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Neutralizing Monoclonal Antibody to Edema Toxin and Its Effect on Murine Anthrax[▽]

Lisa Winterroth,[1]† Johanna Rivera,[1]† Antonio S. Nakouzi,[1] Ekaterina Dadachova,[1,3] and Arturo Casadevall[1,2]*

Departments of Microbiology and Immunology,[1] Medicine (Division of Infectious Diseases),[2] and Nuclear Medicine[3] of the Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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Edema factor (EF) is a component of an anthrax toxin that functions as an adenylate cyclase. Numerous monoclonal antibodies (MAbs) have been reported for the other *Bacillus anthracis* toxin components, but relatively few to EF have been studied. We report the generation of six murine hybridoma lines producing two IgM and four IgG1 MAbs to EF. Of the six MAbs, only one IgM neutralized EF, as assayed by an increase in cyclic AMP (cAMP) production by Chinese hamster ovary (CHO) cells. Analysis of the variable gene elements revealed that the single neutralizing MAb had a different binding site than the others. There was no competition between the neutralizing IgM and the nonneutralizing IgG MAbs indicative of different specificity. MAb-based capture enzyme-linked immunosorbent assay (ELISA) detected EF in liver lysates from mice infected with *B. anthracis* Sterne 34F2. Administration of the neutralizing IgM MAb to A/JCr mice lethally infected with *B. anthracis* strain Sterne had no significant effect on median time to death, but mice treated with the MAb were more likely to survive infection. Combining the neutralizing IgM to EF with a subprotective dose of a neutralizing MAb to protective antigen (PA) prolonged mean time to death of infected mice, suggesting that neutralization of EF and PA could produce synergistic beneficial effects. In summary, the results from our study and literature observations suggest that the majority of Abs to EF are nonneutralizing, but the toxin has some epitopes that can be targeted by the humoral response to generate useful Abs that may contribute to defense against anthrax.

Bacillus anthracis is a Gram-positive, spore-forming bacterium and the causative agent of anthrax (4, 24). *B. anthracis* spores are naturally found in the soil, and the disease primarily affects animals such as sheep and cattle that ingest or inhale spores while grazing. Human anthrax is rare, and most cases occur in individuals who contract the disease via contact with farm animals or spore-infested animal hides. However, in recent years, *B. anthracis* has emerged as a potent biological weapon and there has been great interest in understanding its pathogenesis and developing new therapies (12). *B. anthracis* produces two large plasmids, pXO1 and pXO2, which encode the genes necessary for toxin production and formation of a poly-D-glutamic acid capsule, respectively. A major virulence factor, the toxins are made up of three protein components known as protective antigen (PA), lethal factor (LF), and edema factor (EF). PA interacts in a binary fashion with EF to produce edema toxin (EdTx) and LF to produce lethal toxin (LeTx) (4). PA interacts with EF and LF via a heptameric structure that facilitates their entry into the cell. EdTx acts as a calcium- and calmodulin-dependent adenylate cyclase that leads to cellular edema, whereas LeTx is a zinc metalloprotease, which cleaves mitogen-activated protein kinase kinases activating various cellular pathways and ultimately leading to cell death (4, 16). In considering the various treatment options for anthrax, the delivery of preformed antibodies (Abs) has some advantages over other measures of postexposure prophylaxis, such as antimicrobial agents (5). Due to their low side effect profile, high specificity, lack of selection for antimicrobial drug resistance, and ability to bind to preformed toxins, Abs could reduce damage during infection. Several monoclonal antibodies (MAbs) to PA were isolated and determined to show protective effects against LeTx in mice (1, 18, 31). Other studies have shown that LF MAbs are also able to protect against LeTx activity in rats (17) and mice (38); however, relatively few attempts have been made to isolate neutralizing MAbs to EF. Little et al. described 10 MAbs to EF; however, only 2 had a moderate effect in disrupting ^{125}I-EF binding to cell-bound PA *in vitro*, with just one of the two inhibiting the rounding of CHO cells, a feature known to result from exposure to EdTx (19). Recently, a neutralizing MAb to EF was described that mediated protection by interfering with the toxin interaction with calmodulin (3).

Apart from their potential usefulness as therapeutic reagents, MAbs can also be used to define protective and nonprotective epitopes and for serological assays. Since there has been relatively little work done in the area of neutralizing MAbs against EF, there is an interest in trying to isolate additional MAbs to evaluate the efficacy of humoral immunity and for the design of toxin detection assays. This study describes the isolation of six MAbs against EF, one of which has potent neutralizing activity *in vitro* and modest protective abilities *in vivo*.

MATERIALS AND METHODS

***Bacillus anthracis*.** *B. anthracis* strain Sterne 34F2 (pXO1$^+$, pXO2$^-$) was obtained from Alex Hoffmaster at the Centers for Disease Control and Prevention (Atlanta, GA). *B. anthracis* Sterne 34F268 DeltaT (pXO1$^-$, pXO2$^-$) was ob-

* Corresponding author. Mailing address: Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Phone: (718) 430-3730. Fax: (718) 430-8968. E-mail: casadeva@aecom.yu.edu.
† L.W. and J.R. share first authorship.
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tained from Stephen Leppla at the NIAID (Bethesda, MD). Bacterial cultures were grown in brain heart infusion (BHI) broth (Difco, Detroit, Mich) at 37°C for 18 h with shaking. For some experiments, recombinant protective antigen (rPA) and edema factor (rEF) proteins were obtained from Northeast Biodefense Center Expression Core (K. Chave), NYS Department of Health (Albany, NY). Histidine-tagged PA and EF were expressed in *Escherichia coli* and purified by affinity chromatography (HisTrap HP) (GE LifeSciences, Piscataway, NJ). Expressed His-tagged proteins can be purified easily because of the string of histidine residues under specific buffer conditions using a HisTrap HP ready-to-use column, prepacked with precharged high-performance nickel-Sepharose. Proteins were further purified by ion-exchange (MonoQ) chromatography (GE Life Sciences, Piscataway, NJ). MonoQ ion-exchange chromatography purifies proteins based on charge-charge interactions between protein sample and the immobilized resin. Proteins were then quantitated using the colorimetric Bradford Reagent (ThermoScientific Pierce, Rockford, IL). SDS-PAGE analysis revealed more than 75% of the protein in one band at molecular masses of 83 kDa (PA) and 90 kDa (EF).

