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.
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\textsubscript{H} and V\textsubscript{L} genes fused to human V\textsubscript{H} and K\textsubscript{L} constant regions, respectively. In addition, the V\textsubscript{H} and V\textsubscript{L} segments were subjected to further modification to reduce immunogenicity (Bio-Vation, Aberdeen, United Kingdom), a strategy referred to as DeImmunisation, which entails modifying canonical human T-cell stimulatory motifs to reduce immunogenic potential.

An Nso 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 coated on BiaCore sensorsgrams. The binding of antibody to PA was monitored by using protein A-conjugated peroxidase (Amersham Biosciences) for the detection of the antigen-antibody complex. The sensograms generated were analyzed with BIAevaluation software (Biacore Inc.) to yield the on rate (k\textsubscript{on}) in molar\textsuperscript{-1} second\textsuperscript{-1}, off rate (k\textsubscript{off}) in second\textsuperscript{-1}, and dissociation constant (K\textsubscript{D}, in nanomolar).

Lethal toxin neutralization assay. Neutralization of lethal toxin (LeTx) cytotoxicity by anti-PA Mabs and rabbit serum was performed as previously described (20) with a few modifications. Recombinant PA and LF were produced 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, 34B7, 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 90 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-dimethylthiazol-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\textsubscript{50}] or concentration value of the first time point for PK calculations. The maximal serum concentration, C\textsubscript{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 investigation of survival studies. Survival data were analyzed using 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

<table>
<thead>
<tr>
<th>Sample</th>
<th>$k_{on}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_D$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine 14B7</td>
<td>$5.23 \times 10^5$</td>
<td>$1.96 \times 10^{-3}$</td>
<td>3.74</td>
</tr>
<tr>
<td>Chimeric 14B7 (human IgG1 14B7)</td>
<td>$4.35 \times 10^5$</td>
<td>$1.53 \times 10^{-3}$</td>
<td>3.51</td>
</tr>
<tr>
<td>ETI-204</td>
<td>$4.57 \times 10^5$</td>
<td>$1.50 \times 10^{-4}$</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*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).

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$_{50}$).

![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.](http://iai.asm.org/article-figures/)

![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$_{50}$).](http://iai.asm.org/article-figures/)
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; \( P < 0.001 \)), 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 strain 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\(_{50}\); 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 \( \geq 1:800 \) by anti-PA ELISA (Fig. 4A). The anti-PA response for eight of the nine rabbits started between days 7 and 10, a time

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

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals positive for bacteria in blood/total no. of animals</th>
<th>No. of animals positive for bacteria in organs at day 28/total no. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>ETI-204, 10 mg</td>
<td>0/9</td>
<td>0/5</td>
</tr>
<tr>
<td>ETI-204, 5 mg</td>
<td>1/9</td>
<td>1/8</td>
</tr>
<tr>
<td>ETI-204, 2.5 mg</td>
<td>1/8</td>
<td>1/8</td>
</tr>
<tr>
<td>ETI-204, 1.25 mg</td>
<td>0/8</td>
<td>0/5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals positive for bacteria in blood/total no. of animals (bacteremic animal ID)</th>
<th>No. of animals positive for bacteria in organs at day 28/total no. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>ETI-204, 10 mg</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>ETI-204, 5 mg</td>
<td>1/8</td>
<td>1/8</td>
</tr>
<tr>
<td>ETI-204, 2.5 mg</td>
<td>1/8</td>
<td>1/8</td>
</tr>
<tr>
<td>ETI-204, 1.25 mg</td>
<td>0/8</td>
<td>0/8</td>
</tr>
</tbody>
</table>

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\( a \) 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.
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).

The levels of ETI-204 administered i.v. (10 mg/rabbit) reached a maximum serum concentration of \( 66 \mu g/ml \) immediately after administration, while ETI-204 injected via the i.m. route peaked at \( 18 \mu g/ml \) and \( 43 \mu g/ml \) 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 studya

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
<th>At death</th>
<th>Lungs</th>
<th>Lung-associated lymph nodes</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETI-204 24 h</td>
<td>0/10</td>
<td>0/10</td>
<td>1/9</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>ETI-204 36 h</td>
<td>0/10</td>
<td>3/10</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
<td>3/5</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>ETI-204 48 h</td>
<td>0/7</td>
<td>4/7</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>4/4</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>PBS 48 h</td>
<td>0/9</td>
<td>2/9</td>
<td>8/8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 \( B.\) \( anthracis \).
LD50). While all of the control animals died by day 4 (Fig. 5B), spores (10^6 to 435 times the LD50; median, 268 times the LD50) prior to aerosol challenge with B. anthracis with PBS i.v., ETI-204 i.v. (10 mg), or ETI-204 i.m. (20 mg) 30 min before aerosol challenge with B. anthracis Ames spores (including mediastinal), and spleens for ETI-204-treated animals (i.m. route) showed that four of eight, zero of eight, and 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 Ames spores (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**

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>i.v.</th>
<th>i.m.</th>
<th>i.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg)</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>C_max (µg/ml)</td>
<td>66 ± 3</td>
<td>18 ± 1</td>
<td>43 ± 7</td>
</tr>
<tr>
<td>T_max (days) b</td>
<td>N/A</td>
<td>2.0 (1–2)</td>
<td>1.3 (1–4)</td>
</tr>
<tr>
<td>AUC (µg day/ml)</td>
<td>238 ± 20</td>
<td>121 ± 28</td>
<td>302 ± 3</td>
</tr>
<tr>
<td>Clearance (liters/day)</td>
<td>0.04 ± 0.004</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>V_d (liters)</td>
<td>0.24 ± 0.024</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>T_1/2 (days)</td>
<td>3.8 ± 0.5</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>F (%)</td>
<td>N/A</td>
<td>51</td>
<td>63</td>
</tr>
</tbody>
</table>

a T_max, median time taken to reach C_max; AUC, area under the curve; Clearance, systemic clearance; V_d, 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 (EC50 < 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 advantage, since it is thus 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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