effects. The association (kₐ) and
dissociation rates (k₅), and equilibrium dissociation constants (Kᵤ) obtained are given in Table 1.
Affinities ranged from 5.3 nM to 388 pM, with on-rate and off-rate varying greatly among these
IgGs. Clone 3F2 possessed the slowest off-rate (7.5 x 10⁻⁵ s⁻¹) while 7F10 had the fastest on-rate
(4.88 x 10⁵ M⁻¹s⁻¹).

Epitope mapping

Because the individual IgGs were immobilized and EF is monomeric in solution, epitope
mapping by SPR was straightforward, as described in Materials and Methods. This experiment
revealed that all antibodies appeared to bind to independent epitopes, and the binding of one IgG to EF did not appear to hinder binding of the subsequent IgG.

To further define the binding sites of this panel of IgGs, ELISAs were carried out using truncated variants of EF. These experiments, which were reproducible at a wide range of EF dilutions, revealed that this panel of antibodies targeted several different domains of EF (Fig. 1). Clones 4A6 and 7F10 bind the N-terminal PA binding domain of EF. Clone 3F2 binds to the catalytic domain C_b, while clone 1D4 appears to target the C-terminal helical domain of EF.

**Western blot analysis and EF capture**

The abilities of the MAb to detect EF on Western blots were assessed. Under the conditions used, all antibodies detected EF bound on a membrane, and they did so without cross-reacting with LF or PA (Fig. 2). Further studies by SPR and in RAW264.7 cells treated with lethal toxin (LT, a combination of LF and PA) showed that the anti-EF antibodies had no reactivity with PA or LF at the concentrations used in these studies (data not shown).

To assess the potential utility of these MAb in diagnostic assays, an EF capture assay was constructed. Each antibody was coated onto EIA plates, and different concentrations of purified EF were incubated in each well. Detection of the captured EF was based on the catalytic activity of EF; cAMP levels generated by incubation in a reaction mixture with calmodulin, Mn^{2+}, and ATP were measured using a FRET-based time-resolved fluorescence assay (Fig. 3). In this initial, un-optimized assay, the limit of detection (LOD, signal at 3 standard deviations above the mean of the negative control) for 3F2 was 8 pM; 1D4 and 7F10 had LODs of 25 pM, and 4A6 had an LOD of 250 pM.

**RAW264.7 cell neutralization and in vivo studies**
The abilities of these MAb to protect against EF activity were assessed in RAW264.7 cells. ET was pre-incubated with different concentrations of the MAb for 30 min at room temperature prior to exposure to cells for 1 h at 37 °C. Cells were then lysed in the presence of EDTA to inactivate EF, and cAMP levels were assessed as described in Materials and Methods (Fig. 4). Clone 7F10 exhibited excellent neutralization activity, with an IC$_{50}$ of approximately 10 nM. Clones 3F2 and 4A6 both exhibited partial neutralization of adenylyl cyclase activity with IC$_{50}$ values similar to that of 7F10; IC$_{50}$ values appear to be approximately 10 and 20 nM for 3F2 and 4A6, respectively. The reason for partial neutralization is unclear. Clone 1D4 exhibited marginal neutralizing activity at the concentrations tested.

To further characterize the protective effects of these antibodies, studies were performed in mice. The first model used to characterize the MAb was an edema footpad model in which toxin was pre-mixed with antibodies prior to administration to mice. Mice given only ET showed substantial edema at 24 h post-injection (a 200% increase, Fig. 5). The 7F10 MAb was quite effective in preventing edema (limiting the increase to 65%), but it was not as effective as the well-characterized PA neutralizing MAb 14B7. The other three antibodies appeared to show slight protection that was only marginally different from the no antibody group (p = 0.05)

