

Combinations of Monoclonal Antibodies to Anthrax Toxin Manifest New Properties in Neutralization Assays

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Monoclonal antibodies (MAbs) are potential therapeutic agents against *Bacillus anthracis* toxins, since there is no current treatment to counteract the detrimental effects of toxemia. In hopes of isolating new protective MAbs to the toxin component lethal factor (LF), we used a strain of mice (C57BL/6) that had not been used in previous studies, generating MAbs to LF. Six LF-binding MAbs were obtained, representing 3 IgG isotypes and one IgM. One MAb (20C1) provided protection from lethal toxin (LeTx) in an *in vitro* mouse macrophage system but did not provide significant protection *in vivo*. However, the combination of two MAbs to LF (17F1 and 20C1) provided synergistic increases in protection both *in vitro* and *in vivo*. In addition, when these MAbs were mixed with MAbs to protective antigen (PA) previously generated in our laboratory, these MAb combinations produced synergistic toxin neutralization *in vitro*. But when 17F1 was combined with another MAb to LF, 19C9, the combination resulted in enhanced lethal toxicity. While no single MAb to LF provided significant toxin neutralization, LF-immunized mice were completely protected from infection with *B. anthracis* strain Sterne, which suggested that a polyclonal response is required for effective toxin neutralization. In total, these studies show that while a single MAb against LeTx may not be effective, combinations of multiple MAbs may provide the most effective form of passive immunotherapy, with the caveat that these may demonstrate emergent properties with regard to protective efficacy.

Bacillus anthracis, the causative agent of anthrax, has been studied extensively, especially after it was demonstrated to be a powerful biological weapon during the bioterrorism attacks of 2001. *B. anthracis* virulence is largely due to its ability to produce a tripartite toxin consisting of protective antigen (PA), edema factor (EF), and lethal factor (LF). EF is an adenylate cyclase (1), which binds with PA to form edema toxin, while LF is a zinc metalloprotease that disrupts host cell signaling via cleavage of mitogen-activated protein kinase kinases (as reviewed in reference 2) and combines with PA to form lethal toxin (LeTx).

Anthrax vaccine adsorbed (AVA) has long been the only vaccine available for protection against *B. anthracis* in the United States. This vaccine consists of an acellular filtrate from an acapsular strain of *B. anthracis* (3). Albeit effective, the exact antigenic composition of this vaccine remains unknown and varies from batch to batch (4). Although vaccine-elicited antibodies to PA are thought to be the major mediators of protection, it is unclear whether immune responses to other toxin components also contribute to induce immunity (5–7). The vaccine has several other shortcomings, including a burdensome schedule of vaccinations and a requirement for annual boosts (8). In addition, while antibiotics such as ciprofloxacin can control the bacterial infection, there is currently no effective treatment to counter the effects of anthrax toxin. Antimicrobial therapy can clear the infection but does not affect toxemia.

Over the past 2 decades, passive immunotherapy has been widely explored as an alternative approach to protection from and treatment of *B. anthracis* infections and other microbial pathogens and their toxins and has been reviewed extensively (7, 9–13). Specifically, there have been many studies reporting the generation and characterization of monoclonal antibodies specific to the individual components of anthrax toxin (for a comprehensive summary of these studies, see references 11 and 13). Most of these studies have focused on monoclonal antibodies (MAbs) to PA. There have been several MAbs to LF generated from splenocytes

derived from BALB/c or A/J mice (14–18). Consequently, a goal of our study was to use a genetically different mouse strain (C57BL/6) with the hope of isolating novel MAbs to LF, since the genetic background affects the susceptibility to anthrax toxins (19). In addition, we sought to further characterize the protective efficacy of these MAbs to LF in combinations, since serum is a polyclonal mix of antibodies and the context of a MAb in the presence of other antibodies may affect its interactions with LeTx. To our knowledge, only one study has explored antibodies to LF in combinations with MAbs to PA (20). Together, the combination of these two MAbs provided increased protection against *B. anthracis* Sterne challenge in mice. A subsequent study (21) tested two LF MAbs with one PA MAb in a Fischer F344 rat model and showed “synergistic” protection with one of the two combinations. Here we show that combinations of MAbs to LF can manifest properties different from those of their individual components to enhance or abrogate MAb-mediated LeTx protection both *in vitro* and *in vivo*.

MATERIALS AND METHODS

***B. anthracis* and toxin components.** *B. anthracis* Sterne 34F2 (pXO1+, pXO2–) was obtained from Alex Hoffmaster at the Centers for Disease Control and Prevention (Atlanta, GA). Bacterial cultures were grown from frozen stock in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C for 18 h with shaking. Recombinant, endotoxin-reduced protective antigen (rPA), edema factor (rEF), and lethal factor (rLF) proteins

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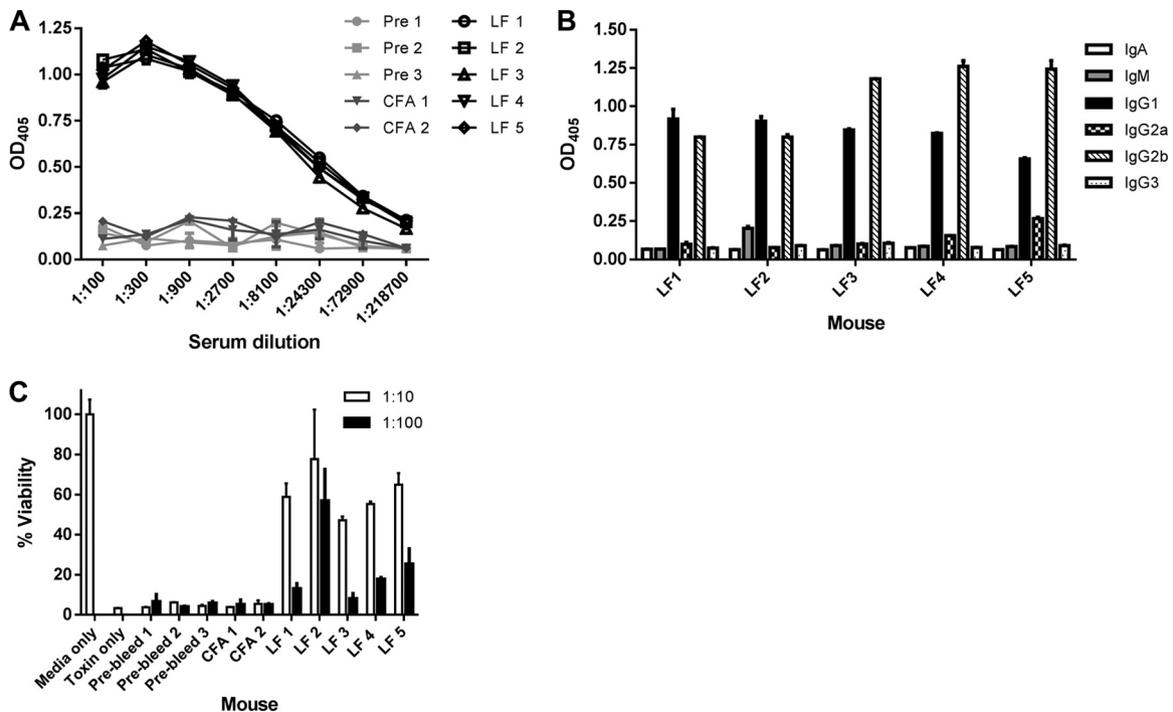


