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Human Monoclonal Anti-Protective Antigen Antibody Completely Protects Rabbits and Is Synergistic with Ciprofloxacin in Protecting Mice and Guinea Pigs against Inhalation Anthrax

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Prevention of inhalation anthrax requires early and extended antibiotic therapy, and therefore, alternative treatment strategies are needed. We investigated whether a human monoclonal antibody (AVP-21D9) to protective antigen (PA) would protect mice, guinea pigs, and rabbits against anthrax. Control animals challenged with *Bacillus anthracis* Ames spores by the intranasal route died within 3 to 7 days. AVP-21D9 alone provided minimal protection against anthrax in the murine model, but its efficacy was notably better in guinea pigs. When Swiss-Webster mice, challenged with five 50% lethal doses (LD_{50}s) of anthrax spores, were given a single 16.7-mg/kg of body weight AVP-21D9 antibody dose combined with ciprofloxacin (30 mg/kg/day for 6 days) 24 h after challenge, 100% of the mice were protected for more than 30 days, while ciprofloxacin or AVP-21D9 alone showed minimal protection. Similarly, when AVP-21D9 antibody (10 to 50 mg/kg) was combined with a low, nonprotective dose of ciprofloxacin (3.7 mg/kg/day) and administered to guinea pigs for 6 days, synergistic protection against anthrax was observed. In contrast, a single dose of AVP-21D9 antibody (1, 5, 10, or 20 mg/kg) but not 0.2 mg/kg alone completely protected rabbits against challenge with 100 LD_{50}s of *B. anthracis* Ames spores, and 100% of the rabbits survived rechallenge. Further, administration of AVP-21D9 (10 mg/kg) to rabbits at 0, 6, and 12 h after challenge with anthrax spores resulted in 100% survival; however, delay of antibody treatment by 24 and 48 h reduced survival to 80% and 60%, respectively. Serological analysis of sera from various surviving animals 30 days postprimary infection showed development of a species-specific PA enzyme-linked immunosorbent assay antibody titer that correlated with protection against reinfection. Taken together, the effectiveness of human anti-PA antibody alone or in combination with low ciprofloxacin levels may provide the basis for an improved strategy for prophylaxis or treatment following inhalation anthrax infection.

The severest form of anthrax results from inhalation of spores from *Bacillus anthracis*. The organism secretes three protein toxin components encoded by the genes *cya*, *lef*, and *pag* located on the pX01 plasmid (6, 20, 23). The virulence of *B. anthracis* is in part attributed to two separate bacterial proteins, edema factor (EF) (encoded by the *cya* gene) and lethal factor (LF) (encoded by the *lef* gene), both of which interact with protective antigen (PA) (encoded by the *pag* gene), a third protein that binds to receptors (4, 16, 28, 30) on the host cell surface, forming edema toxin and lethal toxin (LeTx). EF, an 88.9-kDa protein, is a calmodulin-dependent adenylyl cyclase enzyme, while the 90.2-kDa LF is a Zn^{2+} metalloprotease (6, 23). The 82.7-kDa receptor-binding PA is nicked by a furin-like protease produced by target cells and heptamerizes, forming binding sites for EF and LF. The membrane-bound holotoxin is then transported into the host cell by receptor-mediated endocytosis. Acidification of the endosome causes a conformational change in PA so that the protein forms a channel in the endosomal membrane, thus facilitating EF and LF entry into the cytosol, resulting in their respective biological effects (6, 23).

PA possesses distinct antigenic epitopes that elicit neutralizing antibodies capable of protecting experimental animals against inhalation anthrax (18). However, not all PA antibodies are effective in neutralizing the toxin complex (22). In vivo evaluation of protection provided by neutralizing anti-PA antibodies can be performed in Fisher 344 rats injected with LeTx. The monoclonal antibody AVP-21D9, derived from human blood lymphocytes from an anthrax vaccine adsorbed (AVA)-immunized individual, has been shown to be highly effective at LeTx neutralization (29). Protection against *B. anthracis* infection, however, is a more complex and stringent test of PA antibodies that block only the action of the anthrax toxins, and the small-animal models of inhalation anthrax vary in the extent to which anti-PA antibodies confer protection (9, 11, 19, 26, 27, 38). Nonhuman primates and rabbits are often considered the best models because they can be most effectively protected by the AVA vaccine, which contains PA and

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other *B. anthracis*-derived components (11, 26, 27, 38). In contrast, guinea pigs and mice appear more difficult to protect with the AVA vaccine (2, 9, 19).