To extend these results, a systemic model of intoxication was used (Fig. 6). A lethal dose of 25 µg ET (approximately 280 pmol EF) was given IV 1 h after administration of MAb by the same route. At doses of 35.7 µg IgG (238 pmol), MAb 3F2, 4A6, and 7F10 afforded complete protection out to 7 days, while controls succumbed in 2-3 days (Fig. 6A). Antibody 1D4 was not protective. When administered at substoichiometric doses of 10.7 µg IgG (approximately 71 pmol, Fig. 6B), 4A6 and 7F10 remained highly protective while the efficacy of 3F2 was lost.
To determine the efficacy of these anti-EF antibodies in an infection model, the MAb were administered 10 min prior to subcutaneous spore challenge in forelimbs, and localized edema was assessed at the site of injection 24 h later (Fig. 7A). MAb 1D4 was not tested in this model, due to poor neutralization performance in previous assays. Animals treated with PBS exhibited extensive edema (>200% increase) 24 h after spore injection. Animals that also received MAb 7F10, 3F2, 4A6, or 14B7 showed only 30-60% increases. Thus, the MAb 7F10, 3F2, and 4A6 all had a potent protective effect on localized edema, at roughly the same level as the anti-PA antibody 14B7. Furthermore, a combination of three of the anti-EF antibodies (7F10, 3F2, and 4A6, 12.5 µg of each) given 18 h and 1 h prior to SC infection with Ames 35 spores caused a highly significant shift in time to death relative to untreated controls. The previously described chimpanzee anti-EF antibody EF13D (4) also significantly delayed time to death (when used at 25 µg) while eliciting 50% survival (Fig. 7B). Each of the three mouse monoclonal antibodies was not protective when given alone at a dose of 25 µg (data not shown).

Median time to death for the PBS treated controls was 64 h and was significantly extended to 144 h for both EF13D and the combination of mouse monoclonal antibodies.

**Discussion**

We developed and characterized four MAb to EF, and showed that three of the four have neutralizing activity *in vitro* and *in vivo*. These antibodies can be produced at high levels from hybridomas and can be purified to high purity with minimal effort. We have characterized the different affinities and domains of EF to which each antibody binds. These antibodies are highly specific for EF, and do not cross-react with LF or PA. Furthermore, these antibodies can significantly decrease edema levels and progression of disease in several mouse models.
We found that EF in the absence of adjuvant is very immunogenic, resulting in high anti-EF titers. It has been observed that ET (EF + PA) can act as an adjuvant for other immunogens (9), although this requires cell entry via PA and adenylyl cyclase activity. Therefore, unless some EF is gaining entry into cells through a nonconventional mechanism in the absence of PA, it is unlikely that EF is functioning as an adjuvant for itself. A tractable number of antibodies were selected for characterization through the two-tiered screening approach of ELISA followed by SPR. These antibodies bound to several different domains of EF (PA binding domain, catalytic Cβ domain, and helical domain), which further illustrates the previously described diverse response of the mouse immune system to EF (22).

Another goal in producing these antibodies was to create highly specific reagents that could be used for molecular studies in future work. To that end, all antibodies were useful in a Western blot analysis, and they were specific for EF and not cross-reactive with LF or PA. This is useful from a reagent perspective, because individual IgGs or an oligoclonal cocktail of the four together could be used for specific detection of EF in a complex sample with all three toxins present. Along similar lines, several of these antibodies were amenable to EF capture, which could act as a platform for diagnostic detection of toxin. In the assay described here, levels of EF detection were strongly tied to MAb affinity, with 3F2 allowing detection of EF at a LOD of 8 pM.