FIG 1 Characterization of serum from LF-immunized mice 6 weeks after initial immunization. Five C57BL/6 mice were immunized with 10 μ g of LF and boosted every 2 weeks with 10 μ g for 6 weeks. (A) Titers of antibodies to LF in 6-week serum. (B) Subtyping of 6-week sera. LF-coated polystyrene plates were incubated with sera diluted 1:1,000 and then assayed with isotype-specific, alkaline phosphatase-conjugated secondary antibodies. (C) MTT cell viability assay to measure protective ability of 6-week sera on J774 macrophage cells against LeTx. Macrophages were incubated with 1 μ g/ml LeTx with sera diluted 1:10 or 1:100 in duplicate for each mouse. Percent viability was calculated based on cells incubated with medium alone. Prebleed, preimmune mice; CFA, mice immunized with CFA only; LF, mice immunized with LF.

were obtained from the Northeast Biodefense Center Expression Core, New York State Department of Health (Albany, NY).

Murine immunization with purified LF. Female 6- to 8-week-old C57BL/6 mice were obtained from the National Cancer Institute (Bethesda, MD). Five mice were immunized with 10 μ g rLF in Freund's complete adjuvant (CFA) (Sigma, St. Louis, MO). At 2 and 4 weeks after the initial immunization, mice were boosted with 10 μ g of LF in incomplete Freund's adjuvant (IFA). Six weeks following the initial immunization, one mouse was boosted once a day for 2 days with 100 μ g of rLF in IFA and was then sacrificed 2 days later to collect splenocytes for the hybridoma fusion assay. As a control, two mice were immunized with CFA alone. Sera from the mice were collected by retro-orbital bleeding and stored at -20°C . Antibody titers were determined by standard enzyme-linked immunosorbent assay (ELISA), as described below.

Generation of MAbs to LF. Splenocytes from mouse LF5 were recovered and fused with myeloma cells by standard techniques (22) to generate hybridomas, which were then plated on 96-well plates and screened for reactivity to LF by ELISA. Positive clones were isolated and purified by two rounds of soft-agar cloning and also screened for nonspecific binding to bovine serum albumin (BSA).

ELISA. Binding of Abs to LF, EF, or PA was detected using standardized ELISAs as previously described (23). Briefly, polystyrene plates were coated with 50 μ l of 2 μ g/ml purified rLF, rEF, or rPA, suspended in phosphate-buffered saline (PBS), and blocked for 1 h at 37°C with 1% BSA-PBS. Antibody binding to immobilized toxin components was detected using anti-mouse alkaline-phosphatase (AP)-conjugated secondary antibodies, diluted 1:1,000 (~ 0.5 μ g/ml). For competition ELISAs, plates were coated and blocked as described above and then incubated with 2 μ g/ml of the constant MAb mixed with a 1:3 titration of the variable MAb across the plate starting with 90 μ g/ml. Binding of the primary Abs was detected as described above.

Western blotting. rLF (15 μ g/ml) was submitted to electrophoresis on a 12% SDS polyacrylamide gel, transferred to a nitrocellulose membrane using the iBlot Semi-Dry Transfer system (Invitrogen, Carlsbad, CA), blocked with 5% milk-PBS, and then incubated with each MAb to LF diluted to ~ 0.5 μ g/ml. Primary Ab binding was detected with isotype-matched goat anti-mouse horseradish peroxidase (HRP)-conjugated Abs and chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA).

In vitro cell viability assay. Cell viability assays were performed as described previously (24) using MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide]. In brief, J774 macrophage cells were seeded in 96-well plates at a density of 3×10^4 cells/well and incubated overnight at 37°C . To assay the toxin neutralization capability of polyclonal sera (Fig. 1C), a toxin concentration of 1 μ g/ml was added to each well and cells were incubated for 4 h. For all other MTT assays, a solution containing 0.1 μ g/ml of PA and LF (a concentration that yields approximately 50% cell killing) was added to each well and incubated 4 h. Then, 25 μ l of 5 mg/ml MTT substrate was added to each well, and cells were incubated at 37°C for an additional 2 h. Lastly, culture medium was aspirated, and 200 μ l of 12.5% SDS-45% N,N-dimethylformamide (DMF) was added to each well. Optical densities were measured at 570 nm with a spectrophotometer (Multiskan; Labsystems, Franklin, MA). For the Fc receptor (FcR) blocking experiment, 100 μ l of 10 μ g/ml mouse BD Fc Block (rat anti-mouse CD16/CD32, MAb 2.4G2) (BD Biosciences, San Jose, CA) was added to each well, and wells were incubated for 15 min prior to addition of toxin and MAbs. Fc Block was present in the Ab/toxin media for the duration of the experiment.