In this study, we evaluated the capacity of a fully characterized human monoclonal anti-PA antibody (AVP-21D9), alone and combined with limited ciprofloxacin doses, to protect mice and guinea pigs against lethal doses of *B. anthracis* spores administered by the intranasal route. Further, AVP-21D9 was tested in rabbits for its protective capacity against intranasal challenge. This potent human monoclonal anti-PA antibody was reported to block PA heptamer formation and to neutralize LeTx in vitro and in vivo in a rat toxin neutralization assay (29, 35). In this report, we have demonstrated for the first time that a single dose of AVP-21D9 could delay death in mice and guinea pigs and completely protect rabbits, following lethal challenge by nasal instillation of *B. anthracis* Ames spores. In combination with low ciprofloxacin doses, AVP-21D9 could significantly enhance and prolong the survival of both mice and guinea pigs. Differences in the roles of the *B. anthracis* toxins and the bacterial capsule in the three animal models are thought to account for the variation in protection observed.

MATERIALS AND METHODS

***B. anthracis* toxin proteins.** PA, LF, and EF were from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA).

Human monoclonal antibody to PA. AVP-21D9 (Avanir Pharmaceuticals, San Diego, CA) is a wholly human monoclonal antibody specific for PA and was produced by CHO (Chinese hamster ovary)-K1 cells adapted to growth in serum-free medium in Integra cell culture flasks or in a bioreactor by VaxGen, Inc., Brisbane, CA, for Avanir Pharmaceuticals (29, 35). The protein A-purified AVP-21D9 antibodies showed a purity of >95% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, the binding affinity to PA was within 0.05 ± 0.03 nM (n = 8 different batches) as determined on the BiaCore 3000 instrument (Piscataway, NJ), and the 50% effective concentration in the Centers for Disease Control and Prevention (CDC) standardized toxin neutralization assay using the J774.1 macrophage cell line was previously reported to be 0.02 nM (n = 25) (27a; C. P. Quinn [Centers for Disease Control and Prevention, Atlanta, Ga., personal communication).

Preparation of *B. anthracis* spores. Spores were prepared by inoculating *B. anthracis* Ames strain in 2× SG sporulation medium (pH 7.0) consisting of 16 g Difco Nutrient Broth, 0.5 g $MgSO_4 \cdot 7H_2O$, 2.0 g KCl, and 16.7 g MOPS (morpholinepropanesulfonic acid) per liter (17). Before inoculation, the following supplements were added to the medium after filter sterilization using 0.22-μm syringe filters: 0.1% glucose, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnSO_4$, and 1 μM $FeSO_4$. Cultures were grown in 50-ml aliquots contained in 500-ml Erlenmeyer flasks fitted with Bug Stoppers (Fisher Scientific, Hampton, NH) at 37°C with gentle shaking (80 to 90 rpm) for 24 h, after which 100 ml of sterile distilled water was added to dilute the medium and promote sporulation. After 10 to 11 days of continuous shaking, sporulation was confirmed at >99% via the malachite green spore stain, and the spores were centrifuged at 587 × g in a sealed-carrier centrifuge (Jouan Inc., Winchester, VA) at 4°C for 15 min. The spore pellets were then washed four times in sterile phosphate-buffered saline (PBS) and resuspended in the same buffer. The spore suspensions were homogeneous when examined by phase-contrast microscopy. Plate counts were performed in triplicate to establish the concentration of viable spores in the suspension, which was then adjusted to a concentration of 1×10^9 CFU/ml. Aliquots of the stock spore suspension were stored at −70°C and freshly diluted in PBS to the desired density of CFU immediately before each animal challenge experiment. *B. anthracis* cultures and spores were prepared and stored in a restricted-access biosafety level 2 laboratory registered with the CDC and inspected by the Department of Defense and the United States Department of Agriculture.