Mouse immunization with EF. Female BALB/c (6 to 8 weeks old) mice were obtained from the National Cancer Institute (Bethesda, MD). Mice were immunized intraperitoneally (i.p.) with 2 μg (group A; $n = 3$) or 10 μg (group B; $n = 3$) of EF in complete Freund's adjuvant (Sigma, St. Louis, MO). Two weeks later, the mice were boosted with either 2 or 10 μg of EF in incomplete Freund's adjuvant. Mice were bled from the retroorbital sinus, and sera were collected and stored at −20°C for later analysis of Ab titers by enzyme-linked immunosorbent assay (ELISA). All animal work was done in accordance with regulations of the Institute for Animal Studies at Albert Einstein College of Medicine.

Hybridomas. Hybridomas producing MAbs to EF were generated by standard techniques from splenocytes of EF-immunized BALB/c mice (7). Briefly, splenocytes from EF-immunized mice were fused to NSO myeloma cells at a ratio of 4:1. NSO is the nonproducing mouse myeloma fusion partner, and ascites produced by intraperitoneal injection of NSO cell lines were used as a negative control in some animal experiments. Hybridoma supernatants were screened by ELISA for Ab reactivity to EF, and hybridoma clones were then selected and stabilized by cloning twice in soft agar. The isotypes of the murine MAbs were established by ELISA using goat anti-mouse (GAM) IgM or IgG, heavy-chain specific, labeled or unlabeled with alkaline phosphatase (AP) (Fisher Scientific/Southern Biotechnology, Birmingham, AL).

ELISA. MAb binding to EF was measured by ELISA. Briefly, a solution of rEF (5 μg/ml) in phosphate-buffered saline (PBS) was used to coat 96-well flat-bottom polystyrene (high-binding) plates (Costar; Fisher Scientific). The polystyrene plates were then blocked with 1% bovine serum albumin (BSA)–PBS (BSA, fraction V; MP Biomedical, Solon, OH) and either immune sera or hybridoma supernatants were added. Primary Ab binding was detected using GAM-AP Ab reagents. Competition ELISAs were used to investigate the specificity of EF MAbs as described previously (2). Briefly, a variable amount of one MAb was mixed with a constant amount of a second MAb and allowed to bind to EF in a polystyrene plate. Binding of the Abs was detected by isotype-specific GAM-AP reagent. Incubations were done at 37°C for 1 h, and absorbance at 405 nm (A_{405}) was measured in a microtiter plate reader (Labsystems Multiskan, Franklin, MA).

Determination of V_H and V_L sequences. Total RNA was isolated from hybridoma cell lines producing MAbs to EF using Trizol reagent (Gibco BRL, Gaithersburg, MD) as per the manufacturer's instructions. Briefly, 1 ml Trizol reagent was used per 10^6 hybridoma cells and 50 ng of RNA was used for cDNA synthesis with oligo(dT) primer and Superscript II reverse transcriptase (Qiagen, Valencia, CA). Universal 5′ (sense) variable region and specific 3′ (antisense) constant region primers were used in a PCR to generate cDNA encoding the variable domains of MAbs. The primers are as follows: 3′MsCγ: AGACCTATGGGGCTGTTGTTTTGGC; 3′MsCμ, GACATTTGGGAAGGACTGACTCTC; 3′MsCκ, TGGATACAGTTGGTGCAGCATCAGC; 5′$V_H$uni, TGAGGTGCAGCTGGAGGAGTC; 5′Vκuni, GACATTCTGATGACCCAGTCT. The PCR was performed using 1 μg of the cDNA template, with 2.5 mM (each) deoxynucleoside triphosphate and 125 nM each primer under the following conditions with *Taq* polymerase (Roche, Mannheim, Germany): 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min for 40 cycles, followed by a final 10-min extension at 72°C. The amplified cDNAs were gel purified (Qiagen, Valencia, CA) and then sequenced at the sequencing facility of the Cancer Center at the Albert Einstein College of Medicine.

Scatchard analysis of MAb binding to *B. anthracis* Sterne. *B. anthracis* cells (10^7) were incubated with 0.12 to 0.48 nM concentrations of ^{188}Re-labeled MAbs 8F3, 9F9, 21B9, and 9F3 for 1 h at 37°C, and the tubes were counted in a gamma counter. Cells were collected by centrifugation, and the pellets were counted again. Scatchard analysis was used to determine MAb binding constant and binding sites per cell. The ratio of bound MAb to reactive free MAb is plotted against specific binding values. The slope and intercept values are obtained via linear regression and used to calculate the binding constant and the number of binding sites (30).

Immunoelectron microscopy (IEM). Bacterial cells (10^7) were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer. A saturated solution of sodium metaperiodate was used to etch sections on nickel grids for 60 min. Grids were then washed with H_2O and blocked with 1% BSA–0.5% Tween 20 in PBS (blocking solution) overnight at 4°C for intracellular localization of EF in bacterial cells. Grids were incubated with 10 μg of MAb 9F3 IgM diluted in blocking solution at room temperature for 2 h. Grids were then washed three times with PBS and three times with 1% BSA in PBS and finally incubated with goat anti-mouse IgG conjugated to 10-nm gold particles (Goldmark Biologicals, Phillipsburg, NJ) for 2 h. Grids were washed with PBS, fixed in 2.5% glutaraldehyde, and again washed with PBS and H_2O. Grids were stained with 4% uranyl acetate and washed several times with H_2O. Grids were examined with a JEOL 1200 EX transmission electron microscope (JEOL USA, Peabody, MA).

Mouse infection for *in vivo* EF detection. Female A/JCr (6- to 8-week old) mice were infected intravenously (i.v.) as previously described (25) with either 10^5 ($n = 4$) or 10^6 ($n = 4$) *B. anthracis* Sterne cells or PBS ($n = 3$). Bacterial cells were washed two to three times with sterile PBS, and the inoculum was suspended in PBS before i.v. infection. At day 2 postinfection, mice were bled from the retroorbital sinus and sera were isolated by centrifugation and stored at −20°C for later analysis of toxin by capture ELISA as described below. Mice were sacrificed, and lungs and livers were removed and homogenized in 2 ml of PBS in the presence of protease inhibitors (Complete Mini; Boehringer Mannheim, Indianapolis, IN). The homogenates were centrifuged at 4°C at 6,000 × *g* for 10 min to remove cell debris, and the supernatant was frozen at −80°C until tested for the presence of toxin by capture ELISA as described below.