SPR. Binding constants of MAbs to LF were measured by surface plasmon resonance (SPR) using a BIAcore 3000 analyzer (Biacore, GE Healthcare, Piscataway, NJ). Immobilization of MAbs to a CM5 sensor chip was achieved as described previously (25). Serially diluted rLF was run over the immobilized MAbs.

Sequence analysis of immunoglobulin variable genes. Hybridoma cell lines 12H11 and 20C1 were grown to confluence in 6-well plates and then collected in TRIzol reagent (Invitrogen), and RNA was isolated according to the manufacturer's specifications. cDNA was generated using the Superscript III First-Strand Synthesis system (Invitrogen). PCR amplification of the variable light (V_L) and heavy (V_H) genes was performed with primers and under reaction conditions previously described (26). Sequencing was performed at the Genomics Core at Albert Einstein College of Medicine.

Mouse infections with *B. anthracis* strain Sterne. C57BL/6 mice ($n = 10$) were immunized with rLF or CFA as described above. Mice were boosted with rLF every 2 weeks for 6 weeks and then challenged with 10^5 cells of strain Sterne per mouse. For passive transfer experiments, immunized mice were serially bled retro-orbitally and the sera from each group pooled. Mice were given 200 μ l sera from the LF-immunized mice 2 h prior to infection with strain Sterne. To establish whether MABs to LF alone or in combination were protective *in vivo*, five C57BL/6 mice/group were given 10, 50, or 100 μ g MAB intraperitoneally (i.p.) 2 h before challenge with 10^5 cells of strain Sterne/mouse. Mice were monitored daily for mortality. All animal work was done in accordance with the regulations of the Institute for Animal Studies at Albert Einstein College of Medicine.

Screen for interactions between MABs to LeTx. MTT assays were performed as described above, combining two MABs to LF. One MAB was kept at a constant concentration of 30 μ g/ml and mixed with another MAB to LF, which was serially diluted 1:3 across the plate, starting with 90 μ g/ml. MTT assays were also performed to screen for interesting multiple MAB interactions between the LF MABs generated in this study and MABs previously generated in our laboratory (24, 39). For each combination, one MAB was kept at a constant concentration of 5 μ g/ml, while the other was serially diluted 1:3 across the plate, starting with 30 μ g/ml. Results of these assays were used to calculate fractional inhibitory concentration (FIC) indices, as described below.

Statistical and mathematical analyses. To characterize multiple MAB interactions, we used the following equation for calculating the FICs: MAB concentration yielding 50% of the maximum cell survival for two Abs in combination divided by the MAB concentration yielding 50% of the maximum cell survival of an antibody alone, as adapted from references 27, 28, and 29). The FIC index is a sum of the FICs for each antibody, and the interaction between two Abs was considered synergistic if the FIC index was <1.0 , additive if the FIC index was equal to 1, indifferent if the FIC index was between 1 and 2, and antagonistic if it was >2 . The Student *t* test was used when appropriate, and the log rank test was used for mouse survival experiments (GraphPad Prism version 5.04; GraphPad Software, San Diego CA, USA).

Nucleotide sequence accession number. The V_H and V_L gene sequences of MABs 12H11 and 20C1 determined in this study have been submitted to GenBank under accession numbers [KC592294](#) and [KC592295](#).

RESULTS

Murine antibody response to immunization with LF. All five LF-immunized mice demonstrated similar, robust antibody responses to LF (Fig. 1A), and as expected, the CFA and preimmune mice showed no reactivity to LF. Immunoglobulin subtyping of 6-week sera revealed that the majority of the anti-LF response was IgG, with little to no IgA or IgM present in the sera (Fig. 1B). The IgG response consisted of a mix of IgG1 and IgG2b and very low or absent levels of IgG2a and IgG3 (Fig. 1B). Next, we tested the sera's ability to neutralize lethal toxin *in vivo* using a macrophage viability assay. Sera from the preimmune and CFA mice offered no protection, while the sera from the LF-immunized mice all showed similar levels of toxin neutralization of about 50% or more (Fig. 1C). These results show that over the course of 6 weeks,

the mice mounted a strong, specific polyclonal Ab response to the rLF antigen and produced toxin-neutralizing antibodies.

Generation and characterization of LF-specific MABs. When titers of the LF-immunized mice were deemed sufficiently high (at least 2.5 times greater than baseline titers of CFA-only immunized mice), mouse 5 was sacrificed, and splenocytes were collected and subjected to a hybridoma fusion assay. We recovered six hybridomas producing 6 LF-specific MABs: 17E7, 17F1, 19C9, 20C1, 12H11, and 11D7. Isotyping revealed that this set consisted of one IgG1 (19C9), one IgG2a (17F1), three IgG2b (17E7, 12H11, and 20C1), and one IgM (11D7). Since there are regions of homology between LF and EF (30) and some MABs have been reported to bind both antigens (15, 18), we evaluated the ability of our MABs to bind to all three anthrax toxin components. ELISA results showed that while all MABs strongly bound to LF (Fig. 2B), 17E7, 17F1, and 19C9 had reactivity for all three antigens. All MABs were also reactive with LF by Western blotting (Fig. 2A), but binding to PA and EF could not be detected by immunoblotting (data not shown). The relative binding affinity of these MABs for LF was measured by ELISA, which showed that 17E7 bound the strongest, followed by 20C1, 12H11, 17F1, and 19C9 (Fig. 2C). To further investigate the binding properties of these MABs, SPR techniques were employed on 12H11, 17F1, and 20C1. The two-state kinetic reaction model yielded results that corresponded with the binding ELISA: 12H11 and 20C1 showed similar affinities, both higher than that of 17F1 (Table 1).

Next, we determined the toxin neutralization capacity of these MABs by macrophage cell viability assay. These results showed that MABs 12H11 and 20C1 conferred the highest levels of protection and 17F1 showed slight protection while the remaining MABs showed little to no toxin neutralization (Fig. 2D). Hence, we recovered at least two LF-specific MABs that can neutralize LeTx *in vitro*. To determine whether MAB toxin neutralization was FcR dependent, a cell viability assay was performed using Fc Block (MAB 2.4G2). Cells were incubated with or without Fc Block prior to treatment with toxin and MABs to LF. These results suggest that toxin neutralization mediated by MABs 17F1 and 20C1 is partially dependent on FcR binding (Fig. 2E).