Challenge of mice, guinea pigs, and rabbits with *B. anthracis* Ames spores. To evaluate the protective efficacy of AVP-21D9 monoclonal antibody in vivo, we challenged 8-week-old (25- to 30-g) female Swiss-Webster mice (Taconic, Germantown, NY) and 250- to 300-g Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) intranasally with 5×10^4 CFU and 6×10^5 CFU of *B. anthracis* Ames spores, respectively. We determined that these doses constituted five 50% lethal doses (LD_{50}s) per species (data not shown). Dutch-belted rabbits (0.7 to 1.1 kg; Myrtle's Rabbitry, Inc., Thompson Station, TN) were challenged using similar procedures with 100 LD_{50}s (1×10^7 CFU) of nasally instilled *B. anthracis* Ames spores (the LD_{50} was established in our laboratory).

Mice and guinea pigs were anesthetized by intraperitoneal (i.p.) injection with a mixture of ketamine-HCl (48 mg/kg of body weight for mice and 30 mg/kg for guinea pigs) and xylazine-HCl (9.6 mg/kg for mice and 7 mg/kg for guinea pigs). The rabbits were anesthetized with ketamine (35 mg/kg) and xylazine (5 mg/kg) by intramuscular injection. For spore instillation, anesthetized animals were suspended vertically, using the upper incisors, as described by Comer et al. (7), with the bulk of the body weight of the larger animals resting on the base of the platform. The spore suspension was instilled slowly for 2 to 3 min onto the anterior opening of each naris. Separate experiments instilling crystal violet dye solution using this technique ensured that the inoculum was deposited in the trachea with a minimal amount in the esophagus. The challenge volume was 20 μl/naris for mice, 25 μl/naris for guinea pigs, and 50 μl/naris for rabbits. PBS (20, 25, and 50 μl/naris) was then used to wash any nonadherent spores from the nasal cavity into the lungs for each animal species. All animal challenges were performed in a select-agent-approved, restricted-access animal biosafety level 3 laboratory under an approved Institutional Animal Care and Use Committee protocol.

Passive immunization and ciprofloxacin administration. Generally, 24 h after intranasal challenge with *B. anthracis* Ames spores (a five-LD_{50} dose), mice were passively immunized with a single subcutaneous (s.c.) dose of 500 μg/mouse (16.7 mg/kg) of AVP-21D9, and some animals were also injected with ciprofloxacin (0.9 mg/day; 30 mg/kg/day/mouse) by the i.p. route twice daily (b.i.d.) for 6 days. The AVP-21D9 contained 20 mM Tris, 150 mM NaCl, and 0.01% Tween 80. Guinea pigs were given a single i.p. injection of 3.1 mg (10 mg/kg), 6.2 mg (20 mg/kg), or 15 mg/guinea pig (50 mg/kg) of AVP-21D9, either 6 h prechallenge or 24 h postchallenge (as indicated) and s.c. injections of 1.12 (3.7 mg/kg/day), 2.25 (7.5 mg/kg/day), or 4.5 mg (15 mg/kg/day/guinea pig) of ciprofloxacin b.i.d. for 6 days starting 24 h after challenge with five LD_{50}s of *B. anthracis* Ames spores. Control groups of animals received AVP-21D9 antibody alone, ciprofloxacin only, or no treatment. Rabbits were given a single s.c. injection of AVP-21D9 monoclonal antibody (0.2, 1, 5, 10, and 20 mg/kg) at the same time as nasal challenge with 100 LD_{50}s of *B. anthracis* Ames spores. In the delayed-treatment experiment, rabbits were injected s.c. with AVP-21D9 (10 mg/kg) at various times (0, 6, 12, 24, and 48 h) following intranasal challenge with *B. anthracis* spores.