Capture ELISA for EF detection. A MAb-based EF capture ELISA was developed to detect EF in serum or organ homogenates from infected mice. Briefly, 96-well microtiter plates were coated with 1 μg/ml of goat anti-mouse IgM unlabeled in PBS, blocked with 1% BSA–PBS, and then incubated with a murine MAb to EF, IgM MAb 9F3, which functioned as the capture Ab. Next, we added rEF (positive control) and either serum or organ homogenates from infected mice or organ homogenates spiked with rEF (1 μg/ml) at different dilutions. EF captured by the IgM was detected with the IgG1 MAb 21B9. Microtiter plates were then incubated with isotype-specific GAM IgG conjugated to AP and developed with *p*-nitrophenyl phosphate (PNPP), and absorbance at 405 nm was measured as described above. All incubations were carried at 37°C for 1 h.

cAMP determinations. The Amersham cyclic AMP (cAMP) Biotrak enzyme immunoassay (EIA) system (GE, Buckinghamshire, United Kingdom) was used to determine the ability of EF-binding MAbs to neutralize EdTx *in vitro*. Briefly, 10^5 Chinese hamster ovary (CHO) cells in a 96-well microtiter plate were incubated with various amounts of the MAbs (0.040 μg/ml to 10 μg/ml), followed by the addition of 100 ng EF and 100 ng of PA in the presence and absence of MAbs to EF. Negative controls included conditions with no MAb added and the inclusion of an irrelevant, isotype-matched control MAb, 14FA (IgG2a), with specificity for *B. anthracis* lethal factor (30, 31). Microtiter plates were then incubated for 2 h at 37°C, and the cells were lysed with lysis buffer (dodecyltrimethylammonium bromide) and resuspended in assay buffer (0.05 M sodium acetate buffer, pH 5.8, containing 0.02% BSA) to release intracellular cAMP. Following lysis, CHO cell extracts and cAMP standards were transferred to 96-well plates precoated with donkey anti-rabbit IgG, and then a specific antiserum composed of rabbit anti-cAMP IgG was added to the standards and CHO cell extracts. Microtiter plates were then incubated at 4°C for 2 h. Next, a peroxidase-conjugated cAMP was added to the wells, followed by incubation at 4°C for 1 h. Finally, the plate was washed with 0.01 M PBS, pH 7.5, containing 0.05% Tween. An enzyme substrate containing 3,3′,5,5′-tetramethylbenzidine (TMB)/hydrogen peroxide was added to the wells, the mixture incubated at 25°C for 1 h on a microtiter plate shaker, and the reaction was then halted by adding 1.0 M sulfuric acid. Optical densities (ODs) were measured at 450 nm (Labsystem Multiskan, Franklin, MA).

Survival analysis. Female A/JCr mice (6 to 8 weeks) (NCI, Bethesda, MD) were infected i.v. by tail injection with 10^4 cells of *B. anthracis* strain Sterne 34F2. Two hours prior to infection with *B. anthracis*, the mice received an i.p. injection of 0.1, 0.5, or 1 mg MAbs ($n = 10$ per Ab group) to EF. In some experiments, we evaluated the benefit of combination passive Ab therapy by administering a subprotective dose of MAb 7.5G (PA). These mice were treated with 1 mg of MAb 9F3 (EF) and 0.025 mg of MAb 7.5G (PA) (31) 2 h prior to i.v. infection with *B. anthracis*. Control mice received either PBS ($n = 10$) or NSO ascites ($n = 10$). Mice were monitored daily for mortality.

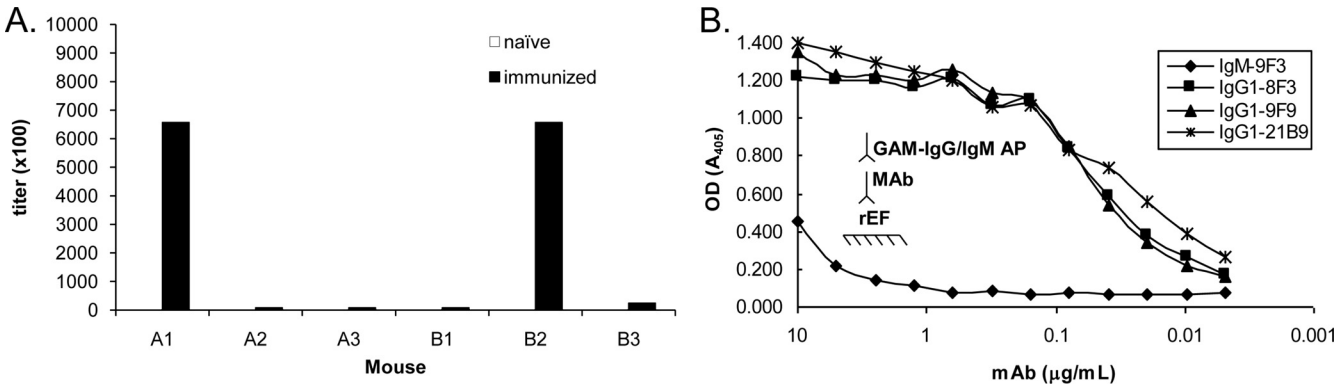

FIG. 1. (A) Ab titers of BALB/c mice immunized with rEF as measured by ELISA. Mice labeled A and B were immunized with 2 and 10 μg of EF, respectively. There were three mice in each group. Mice A1 and B2 were noted to have a significant IgG response to immunization with EF. Mouse A1 was chosen for hybridoma fusion. (B) Relative binding constant of the four MAbs, 9F3, 8F3, 9F9, and 21B9, for EF as measured by ELISA. Inset, ELISA configuration whereby rEF is absorbed on polystyrene plates and detected with AP-GAM-IgG or -IgM.

Statistics. All survival data were analyzed by log rank analysis and Cox regression analysis ($P < 0.05$) (Sigmastat, Chicago, IL).

Nucleotide sequence accession numbers. The GenBank accession numbers for the MAb nucleotide sequences identified in this study are listed in Table 1.

RESULTS

Immunogenicity of EF and hybridoma generation. rEF was immunogenic when injected into BALB/c mice with Freund's adjuvant, but only a minority of mice produced serum antibody responses to EF by 6 weeks after the initial immunization. The most robust IgG response occurred in mice A1 and B2, and mouse A1 was selected for hybridoma generation (Fig. 1). Two splenocyte-myeloma cell fusions were done using spleen cells from mouse A1, yielding six MAbs that bind to EF. The first fusion yielded two MAbs, an IgM (BD3), and an IgG1 (FF7). These MAbs had no neutralizing activity *in vitro*, and we performed a second myeloma-splenocyte fusion with saved frozen cells from mouse A1 spleen that yielded an additional IgM (9F3) and three IgG1s (8F3, 9F9, 21B9) to EF (Table 1). Since MAb 9F3 was found to be neutralizing (see below), our work focused primarily on the four MAbs from the second fusion (Table 1). Comparison of EF binding by the IgM and the three IgG1s revealed that the IgM had a relatively low binding constant despite its neutralizing capacity (Fig. 1B). Immunoelectron microscopy (IEM) with MAb 9F3 IgM revealed immunogold staining throughout the bacterial cell (Fig. 2A, parts i and iii). IEM of toxin-producing bacterial cells with isotype-matched, irrelevant MAbs was negative for gold deposition (Fig. 2A, part iii). IEMs of toxin-deficient Sterne cells with EF MAbs were negative for gold deposition (Fig. 2A, part iv).

