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A High-Affinity Monoclonal Antibody to Anthrax Protective Antigen Passively Protects Rabbits before and after Aerosolized *Bacillus anthracis* Spore Challenge

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We have developed a therapeutic for the treatment of anthrax using an affinity-enhanced monoclonal antibody (ETI-204) to protective antigen (PA), which is the central cell-binding component of the anthrax exotoxins. ETI-204 administered preexposure by a single intravenous injection of a dose of between 2.5 and 10 mg per animal significantly protected rabbits from a lethal aerosolized anthrax spore challenge (~60 to 450 times the 50% lethal dose of *Bacillus anthracis* Ames). Against a similar challenge, ETI-204 administered intramuscularly at a 20-mg dose per animal completely protected rabbits from death (100% survival). In the postexposure setting, intravenous administration of ETI-204 provided protection 24 h (8 of 10) and 36 h (5 of 10) after spore challenge. Administration at 48 h postchallenge, when 3 of 10 animals had already succumbed to anthrax infection, resulted in the survival of 3 of 7 animals (43%) for the duration of the study (28 days). Importantly, surviving ETI-204-treated animals were free of bacteremia by day 10 and remained so until the end of the studies. Only 11 of 51 ETI-204-treated rabbits had positive lung cultures at the end of the studies. Also, rabbits that were protected from inhalational anthrax by administration of ETI-204 developed significant titers of PA-specific antibodies. Presently, the sole therapeutic regimen available to treat infection by inhalation of *B. anthracis* spores is a 60-day course of antibiotics that is effective only if administered prior to or shortly after exposure. Based upon results reported here, ETI-204 is an effective therapy for prevention and treatment of inhalational anthrax.

Bacillus anthracis is a spore-forming bacterium that can cause illness and death in exposed animals and humans (3). There are three forms of anthrax: cutaneous, gastrointestinal, and inhalational (27). Exposure to aerosolized *B. anthracis* spores may cause inhalational anthrax, the most deadly form of the disease. The anthrax-laced letters that were sent in the wake of the 11 September 2001 terrorist attacks on the World Trade Center and the Pentagon have made tragically clear the urgency of developing effective prophylactic and therapeutic treatments for this infection. A total of 11 confirmed cases of inhalational anthrax and 8 cases of cutaneous anthrax were reported during that incident. Five Americans died of inhalational anthrax despite aggressive antibiotic treatment (14).

B. anthracis secretes three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), which comprise the two exotoxins of anthrax (27). PA (83 kDa), which is the central component of the anthrax toxins, initially binds to ubiquitously expressed cell surface receptors (2, 5, 9, 23, 34, 41). This binding is followed by cleavage of PA by cell-associated furin-like proteases, releasing a 20-kDa fragment (15, 18) to produce the activated form, PA_{63} (63 kDa). The next steps are formation of a heptamer of PA_{63} molecules and binding of LF (or EF) to PA_{63} (25, 28, 31, 36). The PA_{63}-LF (or PA_{63}-EF) complexes are internalized, likely via a lipid raft-mediated process, and within the acidic environment of the endosomes, LF and EF are translocated into the target cell cytoplasm (8, 26) where they exert their toxic effects (4, 17, 37). PA by itself has no known deleterious effects. Anthrax toxins are required for massive bacteremia, since the toxins exert strong antiphagocytic effects that appear to favor the growth and spread of vegetative bacilli (29).

There is presently an unmet need for an antitoxin therapeutic as a stand-alone agent or as an adjunct to therapy with antibiotics and/or vaccination. Antibiotic treatment of inhalational anthrax victims is effective if started shortly after exposure but may be less effective if delayed even by hours (12). Use of an antitoxin antibody could be an important stand-alone therapy against antibiotic-resistant strains of anthrax.

The central role of PA in the pathophysiology of anthrax makes it an excellent therapeutic target. Vaccination with the PA-based human anthrax vaccine (6) or purified PA (13, 35, 40) results in the development of a protective immune response. Passive immunization with polyclonal antibodies against toxin proteins, particularly PA, is highly protective from challenge with *B. anthracis* spores (1, 16, 21). Moreover, antibody titers against PA correlate with protective immunity against spore challenge (22, 32, 33). Antibody-based treatment for anthrax will almost certainly need to be monoclonal in origin due to difficulties with the large-scale manufacture and quality control of polyclonal preparations.

Here, we report the activity of an affinity-enhanced, chimeric, deimmunized human immunoglobulin G1 (IgG1) monoclonal antibody (MAb) that targets and neutralizes PA in passive protection of animals against inhalational anthrax. The MAb as a stand-alone agent protects rabbits from death when it is administered before or after exposure to *B. anthracis* spores.

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MATERIALS AND METHODS

MAb engineering. The anti-PA MAb ETI-204 is an affinity-enhanced, chimeric deimmunized MAb that was generated from murine MAb 14B7. The generation of 14B7 has been described previously (19). Mutations that enhance affinity of 14B7 single-chain Fv (scFv) have been reported (24), comprising three amino acid substitutions within the variable region that enhanced scFv affinity greater than 50-fold compared to a wild-type 14B7 scFv and correspondingly increased lethal toxin neutralization in a rat model. We have generated a chimeric antibody containing the enhanced versions of the murine 14B7 V_H and V_L genes fused to human $\nu 1$ and K constant regions, respectively. In addition, the V_H and V_L segments were subjected to further modification to reduce immunogenicity (BioVation, Aberdeen, United Kingdom), a strategy referred to as DeImmunisation, which entails modifying canonical human T-cell stimulatory motifs to reduce immunogenic potential.