Serological assay. Antibody capture enzyme-linked immunosorbent assays (ELISA) for PA were used to determine the adaptive immune responses of mice, guinea pigs, and rabbits to antigens from the *B. anthracis* Ames spore infection. Briefly, blood samples were collected in serum separator microtubes containing silicone (Becton Dickinson, Franklin Lakes, NJ) 30 days postchallenge, and the sera were harvested by centrifugation at 519 × g for 5 min. For the ELISA, twofold serial dilutions of the sera were made beginning at 1:20 in 96-well flat-bottom cell culture plates (Corning Inc., Acton, MA) coated with 5 μg/ml PA (BEI Resources) diluted in sodium carbonate buffer (pH 9.6). The plates were then washed with PBS containing 0.05% Tween 20 and blocked for 1 h with PBS containing 10% fetal bovine serum. After 1 h of incubation at room temperature, the plates were washed three times with PBS plus 0.05% Tween 20, and mouse, guinea pig, or rabbit anti-PA immunoglobulin G (IgG) was detected by adding 0.5 μg/ml goat anti-mouse, anti-guinea pig, or anti-rabbit IgG, respectively, conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA). The plates were developed with ABTS substrate [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid] (Sigma Chemical Co., St. Louis, MO) for 20 to 60 min, and absorbance at 405 nm was measured with a BioTek Synergy HT spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, VT). The final anti-PA titers were established by the dilution that yielded one-half of the maximum absorbance in the ELISA anti-PA control (optical density at 405 nm).

In vitro toxin neutralization assay. We measured lactate dehydrogenase (LDH) enzyme release from a murine monocyte-macrophage cell line (RAW 264.7; American Type Culture Collection, Manassas, VA) to assess the protection afforded by sera from guinea pigs and rabbits that were challenged with *B. anthracis* Ames spores. Briefly, the RAW 264.7 cells were propagated in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin-streptomycin, and 2 mM L-glutamine (Mediatech, Inc., Herndon, VA) at 37°C with 5% CO_2 using tissue culture flasks. Subsequently, the cells were plated in 96-well flat-bottom tissue culture plates (Corning) at a density of 1×10^6 cells/ml and incubated overnight at 37°C in 5% CO_2. The monolayers were washed twice with Dulbecco's modified essential medium devoid of serum or phenol red. LeTx-mediated cytotoxicity was measured as a function of the amount of LDH enzyme released from the macrophages into the cell culture supernatants. Various dilutions of the infected (for 30 days) guinea pig and rabbit sera (starting from 1:10 dilution, followed by twofold serial dilu-

tions) were preincubated with 12 nM PA (1.0 μg/ml) and 2 nM LF (0.2 μg/ml) for 1 h at 37°C and added to the culture media. After incubation for 4 h, LDH release in the supernatant of the macrophage cells was measured using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) and quantitated by measuring wavelength absorbance at 490 nm. The final neutralizing antibody titers of sera were reported as the reciprocal of the dilution that resulted in 50% of the difference between the relative amounts of LDH release in the untreated culture media and LeTx controls.

Pharmacokinetic studies. To determine the serum concentrations of AVP-21D9 antibody in Swiss-Webster mice over time, a group of five 20-g mice each received 1 mg (50 mg/kg) AVP-21D9 by i.p. injection, either as a bolus at time zero or as two injections of 0.5 mg (25 mg/kg) each at time zero and 72 h later. Serum samples were collected by retro-orbital bleeding at 1, 6, 12, 24, 48, 72, 73, 78, 84, 96, 120, 144, 168, 192, 216, and 240 h.

To assess the serum concentrations of AVP-21D9 antibody in Hartley guinea pigs (250 to 300 g) over time, two groups of three guinea pigs each received a single i.p. injection of 1.5 (5 mg/kg) or 15 mg (50 mg/kg) of the antibody. Blood samples were collected by toenail bed snips at 0, 6, 12, 24, 48, 72, 168, and 216 h after injection.

To determine the levels of AVP-21D9 in rabbits, four Dutch-belted rabbits (1.3 to 1.7 kg) were injected with 1 and 10 mg/kg of the antibody by s.c. injection. Blood samples were collected from the central ear artery with a 1-ml syringe fitted with a 25-gauge needle.

The AVP-21D9 antibody concentrations in the sera were measured by surface plasmon resonance using a Biacore 3000. Briefly, a goat anti-human IgG capture antibody (Jackson Immuno Research Inc., West Grove, PA) was coupled to flow cell number 1 of a CM-5 sensor chip (Biacore catalog no. BR-1003-99) at a level of 10,000 response units using an Amine Coupling Kit (Biacore catalog no. BR-1000-50). Human IgG1 (20 μg/ml; Sigma I-5154) was used to generate a standard curve. Antibody serum samples were diluted 1:10 and 1:100 in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant P20). Each sample was assayed in duplicate, and standards were assayed in triplicate. Response unit values were taken 2 min into the injection of the standards and samples, and Biacore evaluation software was used to generate a standard curve from which unknown sample concentrations were calculated.