Scatchard analysis of MAb binding to *B. anthracis* Sterne 34F2. To investigate the phenomenon of good neutralizing activity despite weak reactivity by ELISA, we performed Scatchard analysis of MAb binding to EF using *B. anthracis* Sterne 34F2. This assay was based on our recent observation that lethal toxin components are expressed in the surface of *B. anthracis* Sterne cells and are accessible to binding by MAbs (32). The binding constants of MAbs 21B9 IgG1, 9F3 IgM, and 9F9 IgG1 were relatively low (5.7×10^7, 5.9×10^7, and 8.3×10^7 M^{-1}, respectively), while MAb 8F3 had a binding constant that was approximately a log-fold greater (1.5×10^8 M^{-1}) (Fig. 2B). Additionally, the numbers of binding sites on bacterial cells for all MAbs were similar (6.2×10^4, 5.1×10^4, 4.3×10^4, and 5.3×10^4 M^{-1}, respectively).

V region determination. The V region elements used in the six MAbs to EF were determined (Table 1). MAbs BD3 (IgM) and FF7 (IgG1) utilized different heavy-chain gene elements: V_H (36-60 and 10.2a, respectively) and J_H (J_H4 and J_H1, respectively). Both MAbs used the same light-chain gene element, Jκ1. The IgG1 MAbs 8F3, 9F9, and 21B9 were similar to one another, since they used the same gene elements in immunoglobulin construction. However, the IgM MAb 9F3 utilized different heavy-chain gene elements, V_H (J558.17) and J_H (J_H2), and a different light-chain gene element, Jκ1. All MAbs

TABLE 1. Hybridoma families and MAb V_H and V_L usage

Fusion no.	Hybridoma	Neutralizing status	Accession no.[a]	Variable gene elements[b]			
				V_H	J_H	V_κ	J_κ
1	BD3 (IgM)	No	FJ470002 FJ470003	36–60 (100%) (29)	JH4 (82.6%) (33)	BD2 (92.7%) (34)	JK1 (100%) (21)
	FF7 (IgG1)	No	FJ470004 FJ470005	10.2a (92.5%) (42)	JH1 (87.8%) (37)	BD2 (94.2%)	JK1 (100%)
2	8F3 (IgG1)	No	FJ478144 FJ478140	7183 (97.6%) (15)	JH4 (96.2%)	BD2 (84.7%)	JK2 (87.1%) (21)
	9F9 (IgG1)	No	FJ478137 FJ478141	7183 (99.6%)	JH4 (96.2%)	BD2 (84.0%)	JK2 (89.7%)
	21B9 (IgG1)	No	FJ478138 FJ478142	7183 (99.6%)	JH4 (96.2%)	BD2 (87.5%)	JK2 (86.2%)
	9F3 (IgM)	Yes	FJ478139 FJ478143	J558.17 (98.6%) (11)	JH2 (100%) (37)	BD2 (90.2%)	JK1 (90.6%)

[a] Accession numbers for sequences in GenBank (Bethesda, MD).
[b] Variable gene usage assigned from homology searchers. Shown are percent homology to the stated V region elements (and reference number where appropriate).

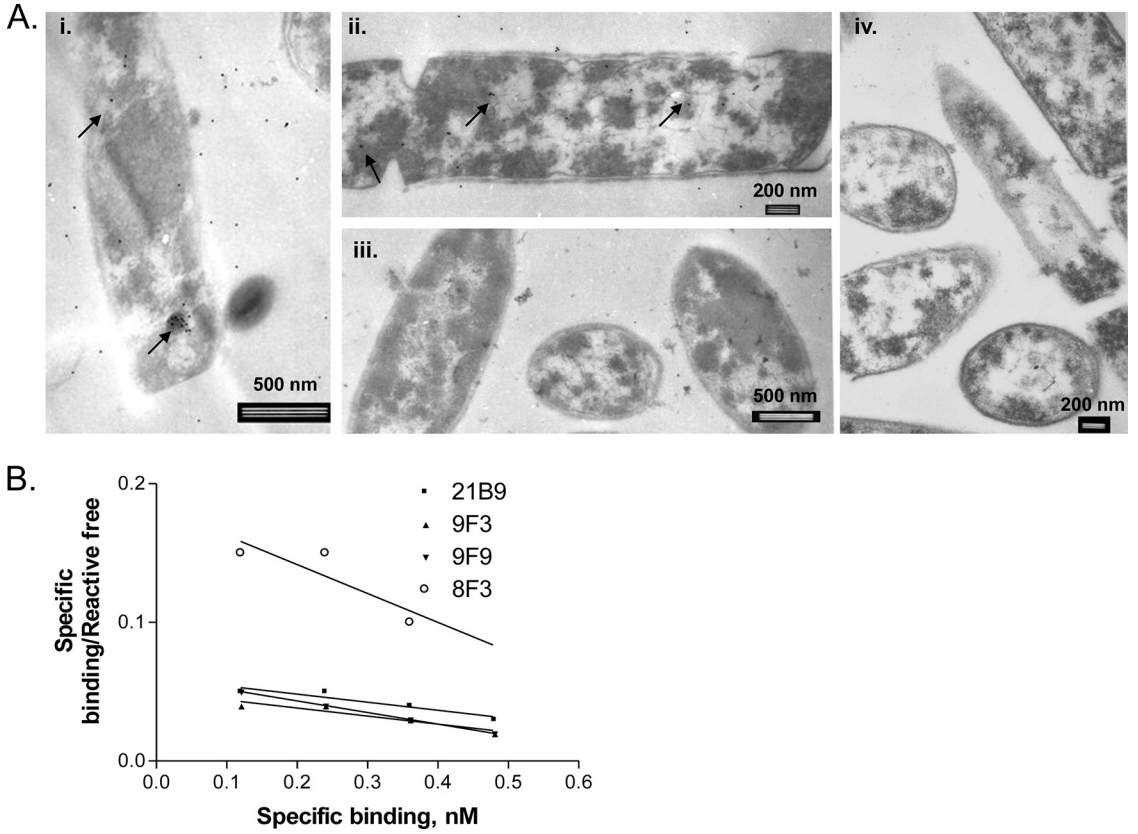

FIG. 2. (A) Immunogold localization of EF in *B. anthracis* Sterne 34F2 cells with MAb 9F3 IgM. IEM revealed gold particles throughout the bacterial cell and in extracellular space (i and ii) as indicated by arrows. (iii) Sterne cells stained with isotype-matched, irrelevant control (MAb IgG or IgM). (iv) Toxin-deficient Sterne cells with MAb 9F3 were negative for gold particles. Scale bar = 500 nm (i), 200 nm (ii), 500 nm (iii) and 200 nm (iv). (B) Scatchard analysis of binding of ^{188}Re-labeled MAbs to *B. anthracis* Sterne 34F2 (pOX1$^+$, pOX2$^-$) cells to determine the number of binding sites and binding constants.