An NS0 cell line producing ETI-204 IgG was grown in stir cells in serum-free medium, and IgG was purified by Protein A affinity chromatography (Amersham Biosciences, Piscataway, N.J.). Purity of the IgG was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was found to be >90% (data not shown). The purified IgG was tested for binding to PA by an enzyme-linked immunosorbent assay (ELISA) using PA-coated plates (data not shown). The purified MAb was also tested for the presence of endotoxin with a commercial kit (Associates of Cape Cod, Cape Cod, Mass.) and was found to have <1 endotoxin unit/ml in the final formulation.

Affinity measurements. Affinities of MAbs were determined by using a Biacore 3000 instrument (Piscataway, N.J.). Rabbit anti-human or rabbit anti-mouse polyclonal antibodies (Jackson ImmunoResearch, East Grove, Pa.) were covalently linked to a CM5 chip via amine coupling per the manufacturer's instructions (Biacore Inc.). The anti-PA MAb (ligand) was captured on the rabbit anti-human- or rabbit anti-mouse-coated chip, and PA (analyte) was passed over the captured MAb at various concentrations (25 to 0.2 nM). All MAbs and PA were diluted in BiaRunning buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20, pH 7.4) (Biacore Inc.). After each MAb-PA cycle, the chip was regenerated by using 20 mM HCl for 30 seconds at 50 μl/min. The sensograms generated were analyzed with BIAevaluation software (Biacore Inc.) to yield the on rate (k_{on}, in molar^{-1} second^{-1}), off rate (k_{off}, in second^{-1}), and dissociation constant (K_D, in nanomolar).

Lethal toxin neutralization assay. Neutralization of lethal toxin (LeTx) cytotoxicity by anti-PA MAbs and rabbit sera was performed as previously described (20) with a few modifications. Recombinant PA and LF were purchased from List Biologicals (Campbell, Calif.). Wells of 96-well tissue culture microtiter plates were seeded with 200,000 RAW 264.7 cells (American Type Culture Collection, Manassas, Va.) per well. LeTx components (PA and LF) were added simultaneously to ETI-204, 14B7, diluted rabbit sera, or tissue culture medium and incubated for 1 h at 37°C prior to addition to RAW 264.7 macrophages. The final concentration of LeTx used was 80 ng/ml (80 ng of PA/ml plus 80 ng of LF/ml). LeTx was not added to control wells. After a 4-h incubation of the LeTx reaction mixture with macrophages at 37°C, MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] was added to the cells for 1 h at 37°C. Cells were then lysed, and the colored formazan product was solubilized by addition of lysing-solubilization buffer (11). After an overnight incubation at 37°C, the plates were read at 570 nm on a plate reader, and the data was analyzed with SoftMaxPro software (Molecular Devices, Sunnyvale, Calif.). The concentration or titer that resulted in 50% neutralization (50% effective concentration [EC_{50}]) was taken as a measure of LeTx neutralization activity.

Aerosol challenge of rabbits with *Bacillus anthracis*. All aerosolized anthrax spore challenge studies in rabbits were performed by Battelle (West Jefferson, Ohio) in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility following Institutional Animal Care and Use Committee (IACUC) review and approval of all protocols. New Zealand White (NZW) rabbits (2.0 to 2.9 kg) were randomized into groups containing equal numbers of male and female animals. For preexposure prophylaxis, rabbits on day 0 received a single dose of ETI-204 administered intravenously (i.v.) (1.25 to 10 mg of IgG/rabbit via the medial ear artery) or intramuscularly (i.m.) (20 mg of IgG/rabbit over multiple sites in the quadriceps muscle) or phosphate-buffered saline (PBS) administered i.v. as a control 30 to 45 min before anthrax spore challenge. To evaluate postexposure efficacy, rabbits were challenged with spores on day 0 and then given single i.v. ETI-204 injections of 10 mg of IgG/rabbit at 24, 36, or 48 h postchallenge or PBS i.v. at 48 h postchallenge. Rabbits were challenged via a muzzle-only exposure system according to standard protocols. The target aerosol exposure for the challenge was 200 times the 50% lethal dose (LD_{50}) of the Ames strain (Ames $LD_{50} = 1.05 \times 10^5$ spores [42]). Actual challenge doses were determined from the starting concentration and a cumulative minute volume gathered throughout the exposure.

All animals were observed twice daily for 28 days postchallenge. Blood and serum were collected prechallenge and on days 1, 2, 7, 10, 14, 21, and 28 postchallenge. When possible, blood and serum samples were collected from animals that were moribund or recently dead ("at death") and analyzed for the presence of *B. anthracis*. On day 28, all survivors were euthanized, and the lungs, spleens, and intrathoracic lymph nodes were harvested and cultured. Serum samples were sterile filtered and deemed noninfectious by culturing prior to ELISA analysis to determine serum ETI-204 levels and rabbit anti-PA titers (described below) and by lethal toxin neutralization (described above).