Since MABs 12H11 and 20C1 were of the same isotype and had similar binding affinity and specificity to LF as well as similar toxin neutralization capability, we determined the V_H and V_L gene sequences of each MAB. Sequence analysis revealed that these MABs shared identical variable gene sequences. Thus, we concluded that these hybridoma cell lines were derived from clonally related B cells that produced the same immunoglobulin and excluded 12H11 from further analysis.

Protective efficacy of anti-LF MABs *in vivo*. To determine whether these MABs are protective *in vivo*, we carried out active and passive immunization to ascertain the level of protection in mice challenged with *B. anthracis*. Three types of experiments were done. First, we tested whether the polyclonal sera from LF-immunized mice conferred protection. LF-immunized mice ($n = 10$), along with the CFA ($n = 10$) and naive ($n = 5$) mice, were each challenged with 10^5 cells/mouse of strain Sterne. LF immunization provided complete protection from *B. anthracis* challenge (Fig. 3A). This experiment was repeated twice with similar results. Next, we tested the efficacy of sera collected from the LF-immunized mice in a passive transfer experiment. Prior to infection, sera were collected and pooled from LF-immunized mice. Naive C57BL/6 mice were administered 200 μ l of undiluted sera 2

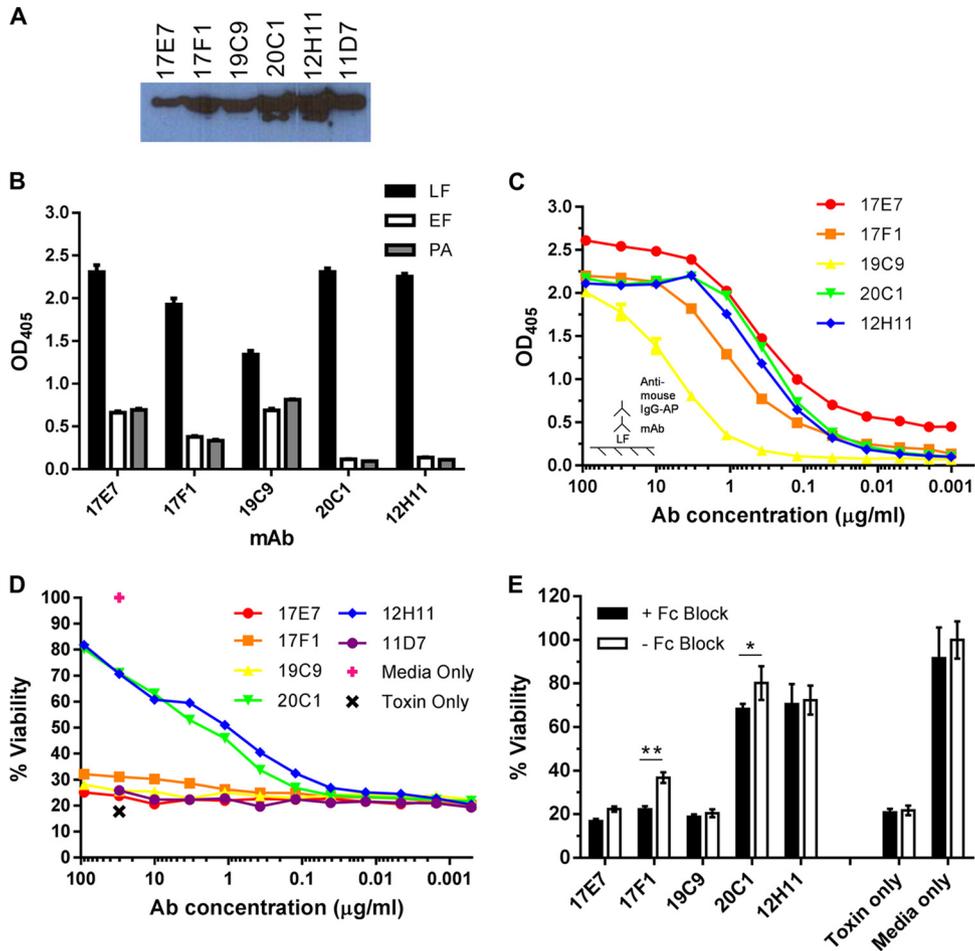


FIG 2 *In vitro* characterization of MABs to LF. Six LF-specific MABs were purified from cell lines generated from a standard splenocyte-myeloma fusion. (A) Immunodetection of LF. MABs, diluted 1:500, were incubated with purified LF on a nitrocellulose membrane and detected by chemiluminescence with HRP-conjugated secondary Abs. (B) IgG MAB reactivity to anthrax toxin components. Binding to LF, EF, and PA was measured by ELISAs in triplicate for each Ab; plates were coated with 2 $\mu\text{g}/\text{ml}$ antigen and incubated with 10 $\mu\text{g}/\text{ml}$ antibody. Ab binding was detected with AP-conjugated secondary Abs. (C) Relative binding capacity of IgG MABs to LF as determined by ELISA. The ELISA plate was coated with 2 $\mu\text{g}/\text{ml}$ LF, and MAB concentrations were titrated 1:3 from 90 $\mu\text{g}/\text{ml}$. (D) *In vitro* cytotoxicity assay to measure MAB-mediated toxin protection. J774 macrophages were incubated with 0.1 $\mu\text{g}/\text{ml}$ LeTx with or without MABs at various concentrations. Cells were incubated with toxin alone or toxin-free medium as negative and positive controls, respectively, for viability. (E) Cell viability assay with Fc receptors blocked. The assay was performed as described above, with 0.1 $\mu\text{g}/\text{ml}$ LeTx, except cells were incubated with 100 μl of 10 $\mu\text{g}/\text{ml}$ Fc Block for 15 min prior to incubation with LeTx and MABs. *, cell viability was significantly lower with Fc Block for 20C1 ($P = 0.0077$); **, $P < 0.0001$ for 17F1.

h prior to infection with strain Sterne. Passive transfer of sera from LF-immunized mice provided significantly ($P = 0.0141$) greater protection than sera from CFA-immunized or naïve sera (Fig. 3B).