utilized Vκ BD2, despite differences in specificity. The sequences were deposited in GenBank under the accession numbers listed in Table 1.

Competition ELISA. Competition assays were used to investigate the epitope specificity of the MAbs to EF. In this assay, absence of competition suggests binding to different epitopes, whereas competition implies either binding to the same epitope or binding to two separate but spatially near epitopes caused by MAb steric hindrance. The IgM MAb BD3 did not inhibit binding of IgG1 MAb FF7 at any concentration to EF (Fig. 3A). Additionally, MAb 9F3 did not inhibit binding of IgG1 MAbs 8F3, 9F9, and 21B9, at any concentration, to EF

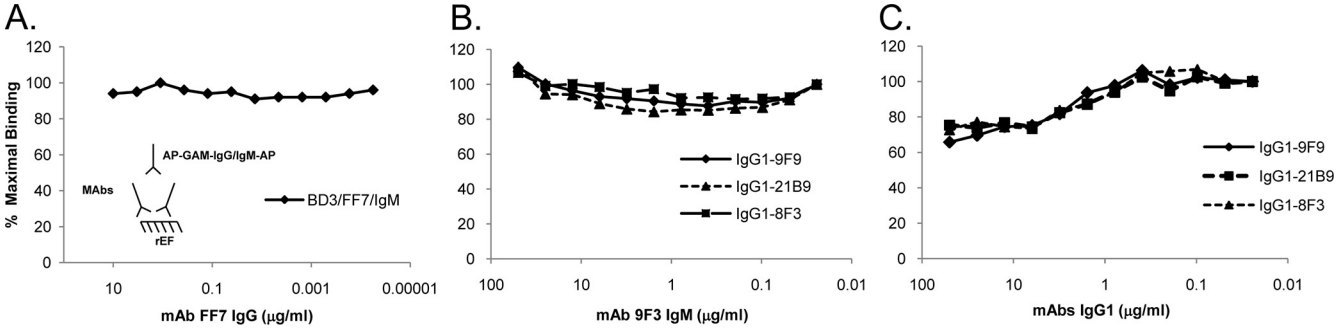

FIG. 3. Analysis of EF-binding MAbs by competition ELISA. (A) IgM MAb BD3 (10 μg/ml) did not compete with IgG1 MAb FF7 at any concentration. Competition assays were done three times with similar results. Data points are the average of results of two absorbance readings. Inset, schematic of competition ELISA configuration used, which applies to all panels. (B) IgM MAb 9F3 (10 μg/ml) did not compete with IgG1 MAbs 8F3, 9F9, and 21B9. Competition assays were done three times with similar results. Data points are the average of results of two absorbance readings. (C) IgM MAb 9F3 (10 μg/ml) competed with IgG1 MAbs 8F3, 9F9, and 21B9 at higher concentrations (50 μg/ml). Competition assays were done three times with similar results. Data points are the average of results of two absorbance readings.

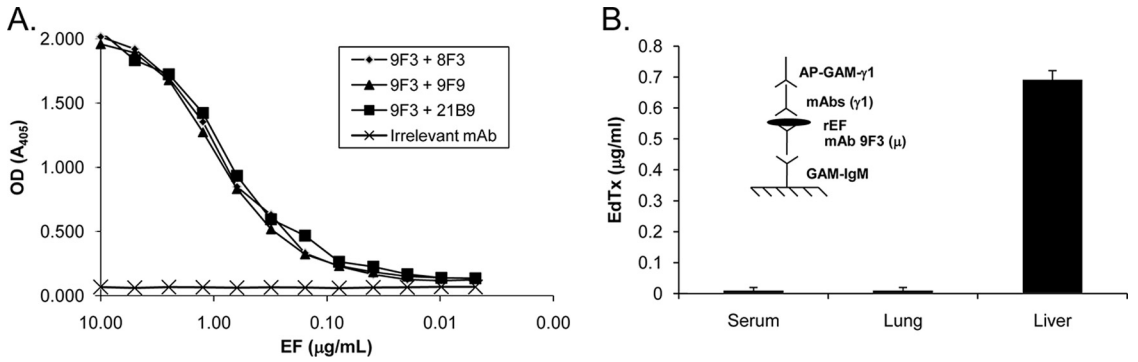

FIG. 4. (A) Capture ELISA using MAbs IgM (9F3) and IgG1 (8F3, 9F9, and 21B9) for the detection of rEF to determine sensitivity of assay. ELISA was developed with 1 mg/ml *p*-nitrophenyl phosphate, and the OD at 405 nm was determined. Isotype-matched, irrelevant MAbs (IgG and IgM) did not bind to EF. Data points are averages of results of two readings. (B) Capture ELISA for the detection of EF in organs of *B. anthracis*-infected mice. EdTx was detected in liver homogenates but not serum or lung homogenates. Experiments were done twice with similar results. The two experiments were combined and bars represent the average of four data points. Inset, schematic of capture ELISA configuration used, which applies to both panels.

(Fig. 3B). However, when the IgG1 MAbs 8F3, 9F9, and 21B9 were varied (beginning with 50 μg/ml), there was some competition with MAb 9F3 for EF binding (Fig. 3C).