Determination of ETI-204 levels in rabbit sera. The concentration of ETI-204 in rabbit serum samples was determined by using a human IgG ELISA. Goat $F(ab')_2$ anti-human IgG Fc (Rockland Immunochemicals, Gilbertsville, Pa.) at a concentration of 1 μg/ml was coated onto wells (100 μl/well) of high binding plates (Costar Corp., Cambridge, Mass.) and incubated overnight at 4°C. The unbound antigen was washed out, and the wells were blocked with Superblock (300 μl/well; Pierce Biotech, Rockford, Ill.) for 1 h at room temperature. The Superblock was then aspirated, and the plates were allowed to dry at room temperature before use. The standard used was a chimeric human IgG1K MAb (rituximab; Biogen IDEC, Cambridge, Mass.). The samples and standard were applied to the anti-human IgG Fc-coated plate for 30 min at 37°C followed by three washes. This process was followed by addition of goat anti-human kappa-horseradish peroxidase (HRP) conjugate for 30 min at 37°C and washing again three times. The anti-human kappa-HRP conjugate was detected with tetramethylbenzidine substrate (100 μl per well) that was allowed to react for 15 min at room temperature, and the reaction was stopped by using 2 N H_2SO_4 (50 μl per well). The plates were read within 10 min at 450 nm on a plate reader (SpectraMax 340 pc; Molecular Devices).

The ELISA was tested for rabbit serum interference and nonspecific binding. A 150-fold dilution of rabbit serum was determined to be adequate to remove any serum interference (data not shown).

Measurement of rabbit anti-PA antibody response. An ELISA was used to measure the immune response against PA in the rabbits challenged with anthrax spores. Costar high binding plates were coated with PA at a concentration of 0.6 μg/ml and incubated overnight at 4°C. The unbound antigen was washed out, and the wells were blocked with Superblock reagent (300 μl/well) for 1 h at room temperature. The blocking solution was then aspirated, and plates were allowed to dry at room temperature before use. A starting dilution of 1:100 of each serum sample was used, since this was determined not to cause any interference in the signal of the assay (data not shown). The diluted serum samples were incubated on the plate for 30 min at 37°C. A goat anti-rabbit IgG HRP conjugate (Santa Cruz Biotech, Santa Cruz, Calif.) was added for 30 min at 37°C, and color was developed by using tetramethylbenzidine substrate (100 μl per well) for 15 min at room temperature. The reaction was stopped by addition of 2 N H_2SO_4 (50 μl per well). The plates were read within 10 min at 450 nm on a plate reader. The serum dilution that resulted in an optical density signal of 1.0 was used as a measure of the response (titer).

Pharmacokinetic parameters. To establish the pharmacokinetics (PK) of ETI-204 injected by the i.v. and i.m. routes, a study was conducted at Perry Scientific (San Diego, Calif.) in an AAALAC-approved facility following an IACUC-approved protocol. NZW rabbits (three per group, 2.3 to 2.7 kg) were injected with ETI-204 at 10 mg/rabbit i.v. via the medial ear artery, 10 mg/rabbit i.m., or 20 mg/rabbit i.m. via multiple injection sites in the quadriceps muscle. Blood samples were collected from the ear artery of each animal at 1, 2, 4, 8, 24, and 32 h preinjection and 2, 3, 4, 5, 6, 8, 10, 12, 15, 17, 19, 21, 23, 25, 28, 30, 32, 35, 37, 39, and 42 days postinjection. Sera were obtained after clotting and centrifugation and stored frozen at −80°C until analysis.

The serum concentration data were analyzed by Microconstants Inc. (San Diego, Calif.). Descriptive PK parameters were determined by standard model-independent methods (30) based on individual serum concentration-time data for ETI-204. The predose time point for i.m. administration was assigned a 0 value, and the predose time point for i.v. administration was assigned the concentration value of the first time point for PK calculations. The maximal serum concentration (C_{max}), time taken to reach C_{max}, area under the curve, systemic clearance, volume of distribution at steady state, terminal half-life, and absolute bioavailability (F) were analyzed for all three data sets.

Statistical analysis of survival studies. Kaplan Meier analysis was used for evaluation of survival studies. Survival data were analyzed with GraphPad's Prism version 4 statistical analysis software (San Diego, Calif.). A two-tailed log rank test was used to determine statistical significance between two groups. A P value of <0.05 was considered to be statistically significant.

TABLE 1. Kinetic constants for murine 14B7, chimeric 14B7, and ETI-204[a]

Sample	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)
Murine 14B7	5.23×10^5	1.96×10^{-3}	3.74
Chimeric 14B7 (human IgG1 14B7)	4.35×10^5	1.53×10^{-3}	3.51
ETI-204	4.57×10^5	1.50×10^{-4}	0.33

[a] The affinity of ETI-204 relative to that of murine MAb 14B7 was 11.3 and relative to that of chimeric MAb 14B7 was 10.6. Values represent the average of results from four separate measurements.

RESULTS

Engineering of ETI-204. We hypothesized that to be an effective therapeutic, an anti-PA MAb would have to bind an epitope of PA that interferes with cellular receptor binding and exhibits a dissociation constant (K_D, k_{off}/k_{on}) close to or below the measured PA-receptor dissociation constant (0.17 to 1 nM [5, 9, 41]). To confirm that ETI-204 had enhanced affinity for PA compared to 14B7, kinetic constants were determined by using a Biacore 3000 instrument (Table 1). The dissociation constant of ETI-204 for PA (K_D = 0.33 nM) was found to be 11.3-fold lower than that of 14B7 (K_D = 3.74 nM). Significantly, the dissociation constant for ETI-204 compares favorably with that of the PA-receptor complex (5, 9, 41).

Presumably due to the affinity enhancement, ETI-204 provides protection from LeTx-mediated cytotoxicity at a significantly lower concentration than 14B7 in an in vitro LeTx neutralization assay (Fig. 1). A fourfold-lower concentration of ETI-204 (0.08 μg/ml) compared to 14B7 (0.32 μg/ml) was required for 50% neutralization. Control mouse or human IgG did not neutralize LeTx cytotoxicity (data not shown).