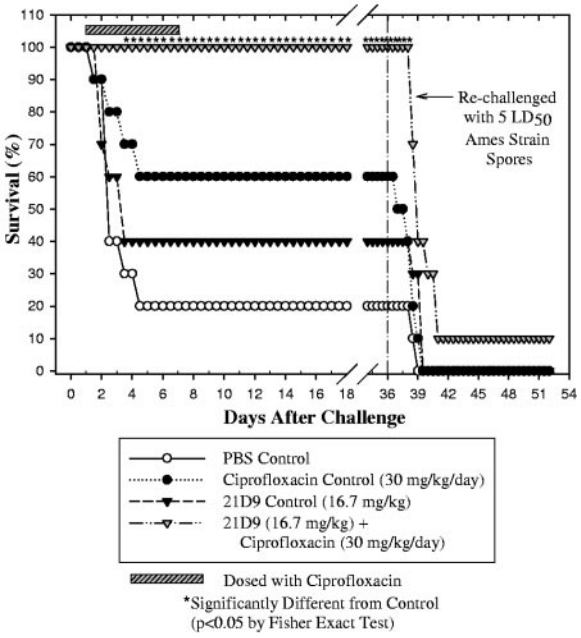

FIG. 1. Protection of Swiss-Webster mice with combinations of AVP-21D9 and a low nonprotective dose of ciprofloxacin after challenge with five LD_{50}s of *B. anthracis* Ames. Human monoclonal antibody to PA (AVP-21D9) (16.7 mg/kg) was administered as a single s.c. dose 24 h after challenge with *B. anthracis* Ames spores, while ciprofloxacin (30 mg/kg/day) was administered by i.p. injection. The number of animals per group was 10. The data were analyzed statistically using the Fisher exact test, and a P value of ≤ 0.05 was considered significant.

RESULTS

Mouse protection experiments. Figure 1 summarizes an experiment in which AVP-21D9 monoclonal antibody was tested for its capacity to protect mice against lethal infection with *B. anthracis* Ames spores (five LD_{50}s) administered by nasal instillation given alone and in combination with ciprofloxacin 24 h after challenge. Combinations of AVP-21D9 and antibiotic were examined, because treatment of human disease with human anti-PA antibody would most likely occur in conjunction with approved antibiotics. Mice dosed i.p. with a single 16.7-mg/kg dose of AVP-21D9 antibody alone showed a minimal, statistically nonsignificant amount of protection compared to animals that were challenged with anthrax spores (PBS control). Our earlier experiments demonstrated that 30 mg/kg/day of ciprofloxacin (b.i.d.) for 6 days was a suboptimal antibiotic dose for protecting mice against *B. anthracis* infection (data not shown). However, when we combined ciprofloxacin treatment (30 mg/kg/day) and passive immunization with a single 16.7-mg/kg dose of AVP-21D9 in anthrax spore-challenged mice, 100% protection was achieved for 38 days, suggesting at least an additive effect, because ciprofloxacin or AVP-21D9 alone protected only 60% and 40% of the mice, respectively. Upon rechallenge, only 5% of the mice survived, indicating that long-term protection with the heterologous human monoclonal antibody was not achieved.

Guinea pig protection experiments. In testing AVP-21D9 in guinea pigs, we determined the amount of protection afforded by administration of 10 mg/kg, 20 mg/kg, and 50 mg/kg of AVP-21D9 6 h before challenge with five LD_{50}s (6×10^5 CFU) of *B. anthracis* Ames spores by nasal instillation (Fig. 2). The results indicated that increasing the dose to 50 mg/kg did not significantly increase the protection observed. As is evident from the figure, administration of anti-PA antibody to guinea pigs delayed their deaths compared to animals infected with the spores alone; however, most animals were still susceptible to lethal infection.