Capture ELISA. Given that the IgM and IgG MAbs did not compete for binding to EF, we used them to design a capture ELISA for measuring EF concentrations. After testing various configurations, we designed a capture ELISA using IgM MAb 9F3 as the capture Ab and each of the IgG1 MAbs (8F3, 9F9, and 21B9) as the detecting MAbs. When a cutoff of two times the background absorbance was utilized, we found the sensitivity of EF detection by this ELISA to be 0.1 μg/ml. The ability of EF MAbs to capture the antigen (Ag) with a known concentration was similar for all three combinations of IgM and IgG1 MAbs (Fig. 4A). We did not detect any binding to EF with isotype-matched, irrelevant MAbs (Fig. 4A). After investigating the efficacy of these MAbs in detecting EF, the combination of the IgM MAb 9F3 and IgG1 MAb 21B9 was chosen to screen serum and lung and liver lysates of mice infected with the *B. anthracis* Sterne strain. EF was not detected in serum or lung lysates, but the assay detected 0.5 to 0.7 μg/ml of EF in liver lysates from infected mice (Fig. 4B). To confirm that organ homogenate did not degrade EF, we added rEF (1 μg/ml) to organ homogenates from naïve mice and recovered 80 to 90% rEF by capture ELISA (data not shown).

Effect of MAb on EdTx toxicity to CHO cells. The cAMP EIA system is based on the fact that unlabeled cAMP released by lysed CHO cells will compete with a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific Ab (Fig. 5A). The ability of MAbs to EF to protect CHO cells from EdTx toxicity was determined by examining the levels of cAMP in the presence and absence of EF-binding MAbs. CHO cells treated with EdTx and MAb 9F3 released low levels of cAMP compared to the levels measured from unprotected CHO cells ($P = 0.021$) (Fig. 5B). This amount of cAMP was steady at concentrations of 1 to 10 μg/ml of MAb 9F3. However, as the MAb concentration was titrated downward, this protective effect decreased (Fig. 5B). The other MAbs did not show a strong protective effect compared to MAb 9F3. However, they all exhibited lower cAMP concentrations at mid-range concentrations (0.5 to 1 μg/ml) ($P = 0.013$), indicating that there may be a prozone effect at work at the higher concentrations of IgG1 MAbs tested (Fig. 5B). Isotype-matched controls as well as irrelevant MAb (LF binding MAb 14FA) did not show protective effects on CHO cells (Fig. 5C).

Survival studies. We evaluated the protective efficacy of the MAbs to EF *in vivo* by administering MAbs i.p. 2 h prior to infection with *B. anthracis* strain Sterne. Six independent passive Ab experiments in mice were performed (Table 2). MAbs 8F3, 9F9, and 21B9 showed no protection *in vivo*. Administration of IgM-neutralizing MAb 9F3 had little or no effect on mean time to death, but we noted that in every experiment, there seemed to be more survivors in the MAb 9F3-treated group. Analysis of the combined results from all survival studies including the different MAb doses demonstrated that more mice treated with MAb 9F3 survived infection (11.5%, 7 of 64) than did control (NSO- or PBS-treated) mice (2%, 1 of 48) ($P = 0.0463$). Although this analysis produced a statistically significant result, we note that this result was obtained by combining data from experiments using different MAb doses. Surmising that this effect represented a modest benefit to EF neutralization *in vivo*, we investigated whether combining MAb 9F3 with a subtherapeutic dose (25 μg) of a protective MAb to PA (MAb 7.5G) would result in synergism. In both experiments done to evaluate synergy (Table 2, experiments V and VI only), the combination of MAbs 9F3 and 7.5G was more effective than either MAb alone in prolonging survival.

DISCUSSION

In this study, we report the generation of six hybridomas producing MAb to EF, of which only one MAb had EdTx-neutralizing activity. The neutralizing Ab was an IgM constructed with a different molecular structure than the nonneutralizing MAbs. This IgM also had a lower apparent binding constant for EF than the nonneutralizing IgG1 MAbs when binding was assayed by ELISA but had binding constants similar to those of the other MAb bacterium-associated toxins as demonstrated by Scatchard analysis. We interpret this discordance as likely to reflect differences in EF conformation in the