Prechallenge administration of ETI-204. The ability of ETI-204 to protect against anthrax in vivo was tested in the NZW rabbit aerosolized spore challenge model (42). NZW rabbits (n = 9) were given a single i.v. injection of 10 mg of ETI-204 (approximately 4 mg/kg of body weight) 30 to 45 min prior to aerosol spore challenge with 107 to 218 times the LD_{50} (median, 139 times the LD_{50}) of the Ames strain of *B. anthracis*. The control group (n = 5) received PBS 30 to 45 min prior to aerosol spore challenge with 96 to 244 times the LD_{50} (median, 182 times the LD_{50}) of *B. anthracis* Ames spores. ETI-204 at a 10-mg dose completely protected rabbits out to day 28, whereas all the rabbits in the control group died by day 5 (P = <0.001) (Fig. 2A).

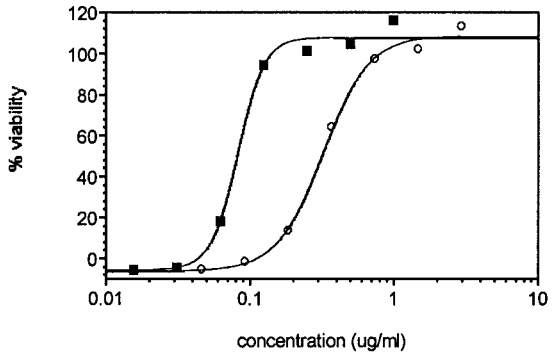

FIG. 1. Neutralization of LeTx cytotoxicity by ETI-204 and 14B7. Increasing concentrations (0 to 3 μg/ml) of either ETI-204 (■) or 14B7 (○) were mixed with LeTx (80 ng of PA/ml and 80 ng of LF/ml) and added to RAW 264.7 macrophages. The percent viability is plotted for each MAb concentration tested.

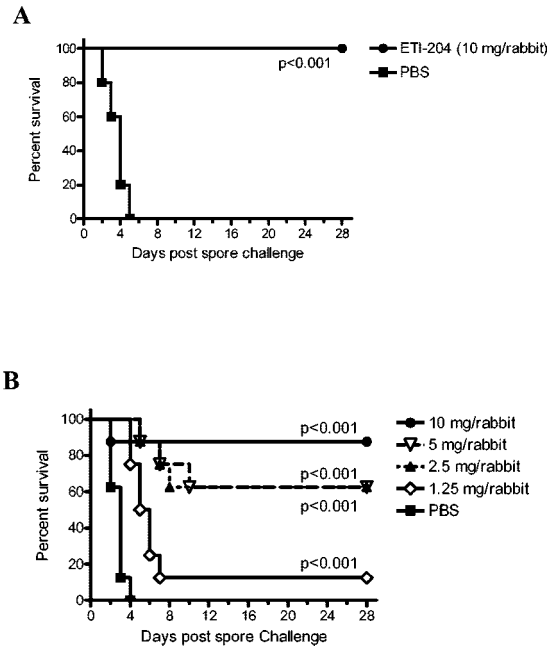

FIG. 2. Protection of rabbits from aerosolized anthrax spore challenge by prechallenge i.v. injection of ETI-204. (A) Kaplan Meier survival curves of NZW rabbits injected i.v. with 10 mg of ETI-204 (n = 9) or PBS (n = 5) 30 to 45 min before aerosol challenge with *B. anthracis* Ames spores (median, 163 times the LD_{50}). (B) Kaplan Meier survival curves of NZW rabbits (n = 8) injected i.v. with 10, 5, 2.5, and 1.25 mg of ETI-204 or PBS 30 to 45 min before aerosol challenge with *B. anthracis* Ames spores (median, 286 times the LD_{50}).

Bacteremia was monitored at multiple time points after challenge, and the presence of *B. anthracis* in the organs was determined for moribund and dead animals as well as for survivors after euthanasia at day 28. While bacilli were detected in the blood of control rabbits on day 2 postexposure and in moribund and recently dead animals, none of the ETI-204-treated animals were positive for bacteremia at any time point (Table 2). The results for lungs, lung-associated lymph nodes (including mediastinal), and spleens on day 28 for ETI-204-treated animals showed that two of nine, one of nine, and zero of nine were positive for *B. anthracis*, respectively (Table 2). Thus, by day 28, all animals that received ETI-204 survived challenge, and the majority were free of infection. A significant finding is that the lungs, the primary site of spore entry, were negative for infection in most of the treated animals, suggesting that ETI-204 treatment not only provides protection but also aids in the efficient clearance of the bacteria.

To determine the minimum effective dose of ETI-204, groups of rabbits were given decreasing doses of the MAb (10, 5, 2.5, and 1.25 mg per rabbit; eight per group) administered i.v. 30 to 45 min prior to aerosol challenge with *B. anthracis* Ames spores (92 to 435 times the LD_{50}; median, 289 times the LD_{50}). The control group (n = 8) received PBS 30 to 45 min prior to aerosol spore challenge with *B. anthracis* Ames spores (163 to 435 times the LD_{50}; median, 295 times the LD_{50}).

TABLE 2. Presence of bacteria in blood and selected organs of rabbits from a prechallenge study[a]

Group	No. of animals positive for bacteria in blood/total no. of animals								No. of animals positive for bacteria in organs at day 28/total no. of animals		
	Day 1	Day 2	Day 7	Day 10	Day 14	Day 21	Day 28	At death	Lungs	Lung-associated lymph nodes	Spleen
ETI-204	0/9	0/9	0/9	0/9	0/9	0/9	0/9	N/A	2/9	1/9	0/9
PBS	0/5	3/5						4/5			

[a] Rabbits were injected i.v. with ETI-204 (10 mg) or PBS 30 to 45 min prior to exposure to aerosolized *B. anthracis* Ames strain spores. Blood samples taken on various days after exposure were cultured for the presence of *B. anthracis.* At the end of the study (day 28), surviving animals were euthanized, and organ homogenates were tested for the presence of *B. anthracis.* N/A, not applicable.