Lastly, we tested each MAB individually in passive protection experiments. For each MAB, five C57BL/6 mice were each given 100 μg of MAB 2 h prior to intravenous challenge with 10^5 cells of strain Sterne. MAB administration was not followed by increased survival for any of the MABs tested relative to the control group (PBS) (Fig. 3C).

TABLE 1 Binding affinities of MABs to LF^a

MAB	K_a (1/Ms)	K_d (1/s)	K (1/M)
12H11	1.25e5	2.26e-3	8.03e9
17F1	1.36e4	3.22e-3	2.75e7
20C1	1.34e5	2.71e-3	4.55e9

^a K_a , binding affinity constant; K_d , dissociation constant; K , equilibrium constant.

Analysis of neutralizing activity of MAB combinations to LF.

Given that under physiological conditions Abs function in combination with other Abs as part of a polyclonal response, we evaluated the neutralization capacity of MABs in combination at a 1:1 ratio against LeTx by MTT assay. One striking Ab combination was 17F1 plus 20C1, which conferred a significant increase in cell viability compared to 17F1 or 20C1 alone ($P < 0.0001$ and $P = 0.0111$, respectively) (Fig. 4A), suggesting that 17F1 potentiated the toxin neutralization activity of 20C1. To further explore these observations, a subsequent MTT assay titrating 17F1 and 20C1 was performed. These results confirmed the previous results that 17F1 in combination with 20C1 was more effective at neutralizing LeTx than either MAB alone (Fig. 4B). The fractional inhibitory concentrations (FICs) were calculated by determining the antibody concentration at 50% of maximum cell survival for each curve. The FIC index is the sum of the FICs and is considered synergistic if the FIC index is < 1 , additive if it is equal to 1, indif-

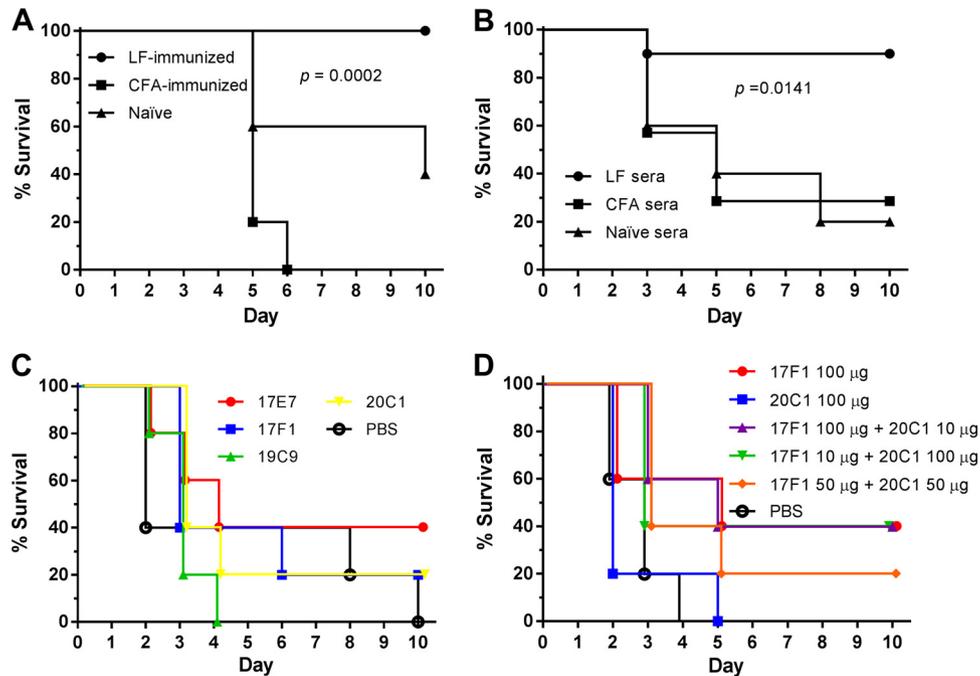


FIG 3 Survival of mice challenged with *B. anthracis* strain Sterne. (A) Challenge of LF-immunized mice with strain Sterne. Mice were given an inoculum of 10^5 cells/mouse ($n = 10$ mice for LF and CFA groups; $n = 5$ for the naïve group). (B) Passive transfer of polyclonal sera. Naïve C57BL/6 mice were administered 200 μ l of polyclonal sera from the LF-immunized mice illustrated in panel A 2 h prior to infection with 10^5 cells of strain Sterne per mouse. $n = 10$ for mice given LF-immunized sera, $n = 7$ for CFA sera, $n = 5$ for naïve sera. (C) Characterization of MABs *in vivo*: 100 μ g of Ab was administered to 5 mice/group 2 h before challenge with 10^5 cells of strain Sterne per mouse. (D) MABs 17F1 and 20C1 in combination. MABs were given alone or in combination to mice 2 h before challenge with strain Sterne. $n = 5$ mice/group.

ferent if the value falls between 1 and 2, and antagonistic if it is >2 . The FIC index for 17F1 and 20C1 was 0.89 (Table 2), suggesting a moderately synergistic interaction.

We then studied whether these two MABs showed a similar interaction *in vivo*. Thus, C57BL/6 mice were given either 17F1 or 20C1 alone or the two in combination at different ratios, and their survival was monitored for 10 days. Survival of mice given 100 μ g 17F1 plus 10 μ g 20C1 was significantly higher than that of mice given PBS alone ($P = 0.037$; Fig. 3D). Interestingly, the addition of 17F1 (10 or 100 μ g) significantly ($P = 0.042$) increased mouse survival compared to use of 20C1 alone (Fig. 3D). These results support the FIC index value and further show that these MABs demonstrate a synergistic interaction.