To distinguish between additive effects and synergy by AVP-21D9 and ciprofloxacin, we challenged guinea pigs intranasally with Ames spores, and 24 h later administered AVP-21D9 (50 mg/kg i.p.) concomitantly with three different doses of ciprofloxacin administered by the s.c. route twice daily for 6 days (Fig. 3A to C). Figure 3A shows a synergistic, rather than an additive, protective effect of combining a suboptimal dose of ciprofloxacin (3.7 mg/kg/day) and AVP-21D9 (50 mg/kg). As ciprofloxacin doses were increased to 7.5 mg/kg/day (Fig. 3B), synergistic protection was reduced to the anticipated additive effects of ciprofloxacin and AVP-21D9. When the ciprofloxacin dose was increased still further to 15 mg/kg/day (Fig. 3C), the protection by AVP-21D9 monoclonal antibody was obscured by the protection afforded by the antibiotic alone. Another experiment, shown in Fig. 4, confirmed the synergistic protection afforded to guinea pigs by low-dose ciprofloxacin (3.7 mg/kg/day) and low doses (5 mg/kg) of AVP-21D9 against nasal instillation of anthrax spores. Analogous to results with the mouse model, long-term survival of the guinea pigs after injection of human monoclonal antibody was not observed following secondary challenge.

Rabbit protection experiments. Protection of rabbits against a lethal challenge of *B. anthracis* spores with AVP-21D9 was

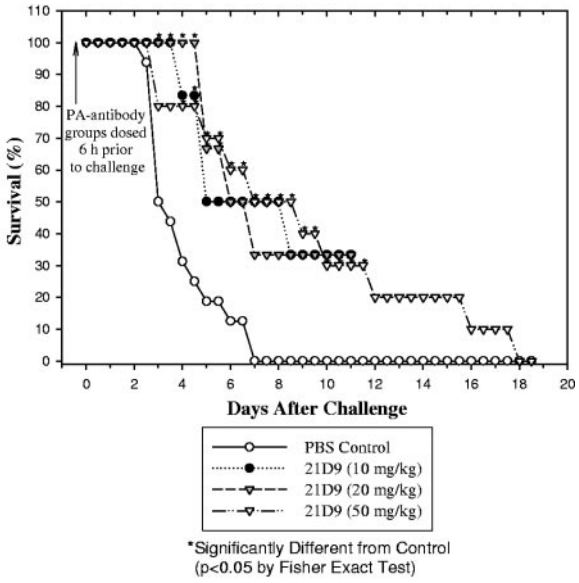

FIG. 2. Protection of Hartley guinea pigs with human monoclonal α-PA antibody (AVP-21D9). The figure shows the protective effects of 10 mg/kg, 20 mg/kg, and 50 mg/kg of AVP-21D9. The numbers of animals per group were as follows: PBS control, $n = 16$; 50-mg/kg PA antibody group, $n = 10$; 10- and 20-mg/kg PA antibody groups, $n = 6$. The data were analyzed statistically using the Fisher exact test, and a P value of ≤ 0.05 was considered significant.

easily demonstrated, thereby showing the human anti-PA antibody to be highly effective (Fig. 5). Unlike the partial protection observed in the mouse and guinea pig models, AVP-21D9 was 100% effective in protecting rabbits for 3 weeks against 100 LD_{50}s of *B. anthracis* spores administered intranasally when given in doses of 1, 5, 10, and 20 mg/kg (Fig. 5). A lower dose of AVP-21D9 (0.2 mg/kg) failed to protect the rabbits from inhalation anthrax, with 80% of the animals dying between 5 and 6 days after infection. In a subsequent delayed-treatment experiment, administration of AVP-21D9 (10 mg/kg) to rabbits at 0, 6, and 12 h after challenge with 100 LD_{50}s of *B. anthracis* Ames spores resulted in 100% survival for 30 days, compared to controls (0% survival). Delay of AVP-21D9 treatment by 24 h and 48 h reduced survival to 80% and 60%, respectively (Fig. 6). For the most part, these animals were protected when subsequently rechallenged with another 100 LD_{50}s of anthrax spores. When all three animal models of inhalation anthrax were compared, it became clear that rabbits were more easily protected by anti-PA antibody than mice or guinea pigs.