There was a significant increase in survival of rabbits that received 2.5 mg (five of eight; $P < 0.001$), 5 mg (five of eight; $P < 0.00l$), and 10 mg (seven of eight; $P < 0.001$) of ETI-204 compared to control rabbits (Fig. 2B). In addition, a significant increase in time to death (TTD) was observed for rabbits that received 1.25 mg of ETI-204 compared to control rabbits (TTD, 5.5 versus 3.0 days; $P < 0.001$). While there was no statistical difference in survival between rabbits receiving the 2.5-, 5-, and 10-mg doses, there was a significant difference between these dose groups and animals treated with the 1.25-mg dose ($P = 0.014$ and 0.007, respectively), suggesting a loss of protective efficacy at the lowest dose.

Bacilli were detected in the blood of all of the control rabbits on days 1 and 2, in moribund or recently dead animals, and in six of eight of the rabbits receiving the 1.25-mg dose of ETI-204 (Table 3). In contrast, only 4 of 33 rabbits receiving higher doses (2.5, 5, or 10 mg i.v.) developed bacteremia, 2 of them transiently (Tables 2 and 3). Thirteen of 15 bacteremic animals died, including controls (Tables 2 and 3). The results for lungs, lung-associated lymph nodes (including mediastinal), and spleens for animals receiving higher doses of ETI-204 (2.5, 5, or 10 mg i.v.) on day 28 showed that 7 of 26, 1 of 26, and 0 of 26 were positive for *B. anthracis*, respectively (Tables 2 and 3).

Postchallenge administration of ETI-204. The efficacy of ETI-204 in protecting rabbits from death due to anthrax infection when administered at various time points postchallenge was also tested. NZW rabbits (10 per group, 5 males and 5 females) were exposed to aerosolized *B. anthracis* Ames spores (62 to 267 times the LD_{50}; median, 172 times the LD_{50}) followed by i.v. administration of a single 10-mg dose of ETI-204 at 24, 36, or 48 h postchallenge. The control group ($n = 10$) received PBS i.v. 48 h after exposure to aerosolized *B. anthracis* Ames spores (104 to 214 times the LD_{50}; median, 182.5 times the LD_{50}). TTD or euthanasia was recorded for 28 days after exposure to spores (Fig. 3).

ETI-204 protected 80% (8 of 10; $P < 0.001$) of rabbits from death through day 28 when administered 24 h after exposure to anthrax spores. When ETI-204 was administered 36 h postexposure, 50% (5 of 10; $P = 0.041$) of the animals were protected. Of the animals treated at 48 h postchallenge, all of the controls died by day 4, and there were three survivors in the ETI-204 group (3 of 7; $P = 0.424$). Several rabbits died before treatment could be administered at the 48-h time point (1 of 10 in the PBS group and 3 of 10 in the ETI-204 group). Of the seven animals that did receive ETI-204, two animals died within hours of being treated. These animals did not display any outward signs of illness before death, consistent with a previous study that reported that rabbits only exhibited brief periods of excitation and hyperactivity within hours or minutes before death (42).

All rabbits in this study that were bacteremic died (Table 4). One of the deaths in the group treated with ETI-204 at 24 h postchallenge was on day 8, 4 days after the latest death in the control group; that animal was bacteremic on day 7. At day 28, all surviving animals were free of detectable bacteria in the blood, lungs, lung-associated lymph nodes (including mediastinal), and spleens. These data demonstrate that ETI-204 is effective in a postexposure setting despite the sensitivity of the rabbit model to rapid lethality from anthrax infection.

Generation of an antibody response in ETI-204-treated rabbits. To examine whether passive protection with ETI-204 would result in the development of immunity in rabbits, sera were tested for anti-PA antibodies. All rabbits treated with 10 mg of ETI-204 (from Fig. 2A) had anti-PA antibody titers ≥1:800 by anti-PA ELISA (Fig. 4A). The anti-PA response for eight of the nine rabbits started between days 7 and 10, a time

TABLE 3. Presence of bacteria in blood and selected organs of rabbits from a prechallenge minimum effective dose study[a]

Group	No. of animals positive for bacteria in blood/total no. of animals (bacteremic animal ID)								No. of animals positive for bacteria in organs at day 28/total no. of animals		
	Day 1	Day 2	Day 7	Day 10	Day 14	Day 21	Day 28	At death	Lungs	Lung-associated lymph nodes	Spleen
ETI-204, 10 mg	0/8	0/8	0/7	0/7	0/7	0/7	0/7	0/1	4/7	0/7	0/7
ETI-204, 5 mg	1/8 (K261)	1/8 (K266)	1/7 (K283)	0/6	0/5	0/5	0/5	1/3 (K283)	0/5	0/5	0/5
ETI-204, 2.5 mg	1/8 (K297)	1/8 (K297)	1/6 (K297)	0/5	0/5	0/5	0/5	1/3 (K297)	1/5	0/5	0/5
ETI-204, 1.25 mg	0/8	0/8	1/2	0/1	0/1	0/1	0/1	6/7	0/1	0/1	0/1
PBS	2/8	8/8						7/8			