MAB 19C9 appeared to exhibit inhibitory effects in combination with other MABs. There was a significant ($P < 0.0001$) decrease in toxin neutralization with the combination of MABs 17F1 and 19C9 in comparison to 17F1 alone; the same effect was observed when MAB 19C9 was combined with 17E7 ($P < 0.0001$) (Fig. 4A). To better characterize this observation, the MTT assay titrating both MABs in combination was repeated. Alone, MAB 19C9 showed no LeTx neutralization, while MAB 17F1 conferred $\sim 60\%$ maximum cell survival (Fig. 4C). When MAB 17F1 was titrated with a constant concentration of MAB 19C9 (30 μ g/ml), the curve mirrored that of MAB 17F1 alone. However, when a constant concentration of MAB 17F1 was mixed with various amounts of MAB 19C9, cell survival increased as the concentration of MAB 19C9 decreased, suggesting that MAB 19C9 interfered with MAB 17F1-mediated toxin neutralization, but MAB 17F1 was able to overcome this inhibition at high concentrations

(Fig. 4C). FIC analysis revealed a strong antagonistic interaction (FIC index = 25.96; Table 2). We hypothesized that this could be due to these MABs sharing the same epitope on LF; a competition ELISA between MABs 17F1 and 19C9 was consistent with that premise (Fig. 4D). To determine whether these two MABs also demonstrated an antagonistic interaction *in vivo*, mice were given 17F1 and 19C9 and challenged with strain Sterne. Although there was a trend toward increased killing with the two MABs in combination, there was no significant decrease in survival over the course of several experiments (data not shown).

A very strong antagonistic reaction was observed between MAB 17E7 and MAB 19C9 (FIC = 1,130; Fig. 4E and Table 2). However, competition ELISA between 17E7 and 19C9 suggested that whereas 17E7 interfered with 19C9 binding to LF, 19C9 did not seem to interfere with 17E7 binding to LF (Fig. 4F).

***In vitro* characterization of multiple anti-LeTx Ab interactions.** Because we observed variation in toxin neutralization efficacy among different combinations of LF-specific MABs, we also wanted to characterize multiple MAB interactions with MABs to LF and PA. Thus, we took advantage of an array of MABs to PA previously generated in our laboratory (24, 39). A cell viability screen was performed, combining the four unique IgG anti-LF MABs generated in this study and 25 MABs to PA: 3 protective, 6 toxin-enhancing, and 16 toxin-indifferent MABs. Most of the antibody combinations did not show any changes in toxin neutralization levels (data not shown), with a few exceptions. Combining the MAB to PA N5C10 with MAB 20C1 resulted in robust toxin neutralization (Fig. 5A). This combination was deemed synergistic based on the FIC index (Table 2). MTT assays combining MAB