Three of the four IgGs could at least partially inhibit production of cAMP by ET in RAW264.7 cells, with 7F10 having the highest efficacy. Within this panel of MAb, those IgGs directed toward the PA binding domain were most effective at protection. Those antibodies having nanomolar affinity (7F10 at 1.97 nM and 4A6 at 4.8 nM) that bind to the N-terminal PA binding domain afforded significant protection even at substoichiometric ratios. This is
consistent with the work of Little and colleagues (18) in which the only antibody with neutralizing activity on CHO cells (9F5) bound to the N-terminal 18 kDa region of EF. However, this result does not rule out the existence or development of highly effective antibodies that react with other domains; in fact, a potent antibody has already been isolated that reacts with the helical domain of EF (4). These two previous reports described neutralizing monoclonal antibodies that bind to the N-terminal PA binding domain and the C-terminal helical domain of EF. It is worth noting the novelty of 3F2, which is the first monoclonal antibody to EF with neutralizing activity whose epitope is on the catalytic C\textsubscript{a} domain. This antibody had the best affinity of the panel developed in this study (388 pM), but it was only protective at stoichiometric ratios with the toxin.

All of the antibodies isolated in this study were mouse IgG1 subtype, and it is possible that modification of these antibodies to alternative subtypes could improve their effects. This was shown to be the case by Abboud and colleagues for the anti-PA antibody 19D9, where subtype variants neutralized lethal toxin activity in an Fc\gamma receptor-dependent manner with efficacy of IgG2a > IgG2b > IgG1 (1).

In a subcutaneous spore challenge with the Ames 35 strain, MAb 7F10, 3F2, and 4A6 were all effective at preventing edema, while in a more stringent model of purified ET injection, only 7F10 had a protective effect. In light of the recent injectional anthrax outbreak in heroin users (2,23,26), where deep tissue edema was a prominent effect, it is possible that anti-EF and anti-PA antibodies might have therapeutic value.

Additionally, three anti-EF antibodies in combination were shown to delay the time of death significantly in an in vivo mouse spore challenge. This appears to be the first evidence that
anti-EF antibodies alone can affect disease progression in vivo. Perhaps this is not altogether unexpected, as previous reports have shown that loss of EF expression can increase the LD50 of spores by an order of magnitude (24), and survival of mice challenged with vegetative bacteria lacking EF can differ significantly from those infected by wild-type strains (19). A recent report using noninvasive imaging to track dissemination of wild-type and single toxin knockout strains of B. anthracis during infection showed that loss of EF expression altered the bacterial dissemination pattern (8). EF expression was shown to correlate with a rapid spreading of bacteria to the spleen (8), suggesting a more prominent role for EF in infection than previously believed. Thus, targeting of EF could lead to therapeutic effects that have not previously been appreciated. In this way, our novel finding of an anti-EF antibody and a cocktail of three monoclonal antibodies that significantly alters the course of infection further highlights the role of EF in anthrax pathogenesis. These findings also strengthen the case for pursuing complementary modalities to antibiotics and PA-directed toxin therapy, as a multi-tiered neutralization strategy should give greater assurance of protection.

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References


Table 1. Kinetic parameters of clones as determined by SPR.

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<th>Hybridoma</th>
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<tbody>
<tr>
<td>1D4</td>
<td>5.34 ± 0.81</td>
<td>0.64 ± 0.07</td>
<td>3.40 ± 0.32</td>
</tr>
<tr>
<td>3F2</td>
<td>0.39 ± 0.11</td>
<td>1.95 ± 0.23</td>
<td>0.75 ± 0.18</td>
</tr>
<tr>
<td>4A6</td>
<td>4.80 ± 2.45</td>
<td>4.43 ± 1.30</td>
<td>19.1 ± 4.73</td>
</tr>
<tr>
<td>7F10</td>
<td>1.97 ± 0.10</td>
<td>4.88 ± 0.02</td>
<td>9.59 ± 0.48</td>
</tr>
</tbody>
</table>
**Figure Legends**

**Figure 1. Domain reactivity mapping of MAb.** The domains of EF include PABD, Protective Antigen Binding Domain; CA, Catalytic A domain; CB, Catalytic B domain; HD, Helical domain. Horizontal bars show EF truncation mutants expressed in *E. coli* and tested for ELISA reactivity with MAb listed at right, giving the indicated results (+ or -). H6 indicates the 6x Histidine tag located at the N-terminus of full length EF and at the C-terminus of each EF truncation variant.