[a] Rabbits were injected i.v. with ETI-204 (10, 5, 2.5, or 1.25 mg) or PBS 30 to 45 min prior to exposure to aerosolized *B. anthracis* Ames strain spores. Blood samples taken on various days after exposure were cultured for the presence of *B. anthracis.* At the end of the study (day 28), surviving animals were euthanized, and organ homogenates were tested for the presence of *B. anthracis.*

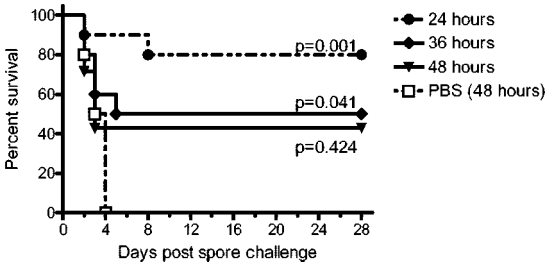

FIG. 3. Protection of rabbits from aerosolized anthrax spore challenge by postchallenge i.v. injection of ETI-204. Kaplan Meier survival curves of NZW rabbits ($n = 10$) injected i.v. with 10 mg of ETI-204 24, 36, or 48 h after challenge or PBS 48 h after aerosol challenge with *B. anthracis* Ames spores (median, 172 times the LD_{50}).

when ETI-204 was almost undetectable in the sera of rabbits (data not shown). For one rabbit, the anti-PA response was first detectable at day 14, but its titer was ultimately the highest of the group. The median titer after 28 days was 1:2,635 (1:800 to 1:12,270). In addition, the anti-PA antibodies present in the sera at day 28 protected macrophages in an in vitro LeTx cytotoxicity experiment, and the protective ability correlated with the anti-PA titer (Fig. 4B; $R^2 = 0.95$). Rabbit sera from naïve animals did not cause any neutralization in this assay (data not shown). Sera from rabbits that survived out to day 28 in the postchallenge study also had a similar anti-PA response (median titer, 1:3,668; range, 1:487 to 1:36,153; data not shown). The LeTx-neutralizing ability (EC_{50}) of those samples was not tested.

ETI-204 pharmacokinetics and prechallenge i.m. administration of ETI-204. In the event of an anthrax attack, the delivery of ETI-204 via the i.m. route would be more rapid and logistically easier for treating larger numbers of people than i.v. administration. To determine the feasibility of administering a dose of ETI-204 i.m. that reached levels in serum equivalent to the i.v. protective dose, pharmacokinetic analysis was conducted. ETI-204 was administered as a single 10-mg dose i.v. or a single 10- or 20-mg dose i.m., and serum concentrations of ETI-204 were determined at various times postchallenge. As expected, ETI-204 injected i.m. peaked in serum at a later time point than when the MAb was injected i.v. (Fig. 5A). The bioavailability (F) of ETI-204 administered i.m. was high (51% for the 10-mg dose and 63% for the 20-mg dose) (Table 5). Interestingly, the rate of clearance of ETI-204 given i.v. in this study was significantly slower than the rate in rabbits that were challenged with anthrax spores (see above; data not shown).

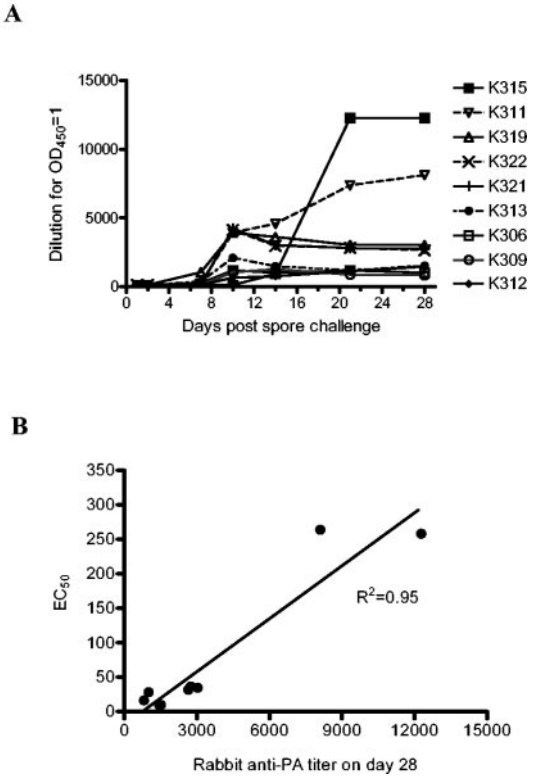

FIG. 4. Development of anti-PA response in surviving ETI-204-administered rabbits. (A) Anti-PA titers from ETI-204-treated animals from Fig. 2A. The titers were measured as the reciprocal of the serum dilution that resulted in an optical density at 450 nm of 1.0 in an anti-PA ELISA. (B) Day 28 sera from all ETI-204-treated rabbits in Fig. 2A were analyzed for lethal toxin neutralization activity. The dilution that neutralized 50% of LeTx activity (EC_{50}) was determined and plotted against the rabbit anti-PA titer at day 28 (panel A). The R^2 value is noted.

The levels of ETI-204 administered i.v. (10 mg/rabbit) reached a maximum serum concentration of 66 ± 3 μg/ml immediately after administration, while ETI-204 injected via the i.m. route peaked at 18 ± 1 μg/ml (10-mg/rabbit dose) and 43 ± 7 μg/ml (20-mg/rabbit dose) by 2 and 1.3 days, respectively. The concentration of ETI-204 in serum 1 day after i.m. administration of the 20-mg dose was greater than that achieved after a 10-mg i.v. administration (Fig. 5A). These data predicted that an effective concentration of ETI-204 could be achieved by i.m. administration and that this dose would provide protection from spore challenge.