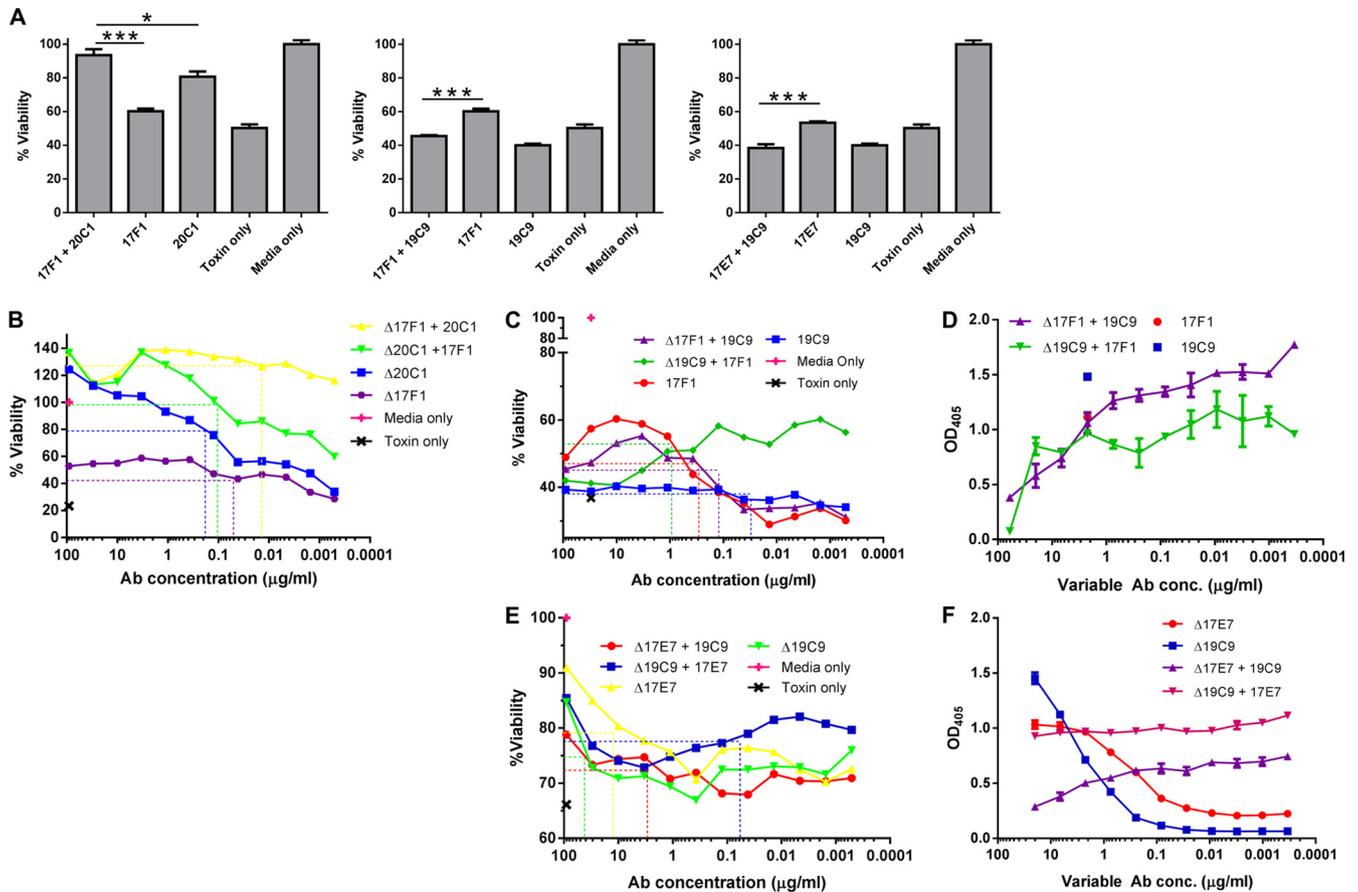


FIG 4 Combinatory effects of LF MAb on lethal toxin neutralization. (A) Macrophage viability assay of anti-LF MAb in pairs. J774 cells were incubated with 0.1 µg/ml LeTx and 10 µg of each Ab. ***, $P < 0.0001$; **, $P < 0.01$; *, $P < 0.05$. (B) Cell survival assay titrating 17F1 in combination with 20C1. Cells were incubated with 0.1 µg/ml LeTx and 30 µg/ml of the constant MAb mixed with titrated amounts of the variable MAb. (C) Cell survival assay of 19C9 and 17F1 in combination. Cells were incubated with 0.1 µg/ml LeTx and 30 µg/ml of the constant MAb and titrated 1:3 with the variable Ab starting with 90 µg/ml. (D) Competition ELISA between 17F1 and 19C9. A 96-well plate was coated with 2 µg/ml LF and then incubated with 2 µg/ml of one MAb (constant concentration) and a variable concentration of the other. Binding of the constant MAb was detected with an isotype-specific AP-conjugated secondary Ab. (E) MTT assay with 17E7 and 19C9. The assay was performed as described for panels B and C. (F) Competition ELISA between 17E7 and 19C9. Dashed lines indicate the MAb concentration at 50% of maximum cell survival for each of the survival curves shown in panels B, C, and E.

20C1 also showed strong synergistic interactions with another MAb to PA, 19D9, with an FIC index of 0.41 (Fig. 5B; Table 2).

Given the experience with botulinum toxin, where the combination of 3 MAb provided greater protection against botulinum toxin than one or two MAb alone (31), we hypothesized that combinations of 3 MAb to LF might also demonstrate synergistic effects. We tested all possible combinations of the 5 IgG MAb by MTT assay, but there were no 3-MAb combinations that showed any distinct differences in cell survival compared to each MAb alone or in pairwise combinations (data not shown).

DISCUSSION

Over the past 2 decades, passive immunotherapeutics have become an increasingly attractive approach to treatment of infectious diseases (10), including that caused by the potential bioterrorism agent *B. anthracis*. Antimicrobial drugs can kill the bacteria but do not affect preformed toxins. Consequently, MAb are an option for the development of new therapies. MAb have the advantage over polyclonal reagents in that they are specific, have high activity per protein amount since all

immunoglobulins bind to the intended target, and can be produced in virtually unlimited amounts. However, while many studies have described the generation of MAb to *B. anthracis* virulence factors (as summarized in references 11 and 13), many are only partially effective. Most MAb generated evaluated to date have been against PA; thus, we sought to produce LF-specific MAb in a mouse strain (C57BL/6) that had not been used in previous, similar studies (14–18). Of the six MAb generated in this study, two showed a moderate level of protection *in vitro* (Fig. 2D), but not *in vivo* (Fig. 3C). That the MAb to LF were not protective *in vivo* is not surprising, as some other MAb generated to LF have not been protective in mice (20). Further, while all the MAb generated in this study reacted with LF, some of the MAb also showed reactivity with PA and EF by ELISA, but not by Western analysis. This phenomenon is likely due to recognition of cross-reactive epitopes that are known to exist in anthrax toxin components (32). Interestingly, the MAb that did not show any cross-reactivity to other anthrax toxin components was 20C1, so perhaps this specificity contributed to its toxin neutralization capability.

TABLE 2 FIC indices for multiple MAb interactions

MAb(s) ^a	Survival (%)			Ab concn (μg/ml) for 50% survival	FIC ^b	FIC index ^c
	Maximum	Minimum	50%			
Δ17F1	58.76	28.74	43.75	0.05		
Δ20C1	124.55	33.88	79.22	0.18		
Δ17F1 + 20C1	139.05	114.26	126.65	0.01	0.28	
Δ20C1 + 17F1	137.07	59.79	98.43	0.11	0.61	0.89
Δ17F1	60.35	28.96	44.65	0.40		
Δ19C9	40.28	34.10	37.19	0.05		
Δ17F1 + 19C9	55.28	31.18	43.23	0.18	0.45	
Δ19C9 + 17F1	60.21	40.63	50.42	1.25	25.50	25.96
Δ17E7	90.98	70.28	80.63	11.10		
Δ19C9	84.71	66.93	75.82	39.80		
Δ17E7 + 19C9	78.81	67.95	73.38	2.37	0.21	
Δ19C9 + 17E7	85.44	72.83	79.14	0.04	1,130.68	1,130.90
Δ20C1	54.30	17.00	35.65	2.95		
ΔN5C10	63.58	15.88	39.73	0.43		
Δ20C1 + N5C10	83.99	60.04	72.01	0.04	0.02	
ΔN5C10 + 20C1	90.58	39.48	65.03	0.08	0.20	0.21
Δ20C1	44.84	11.94	28.39	1.62		
Δ19D9	73.42	12.31	42.86	1.79		
Δ20C1 + 19D9	92.71	61.29	77.00	0.41	0.26	
Δ19D9 + 20C1	96.48	22.79	59.63	0.27	0.15	0.41

^a MAbs preceded by a Greek capital delta (Δ) are “variable antibodies,” i.e., used at various concentrations (see Fig. 4 and 5).

^b FIC was calculated as follows: Ab concentration of the variable antibody A that yields 50% of maximum cell survival when in combination with a constant concentration of antibody B divided by the Ab concentration at the 50% of maximum cell survival with antibody A alone.

^c The FIC index was defined as the sum of the FICs for each antibody pair. An interaction is defined as synergistic at an FIC index of <1, additive at an FIC index of 1, indifferent at an FIC index between 1 and 2, and antagonistic at an FIC index of >2. Adapted from references 27, 28, and 29.

Characterization of this MAb panel shows that the avidity and binding capacity of these MAbs does not determine their toxin neutralization capacity. All of the MAbs tested bound strongly to LF, yet only one appeared to show any effective toxin neutralization. This suggests that the epitope recognized by the MAb is the primary factor of protective efficacy. The same has been found for MAbs directed against other microbial virulence factors, such as MAbs targeting the capsule of *Cryptococcus neoformans* (33). However, a previous study generating Ab fragments to PA showed a strong correlation between antigen affinity and toxin neutralization (34). This raises the possibility that the correlation between binding affinity and neutralization efficiency is antigen-antibody specific.