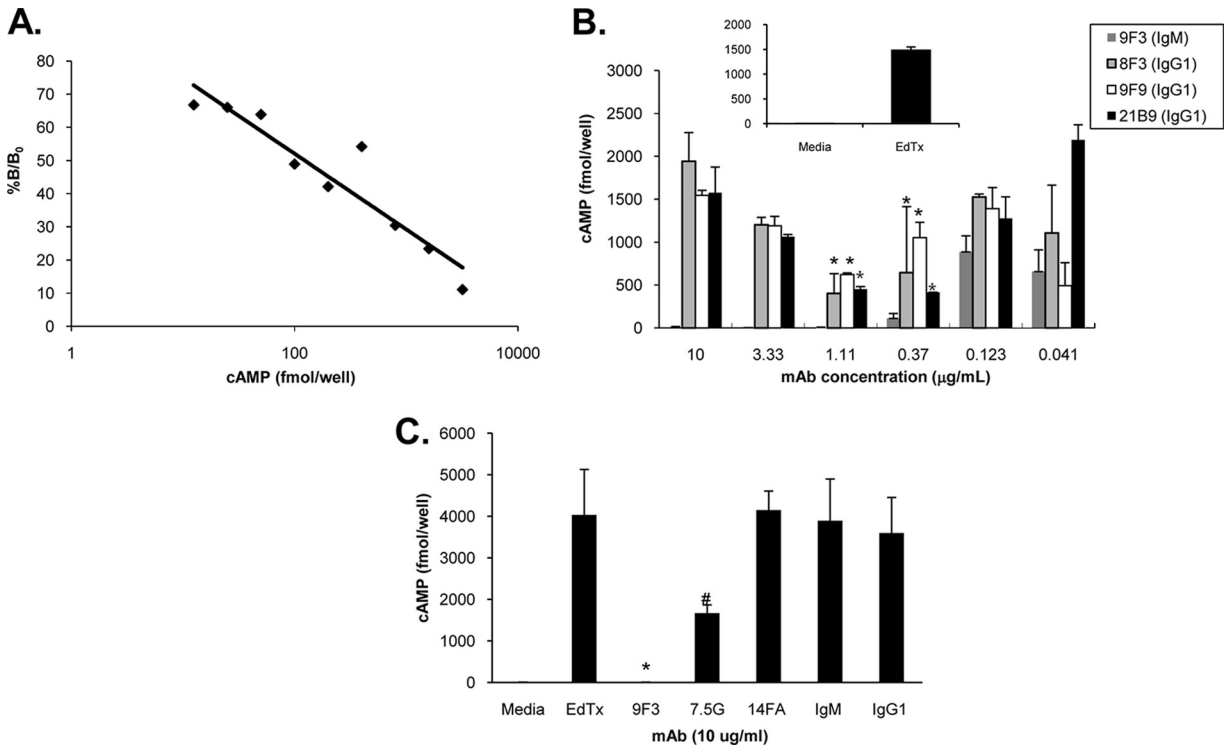

FIG. 5. cAMP produced by CHO cells treated with EdTx in the presence or absence of various concentrations of MAbs. The cAMP EIA system is based on the fact that unlabeled cAMP released by lysed CHO cells will compete with a fixed quantity of peroxidase-labeled cAMP for a limited number of binding sites on a cAMP-specific Ab. (A) Dose-response curve for assay. The percent bound for each standard was calculated using the following relationship: $\%B/B_0$ = (standard OD − nonspecific-binding OD) × 100 / (zero standard OD − nonspecific-binding OD). A standard curve was generated by plotting the percent B/B_0 as a function of the log cAMP concentration. (B) Efficacy of IgM and IgG MAbs in inhibiting EdTx-mediated cAMP release by CHO cells over various antibody concentrations. MAb 9F3, $P < 0.05$ (10, 3.33, 1.11, and 0.37 μg/ml compared to 0.123 and 0.041 μg/ml). Each data point is an average of values of two data points from two experiments. Error bars represent standard deviations. Inset, cAMP release by CHO cells in the absence of EdTx (media) or EdTx alone. *, $P < 0.05$ (1.11 μg/ml compared to 3.33 μg/ml and 10 μg/ml; 0.37 μg/ml compared to 10 μg/ml). (C) Efficacy of EF-binding IgM MAb 9F3 relative to MAb to PA (MAb 7.5G IgG2b) and IgM and IgG isotype-matched irrelevant MAb controls. The condition labeled "media" refers to the absence of EdTx. All conditions used MAbs at 10 μg/ml. Each data point is an average of values for four data points from the two experiments. Error bars represent standard deviations. *, $P < 0.05$ (MAb 9F3 compared to all conditions); #, $P = 0.08$ (MAb 7.5G compared to all conditions).

two assays. ELISA measures binding to EF adsorbed onto polystyrene, where it is likely to undergo significant conformational changes. In this study, we measured binding to EF by Scatchard analysis and IEM, revealing binding to EF in the bacterial surface representing toxin in the midst of secretion or attached by a yet-to-be-determined mechanism (32). Since neither method necessarily reflects solution binding, it is possible that solution interactions are different. The findings that the IgM MAb had a different V region usage than the nonneutralizing IgG MAbs, had no competition to EF, and could be successfully employed in an EF capture ELISA indicate different epitope specificity. This, in turn, implies the existence of domains in the EF molecule that can elicit neutralizing and nonneutralizing Abs. The fact that most Abs to EF reported in this study and in the literature (17, 19) are not neutralizing suggests that epitopes that elicit nonneutralizing Abs are immunodominant, with the caveat that relatively few MAbs to EF have been studied.

The molecular analysis of V region usage was noteworthy in the relative homogeneity of V gene utilization. The observation that all six hybridomas used the Vκ BD2 gene suggests a preference for this light chain sequence in binding to EF domains. It is noteworthy that whereas EF specificity may be conferred by BD2, fine specificity for binding to different epitopes is a function of V_H contributions. Restricted responses in immunoglobulin V region usage are generally associated with polysaccharide antigens, haptens, and/or immunodominant epitopes. The relative homogeneity in Vκ region restriction among this EF-binding MAb set can be interpreted as indicating a requirement for this gene element in constructing Abs that recognize a major toxin Ag. Comparison of MAb V region sequence to germ line V region sequences revealed a significant number of nucleotide changes consistent with somatic hypermutation and affinity maturation of the Ab response. The existence of an immunodominant epitope in EF that elicited nonneutralizing Abs could help explain the relative paucity of protective Abs to EdTx.

EdTx is a powerful adenylate cyclase that has dramatic effects on the morphology of susceptible cells *in vitro*. However, the contribution of EF to the virulence phenotype of *B. anthracis* and its precise role in pathogenesis are uncertain. A recent study that examined the role of EdTx alone in the pathogenesis of *B. anthracis* infections determined that this toxin causes a wide range of destructive effects, including in-

TABLE 2. Survival of A/JCr mice treated with MAb prior to i.v. infection with *B. anthracis* strain Sterne

Expt	Group[a]	MAb dose (mg/mouse)	*n*	Survival (days)		No. of survivors[b]	*P* value[c]
				Mean	Median		
I	PBS		5	2.8	3	0	
	8F3 (IgG1)	0.1	5	2.8	3	0	0.9
	9F9 (IgG1)	0.1	5	2.8	3	0	0.9
	21B9 (IgG1)	0.1	5	3.2	3	1	0.54
	9F3 (IgM)	0.1	5	3.2	3	1	0.54
II	PBS		5	4.6	5	0	
	8F3 (IgG1)	0.1	5	4.0	4	0	0.17
	21B9 (IgG1)	0.1	5	4.4	5	0	0.85
	9F3 (IgM)	0.1	10	15.5	4	2	0.58
III	NSO		10	4.3	3	1	
	9F3 (IgM)	0.5	13	3.69	3	0	0.70
	9F3 (IgM)	1	13	4.0	3	1	0.81
IV	NSO		10	4.60	4	0	
	9F3 (IgM)	1	10	5.30	4	2	0.52
	NSO		8	3	3	0	
V	7.5G (IgG2b)	0.025	8	3.25	3	0	0.14
	9F3 (IgM)	1	8	3.13	3	0	0.31
	9F3 + 7.5G	1 + 0.025	8	3.63	4	0	0.039
VI	NSO		10	3	3	0	
	7.5G (IgG2b)	0.025	10	3.1	3	0	0.31
	9F3 (IgM)	1	10	3.1	3	0	0.31
	9F3 + 7.5G	1 + 0.025	10	4.4	3	1	0.029

[a] PBS and NSO are control groups where the volume was administered i.p. in a 0.5-ml volume.
[b] Number of mice that survived infection.
[c] *P* value for the comparison of survival relative to PBS or NSO by Cox regression analysis.

traluminal fluid accumulation in the gastrointestinal tract, hemorrhaging of the ileum and adrenal glands, other histopathological lesions in the heart, kidneys, bone, and lymphoid organs, and increased levels of several cytokines leading to inflammation and tissue destruction. Physiological changes due to EdTx pathogenesis include hypotension and bradycardia thought to be a result of fluid accumulating within tissues and multiorgan failure (8). Hence, it is reasonable to posit that EdTx-neutralizing Abs could contribute to host defense by minimizing EdTx-mediated damage.