TABLE 4. Presence of bacteria in blood and selected organs of rabbits from the postchallenge study[a]

Group	No. of animals positive for bacteria in blood/total no. of animals								No. of animals positive for bacteria in organs at day 28/total no. of animals		
	Day 1	Day 2	Day 7	Day 10	Day 14	Day 21	Day 28	At death	Lungs	Lung-associated lymph nodes	Spleen
ETI-204 24 h	0/10	0/10	1/9	0/8	0/8	0/8	0/8	1/2	0/8	0/8	0/8
ETI-204 36 h	0/10	3/10	0/5	0/5	0/5	0/5	0/5	3/5	0/5	0/5	0/5
ETI-204 48 h	0/7	4/7	0/3	0/3	0/3	0/3	0/3	4/4	0/3	0/3	0/3
PBS 48 h	0/9	2/9						8/8			

[a] Rabbits were injected i.v. with ETI-204 (10 mg) or PBS at various time points after exposure to aerosolized *B. anthracis* Ames spores. Blood samples taken on various days after exposure were tested for the presence of *B. anthracis.* At the end of the study (day 28), surviving animals were euthanized, and organ homogenates were tested for the presence of *B. anthracis.*

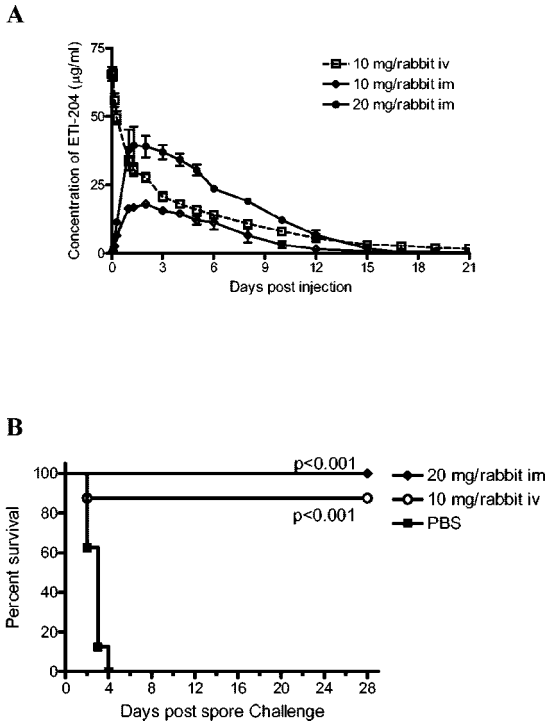

FIG. 5. Pharmacokinetics of ETI-204 and protection of rabbits from aerosolized anthrax spore challenge by prechallenge i.m. administration of ETI-204. (A) NZW rabbits ($n = 3$) were injected with 10 mg of ETI-204 i.v., 10 mg of ETI-204 i.m., or 20 mg of ETI-204 i.m. Serum samples were collected at increasing time points (5 min to 42 days), and ETI-204 levels were determined by using an anti-human IgG ELISA. (B) Kaplan Meier survival curves of NZW rabbits ($n = 8$) injected with 10 mg of ETI-204 i.v., 20 mg of ETI-204 i.m., or PBS i.v. 30 to 45 min before aerosol challenge with *B. anthracis* Ames spores (median, 268 times the LD_{50}).

To test the above hypothesis, NZW rabbits were injected with PBS i.v., ETI-204 i.v. (10 mg), or ETI-204 i.m. (20 mg) 30 to 45 min prior to aerosol challenge with *B. anthracis* Ames spores (106 to 435 times the LD_{50}; median, 268 times the LD_{50}). While all of the control animals died by day 4 (Fig. 5B), significant protection was observed when ETI-204 was injected i.v. (seven of eight; $P < 0.001$) or i.m. (eight of eight; $P < 0.001$). While none of the rabbits that received ETI-204 by the i.v. route (10 mg) were positive for bacteremia (Table 3), only one of eight rabbits that were administered ETI-204 i.m. had bacteremia, and it was transient (positive only on day 2; data not shown). The results for lungs, lung-associated lymph nodes (including mediastinal), and spleens for ETI-204-treated animals (i.m. route) showed that four of eight, zero of eight, and zero of eight were positive for *B. anthracis* (data not shown), respectively, results which are similar to those obtained with 10 mg of ETI-204 administered i.v. (Table 3). These results demonstrate the feasibility of i.m. administration of ETI-204 for protection against inhalational anthrax.

DISCUSSION

B. anthracis, the pathogen for anthrax, has been identified as a top priority biowarfare concern by the United States Department of Defense and as a Category A agent by the Centers for Disease Control and Prevention. A high-affinity monoclonal antibody therapeutic that targets anthrax toxins would be an important therapeutic addition to the options for prophylaxis and treatment of anthrax. We report here results for such a high-affinity MAb, ETI-204, which displays an enhanced ability to neutralize anthrax toxin. Most importantly, ETI-204 showed excellent preclinical efficacy as a therapeutic agent in the prevention and treatment of inhalational anthrax.

ETI-204 was derived from 14B7 (19), one of the first identified anti-PA MAbs that neutralized lethal toxin in vitro, and has a 11.3-fold-higher affinity for PA than 14B7 primarily due to a decrease in the off rate (Table 1), resulting in a longer predicted half-life of the MAb-PA complex. It should be noted that the dissociation constant for ETI-204 is similar to the dissociation constant of PA for its cellular receptors (0.170 to 1.0 nM [5, 9, 41]), perhaps explaining its improved ability to neutralize LeTx-mediated cytotoxicity (Fig. 1).