**Figure 2. Reactivity of MAb with EF, PA, and LF on Western blots.** Samples were run on 4-20% Tris-Glycine SDS gradient gels under reducing conditions, blotted, and tested for reactivity with each MAb and with a mixture of rabbit antisera specific for each protein as the positive control. MW, molecular weight marker (SeeBlue Plus2 prestained marker, Invitrogen).

**Figure 3. EF capture ELISA using MAb.** MAb were coated on EIA plates and used to capture EF from solutions of the indicated concentration. EF catalytic activity was then measured by addition of Mn$^{2+}$, calmodulin, and ATP, with detection of the resulting cAMP done using the HTRF cAMP HiRange kit from Cisbio. Limits of detection (LOD, signal at 3 standard deviations above the mean of the negative control) for MAb were: 3F2, 8 pM; 1D4 and 7F10, 25 pM; 4A6, 250 pM. Inset, same data focused on low cAMP concentrations. 1D4 (p = 0.0045), 3F2 (p = 0.0162), and 7F10 (p = 0.0104) are all statistically significant at their LOD with respect to the no antibody control. 4A6 (p = 0.0780) is not statistically significant, although it is near significance. Statistics are the result of a two-tailed, paired t-test.

**Figure 4. MAb neutralization of ET activity toward RAW264.7 cells.** MAb at the indicated concentration were mixed with 0.5 µg/ml PA + 2.25 µg/ml EF and added to RAW264.7 cells for 60 min. Cells were lysed and the intracellular cAMP concentrations measured. Single data...
points at left (at IgG = 0) are for no ET treatment (open triangles, \( \triangle \)) and for ET alone treated cells (inverted triangles, \( \bigtriangledown \)). 7F10 (p = 0.0007), 4A6 (p = 0.0003), 3F2 (p = 0.0046), and 1D4 (p = 0.0116) are all significantly different from the no antibody control. Statistics are the result of a two-tailed, paired t-test taken at approximately 350 nM IgG concentration relative to the no antibody control.

**Figure 5.** MAb neutralization of ET activity in a mouse edema footpad model. IgGs (3.6 \( \mu \)g) were mixed with 0.5 \( \mu \)g ET and injected in mouse footpads. Data are the percent change in the dorsal/plantar measurements of the footpad 24 h post-injection relative to pre-treatment. Each symbol represents one mouse. Statistics are the result of a two-tailed, paired t-test.

**Figure 6.** MAb protection of mice against ET challenge. MAb were administered to mice 1 h prior to ET challenge and survival followed. (A) 35.7 \( \mu \)g IgG was administered 1 h prior to 25 \( \mu \)g of ET. 3F2, 4A6, and 7F10 are all statistically significant (p = 0.0007) relative to ET only treatment by log rank test. (B) 10.7 \( \mu \)g IgG was administered 1 h prior to 25 \( \mu \)g of ET. 7F10 (p = 0.006), 4A6 (p = 0.003), and 3F2 (p = 0.04) all differ in a statistically significant manner from the ET-only treatment by log rank test.

**Figure 7.** MAb protection against spore challenge in two infection models. (A) MAb (71.4 \( \mu \)g) were administered IV followed by injection of spores into the foreleg and measurement of edema. Data are the percent change of sagittal dorsal/ventral measurements of the limb 24 h after spore injection. Each symbol represents one mouse. All MAb treatments produced effects that differ with statistical significance (p < 0.05) from the PBS treatment, using a two-tailed, paired t-test. (B) A mixture of MAb 7F10, 3F2, and 4A6 (12.5 \( \mu \)g of each, IV) or antibody EF13D (25 \( \mu \)g, IV) was administered 18 h and 1 h prior to SC spore challenge, and survival
followed. The triple antibody treatment group contained 5 mice, and the EF13D group contained 8 mice. Anti-EF treatment delayed the time to death in a statistically significant manner (p < 0.0001 for both antibody treatements relative to PBS treatment using a log rank test).