The efficacy of our MAbs *in vitro* was evaluated by their ability to inhibit cAMP accumulation in EdTx-treated cells. Inspection of the dose-response curves with the various IgG1 MAbs reveals what appeared to be prozone-like effects where lower efficacy against EdTx was observed with higher antibody concentrations as determined by cAMP assay. A prozone effect is a phenomenon whereby Ab efficacy is reduced at higher concentrations relative to that observed at lower concentrations. Prozone-like effects occur both *in vivo* and *in vitro*, and these phenomena are often underappreciated and poorly understood. Prozones have been observed in a variety of systems, including Ab-dependent cellular cytotoxicity against viruses (36), Ab-mediated complement activation (20), and Ab-mediated protection against fungi (35, 39) and tumors (10). Although the mechanism responsible for the shapes of these curves in this study is beyond the scope of this study, we note that prozone-like effects probably exist for Ab-mediated neutralization of EF and could conceivably play a role in this system.

One approach to identifying the contribution of a virulence factor to microbial pathogenesis is to provide the host with neutralizing Abs and observe the outcome of experimental infection. For example, we recently established that anthrolysin O made a contribution to *B. anthracis* Sterne virulence by demonstrating that mice given a neutralizing Ab were more likely to survive infection (26). Administration of MAb 9F3 to mice prior to systemic infection with *B. anthracis* strain Sterne did not affect survival. The inability of MAb 9F3 to significantly prolong survival of mice lethally infected with *B. anthracis* strain Sterne can be interpreted to mean that EdTx makes a relatively small contribution to the lethality outcome of murine anthrax.

Despite the inefficacy of MAb 9F3 administration in prolonging survival in murine anthrax, we did observe that in most experiments, some mice receiving MAb 9F3 survived the infection. When survivors from all groups in the various independent experiments were tallied, there was a suggestion that MAb 9F3 administration was associated with a higher likelihood of surviving infection, implying that in some mice, EdTx neutralization could have tipped the scales in reducing host damage to enhance the likelihood of surviving a lethal bacterial inoculation. Since the magnitude of any protective effect to EdTx neutralization appeared to be small in our mouse protection model, we surmised that such a small effect could affect mean time to death, but demonstrating a significant difference in mean time to death would likely require a prohibitively large number of animals. Consequently, we opted for alternative approaches and investigated whether combining the neutralizing IgM to EF with a subprotective dose of MAb to PA would affect mean time to death. In both experiments, mice receiving MAb to EF and PA had a modest yet statistically significant prolongation in mean time to death relative to mice receiving MAb to EF or PA alone. We interpret this result as strongly suggesting a synergistic beneficial effect to EF and PA neutralization. In this regard, we note reports that EdTx and LeTx can work synergistically to enhance their lethality in mice (9).

On the basis of our results, we infer that neutralizing Abs to EdTx can contribute to host defense against anthrax, albeit in a minor role. A caveat to these results is that *B. anthracis* strain Sterne lacks a poly-D-glutamic acid capsule and, in the absence of a capsule, its toxins could make a relatively larger contribution to the overall virulence phenotype than those of wild-type strains (23). On the other hand, we note that the MAb used in these passive experiments may have been suboptimal for neutralizing EdTx. MAb 9F3 is an IgM, and this isotype has the short serum half-life of 8 to 12 h in mice (27, 41). IgM is also largely confined to the vascular compartment, given its large mass of 800,000 Da. Despite the dodecavalency of IgM, this protein is about 10-fold larger than EF, which implies a significant unfavorable molar ratio relative to those of IgGs with regard to toxin neutralization, especially when infection is progressing, resulting in further increase in EF levels, while IgM levels are declining due to fast clearance from the blood. Furthermore, MAb 9F3 is a relatively low binding constant compared to other anthrax toxin-neutralizing MAbs. In this regard,

we note that some of the most effective PA-neutralizing IgGs had affinities in the nano- and picomolar ranges (13, 14, 28). Given that the Ab binding constant is a critically important variable in determining the efficacy of anthrax toxin neutralization (22), the relatively low binding constant of MAb 9F3 could have been a significant impediment to achieving more impressive protection data in mice. Hence, the combination of short half-life, relatively low tissue penetration, and modest binding constant may be attributed to the relatively lackluster protective effects observed *in vivo* despite strong neutralizing activity *in vitro*. However, these deficiencies might be overcome with a high-affinity IgG to the same epitope recognized by MAb 9F3, and it is possible that more impressive passive protection results would be obtained with an IgG-neutralizing MAb.

The combination of one IgM and an IgG1 MAb was used to design a capture ELISA capable of reliably detecting EF at concentrations of 0.1 μg/ml and higher. Again, the modest sensitivity of this ELISA is probably a reflection of the relatively low binding constants of the capture and detection MAbs. This capture ELISA was used to investigate the concentration of EF in serum and lung and liver homogenates. We measured EF in liver homogenates but did not detect toxin in serum or lung, although this may have been a false-negative result from low ELISA sensitivity. A previous study investigating the pathogenicity of EdTx alone in mice did not report histopathological changes in the liver but determined that the toxin still may have some effects on hepatic function, as mice receiving EdTx had elevated alkaline phosphatase, aspartate transaminase, alanine aminotransferase, bilirubin, and γ-glutamyl transpeptidase levels compared to control mice (8). Given that these MAbs detected EF in an organ that reportedly exhibits few pathological changes, it is possible that the detection of this toxin component in liver lysates reflects liver clearance and accumulation of toxin (8), since we have shown that intravenous administration of EF leads to rapid liver uptake (6). The availability of an assay that determines EF in tissues could prove helpful in future studies determining the role of EdTx in the pathogenesis of *B. anthracis* infections and for studying EF production by *B. anthracis*.

In summary, we report the generation of a neutralizing IgM to EF that modestly enhanced the likelihood of survival when administered to mice lethally infected with *B. anthracis* strain Sterne. The recovery of one IgM with strong neutralizing activity against EdTx yields a potentially useful reagent for evaluating the role of this toxin component in pathogenesis and for investigating the usefulness of Ab-neutralizing activity in host defense. The results provide encouragement for the notion that more effective Abs to EF exist and that humoral immunity to EF can make an important contribution to host defense in anthrax. This, in turn, suggests that vaccines that also elicit neutralizing Abs to EF may be more effective than those focused only on eliciting Abs to PA. Unfortunately, our results showing that most of the MAbs generated were not neutralizing, combined with similar findings in a prior report (19) and the observation that depletion of EF-binding Abs from human immune sera does not significantly affect neutralizing activity (40), suggest that neutralizing Abs to EF may be relatively uncommon. However, epitope mapping of protective and nonprotective epitope domains in EF using MAbs could result in the identification of antigenic sites that elicit useful Abs and the incorporation of such domains in future vaccines.

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