Following the favorable results from in vitro experiments, ETI-204 was tested for its ability to protect rabbits against inhalational anthrax. Administration of ETI-204 at 10 mg/rabbit by the i.v. route prior to aerosol exposure to *B. anthracis* Ames spores provided 94% protection (16 of 17) in two separate experiments (Fig. 2). Lower doses of ETI-204 (up to 2.5 mg/rabbit) also afforded significant protection compared to the vehicle control (Fig. 2B). The fact that a single dose of ETI-204 was able to protect against a robust challenge of spores suggests that high-affinity binding to PA is sufficient to prevent infection.

When ETI-204 was tested in a postchallenge scenario in rabbits, protection from death was observed upon administration of ETI-204 up to 2 days after challenge with spores (Fig. 3). A significant increase in survival (versus PBS controls) was observed when ETI-204 was administered up to 36 h postchallenge. When animals were treated at 48 h postexposure, there was increased survival compared to the PBS control, although the treatment failed to reach statistical significance in this study. This was due in part to deaths in this group prior to and immediately after ETI-204 administration. Given the rapid course of anthrax-induced lethality in rabbits, it is remarkable that a single dose of ETI-204 given as late as 48 h postexposure protected some of the animals from death.

Analysis of bacteremia data demonstrated that only 7 of 43 rabbits in three separate studies receiving protective doses of

TABLE 5. Summary of pharmacokinetic parameters of ETI-204 administration in rabbits by different routes[a]

Route of administration	i.v.	i.m.	i.m.
Dose (mg)	10	10	20
C_{max} (μg/ml)	66 ± 3	18 ± 1	43 ± 7
T_{max} (days)[b]	N/A	2.0 (1–2)	1.3 (1–4)
AUC (μg day/ml)	238 ± 20	121 ± 28	302 ± 3
Clearance (liters/day)	0.04 ± 0.004	N/A	N/A
V_{dss} (liters)	0.24 ± 0.024	N/A	N/A
$T_{1/2}$ (days)	3.8 ± 0.5	N/A	N/A
F (%)	N/A	51	63

[a] T_{max}, median time taken to reach C_{max}; AUC, area under the curve; Clearance, systemic clearance; V_{dss}, volume of distribution at steady state; $T_{1/2}$, terminal half-life; and N/A, not applicable.
[b] The numbers in parentheses for T_{max} values show the range.

ETI-204 were positive for bacteria in the lungs, the initial site of spore entry (Tables 2, 3, and 4). Only 1 of the 43 rabbits was positive for *B. anthracis* in the intrathoracic lymph nodes, and all of the rabbits were negative for *B. anthracis* in the spleen. These results suggest that neutralization and clearance of the toxin prevents the spread of bacteria. It is also possible that the MAb may be able to inhibit early stages of infection by anthrax spores, since previous studies have shown that anti-PA antibodies also have antispore activity (38, 39).

The animals that survived spore challenge after administration of ETI-204 developed an immune response against PA, and sera from these animals were able to neutralize anthrax LeTx in vitro (Fig. 4). This result indicates that while ETI-204 was able to block the lethal effects of the toxin and limit bacterial growth, PA was still presented to evoke an active immune response. Although previous studies suggest that the lower titers observed (EC_{50} < 1:100) may not be protective against a lethal spore challenge (22, 32), it is reasonable to propose that animals would rapidly develop a robust secondary response upon rechallenge, since they had developed toxin-neutralizing antibodies when they were primed by the initial infection. This is significant, since it has been shown that animals treated with antibiotics after anthrax exposure do not develop an immune response (10).

The pharmacokinetic studies show that ETI-204 was detected in the sera of rabbits for more than 10 days whether it was injected i.v. or i.m. (Fig. 5A). In vivo challenge studies demonstrated that i.m. administration of ETI-204 (20 mg/rabbit) provided complete protection of rabbits from death (Fig. 5B). Allometric scaling to humans following Food and Drug Administration guidance (7) predicts that an effective dose in humans could be as low as 100 to 200 mg, a dose that can be easily administered by either the i.v. or the i.m. route. Administration of a therapeutic MAb by the i.m. route has significant value, since it is can be accomplished faster and more efficiently than i.v. administration and would facilitate rapid treatment of a large number of exposed individuals. Moreover, i.m. injections can be self-administered by individuals with limited medical training and therefore could be used by troops on the battlefield or by first responders in the case of a terrorist attack.

The "animal rule" codified as Subpart I of 21 CFR 314 and Subpart H of 21 CFR 601 allows definitive efficacy testing of new therapeutic agents in appropriate animal models when testing in human volunteers is deemed unethical, as is the case for inhalational anthrax. The rabbit aerosolized spore challenge model is a good predictor of vaccine efficacy in nonhuman primates (6). Therefore, the efficacy data in rabbits presented here predict a favorable outcome in the nonhuman primate model, which most closely resembles anthrax infection in humans. We are confident that strong efficacy data in nonhuman primates coupled with human studies in a range of subjects to demonstrate safety will provide sufficient data for Food and Drug Administration approval of ETI-204 as an effective countermeasure against a widespread anthrax attack.

The results presented in this paper demonstrate that an enhanced-affinity monoclonal antibody shows high efficacy in a clinically relevant model of inhalational anthrax. We are currently developing ETI-204 as a therapeutic agent for human use in preexposure prophylaxis, postexposure prophylaxis, and treatment of inhalational anthrax.

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