None of the MAbs to LF generated in this study were protective *in vivo* when used as single agents. Although this result is disappointing, it is consistent with the recent finding that most toxin binding MAbs are not protective (13). In contrast, LF-immunized mice showed 100% survival when challenged with strain Sterne (Fig. 3A). That LF-immunized mice showed significantly greater survival than nonimmunized mice suggested that protection from LeTx intoxication requires a polyclonal response or that the MAbs with protective efficacy were not recovered by our screening technique. To establish that protection in LF-immunized mice was mediated by Ab, a passive transfer experiment was performed using sera from LF-immunized mice. The sera from these mice provided significant protection to naïve mice challenged with strain

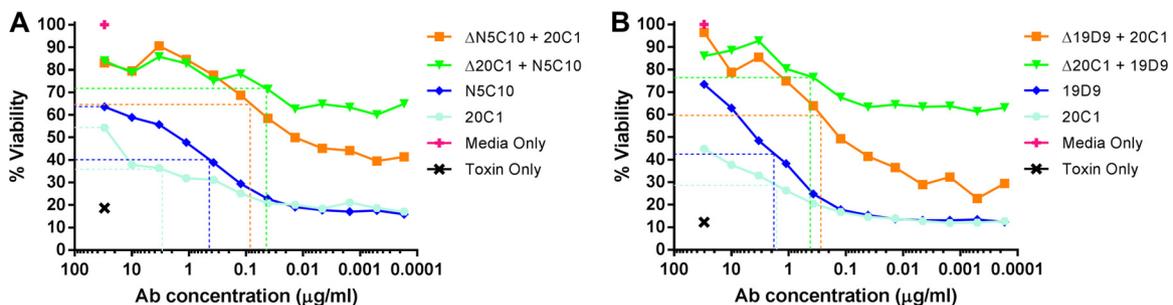


FIG 5 Combinatory effects of anti-LF and anti-PA MAbs *in vitro*. (A and B) MTT assays titrating LF MAb 20C1 with two MAbs to PA, N5C10 (A) and 19D9 (B). Mouse macrophage cells were incubated with 0.1 μg/ml LeTx, along with one MAb at a constant concentration of 5 μg/ml mixed with another MAb serially diluted 1:3 starting with 30 μg/ml. Dashed lines indicate the MAB concentration at 50% of maximum cell survival for each curve.

Sterne (Fig. 3B). A recent review summarizing the progress of anthrax vaccine development (7) argued that neutralization of PA is necessary for protection from anthrax intoxication, and studies in humans (6) have shown that Abs to LF or EF do not contribute to toxin neutralization. However, our results support early studies with rodents (35) in which LF alone was highly protective and suggest that sufficient protection can be achieved by immunization with LF protein and without the presence of PA as an immunogen.

A limitation of MABs as prophylactic and therapeutic agents against infectious diseases is that pathogens are often antigenically variable and have redundant mechanisms of virulence. One option is to combine MABs into therapeutic cocktails, but until recently this option was difficult to implement due to regulatory and cost concerns. However, a MAB cocktail has now been shown to be effective for the prophylaxis of rabies (36), providing an important precedent for future MAB development, and there is now great interest in the efficacy of combinations. While the use of individual MABs to LF generated here is not promising as single therapeutic components, combinations of MABs manifested different protective efficacies, some of which were synergistic. To that end, two LF MABs, 17F1 and 20C1, showed synergy *in vitro* and *in vivo*. Adding 17F1 to 20C1 significantly increased protection from strain Sterne in mice, but the same effect was not observed when 20C1 was added to 17F1, despite 20C1 being the most protective MAB *in vitro*. Further, giving equal amounts of each MAB to mice did not increase survival compared to that provided by each MAB alone, suggesting that an optimal concentration of each MAB is necessary to maximize protection. Conversely, MAB 19C9 demonstrated antagonistic interactions with two other MABs to LF, 17E7 and 17F1, *in vitro*. Again, these results indicate that there is a narrow range of Ab concentrations in which these synergistic or antagonistic interactions can be observed, emphasizing that combinations of MABs must be carefully characterized if considered for therapeutic applications. Furthermore, the finding that blocking FcR reduced the neutralizing efficacy of some of these antibodies shows that their protective activity is partially dependent on this receptor, as recently described (24).

When we screened these MABs to LF with a library of MABs to PA, we discovered several MAB combinations that demonstrated synergy, resulting in increased cell protection *in vitro*. These results indicate that multiple MABs could be a more effective form of therapeutics than individual MABs, as has been observed with MABs to LeTx (20, 21) and with MABs to other bacterial toxins (31). Perhaps the combination of MABs to multiple toxin components, as well as other virulence factors such as the capsule, would provide even greater protection (11).

The FIC index is used for evaluating drug combinations, but this approach has not been applied to antibodies. In this study, we pioneered the use of FIC to investigate the efficacy of MAB combinations. The applicability of the FIC formalism to antibody combinations was uncertain, since antibodies work through various mechanisms, including engagement of Fc receptors, and it was unclear whether the simple mathematics used for drug comparisons will hold with these biological agents. Calculation of FIC indices for MAB combinations that appeared to be synergistic or antagonistic subjected these interactions to a more rigorous analysis than previous studies claiming such MAB-MAB interactions (20, 21, 37, 38). However, FIC calculations must be carefully cal-

culated from curves with a significant slope, otherwise the calculated value will not accurately reflect the nature of the interaction.

In summary, our results provide additional evidence that antibodies to LF can contribute to protection against anthrax and support the development of this antigen as a component of the next generation of vaccines against anthrax. Moreover, the synergy demonstrated between the LF and PA MABs suggests that greater toxin-neutralizing efficacy can be achieved by combining antibodies with different toxin specificities. However, the protective efficacy of MAB combinations was unpredictable, suggesting that the outcome of mixing MABs must be determined empirically. Our results illustrate a daunting complexity in Ab combinations and imply that these interactions should be carefully studied in order to generate the most effective monoclonal antibody-based therapeutics.

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