Serological analysis of infected animals. Passive administration of AVP-21D9 conferred protection against inhalation anthrax, although in mice and guinea pigs, concomitant administration of ciprofloxacin was necessary to demonstrate the additive/synergistic protective response. To evaluate the role of the adaptive immune response in mice to PA following nasal rechallenge, we evaluated the sera of mice surviving challenge with five LD_{50}s of *B. anthracis* Ames spores for 30 days post-primary challenge by murine-specific PA ELISA. The results revealed that most of the mice (18/21) dosed with AVP-21D9 and/or ciprofloxacin failed to build a murine anti-PA antibody response (<1:20) post-primary challenge, and all but one died following nasal rechallenge (data not shown).

We measured low levels of residual human IgG antibody specific for PA (AVP-21D9) by species-specific PA ELISA in sera from 22 guinea pigs surviving 30 days post-primary challenge (geometric mean titer, 1:41, or 40-ng/ml AVP-21D9 equivalents). This residual human anti-PA antibody could have provided some protection to the guinea pigs upon rechallenge; however, we also sought to examine the animals' adaptive immune response to PA post-primary challenge. Serum samples were collected from the guinea pigs surviving 30 days (Fig. 3 and 4) post-primary challenge with five LD_{50}s of *B. anthracis* spores. In our analysis, guinea pig-specific anti-PA ELISA serum titers and LeTx neutralization titers, illustrated in Fig. 7A and B, from two experiments (Fig. 3 and 4) were combined. Of the guinea pigs that survived secondary challenge, 41.7% (15/36) had developed species-specific anti-PA ELISA titers of ≥1:300, with a geometric mean titer of 1:3,860 (range, 1:300 to 1:40,000) by 4 weeks post-primary challenge (Fig. 7A). Further, 89.5% (17/20) of the guinea pigs that died after secondary challenge had no detectable species-specific anti-PA ELISA titer (≤1:20). One animal with a species-specific anti-PA ELISA titer of 1:160 and another with a titer of 1:5,000 did not survive rechallenge, and one animal with a titer of ≤1:20 survived the secondary challenge. Despite these exceptions, the results (Fig. 7A) indicated a strong relationship between survival after rechallenge and an animal's capacity to develop an anti-PA antibody response following initial spore challenge. These results were supported by additional titration of the sera by an LeTx neutralization assay in RAW 264.7 cells (Fig. 7B), although the neutralization titers were low and the difference in titers between the two groups of guinea pigs (those that survived [1:47] versus those that did not survive [1:11]) was not as prominent.

Sera from rabbits surviving 3 weeks after primary challenge with 100 LD_{50}s (1×10^7 CFU) of *B. anthracis* Ames spores by nasal instillation (Fig. 5) were assayed for residual human anti-PA antibody titers (AVP-21D9) by a species-specific PA ELISA. The geometric means of human anti-PA ELISA titers from sera from rabbits dosed 3 weeks earlier with 5, 10, or 20 mg/kg of AVP-21D9 were 1:118, 1:373, and 1:691, which corresponded to 118-, 373-, and 691-ng/ml AVP-21D9 equivalents, respectively. LeTx neutralization titers of sera from rabbits dosed with 10 and 20 mg/kg of AVP-21D9 were 1:51 and 1:106, respectively. All rabbits dosed with 1, 5, 10, and 20 mg/kg of AVP-21D9 survived rechallenge and were euthanized 2 weeks later.

The same sera from rabbits surviving 3 weeks after primary challenge were also assayed for newly formed rabbit anti-PA antibodies by species-specific PA ELISA. We observed that the rabbits dosed with 20 mg/kg of AVP-21D9 3 weeks earlier built species-specific geometric mean anti-PA titers of 1:146 ($n = 7$), while rabbits that had been dosed with 10 mg/kg of AVP-21D9 had formed rabbit anti-PA titers of 1:80 ($n = 6$). Rabbits receiving lower doses of AVP-21D9, specifically 5 or 1 mg/kg, had titers of 1:288 ($n = 7$) and 1:260 ($n = 7$), respectively. The human IgG (AVP-21D9 control) in these assays did not cross-react with the goat anti-rabbit IgG conjugate used in the ELISA (data not shown).

Finally, we examined sera from mice, guinea pigs, and rab-

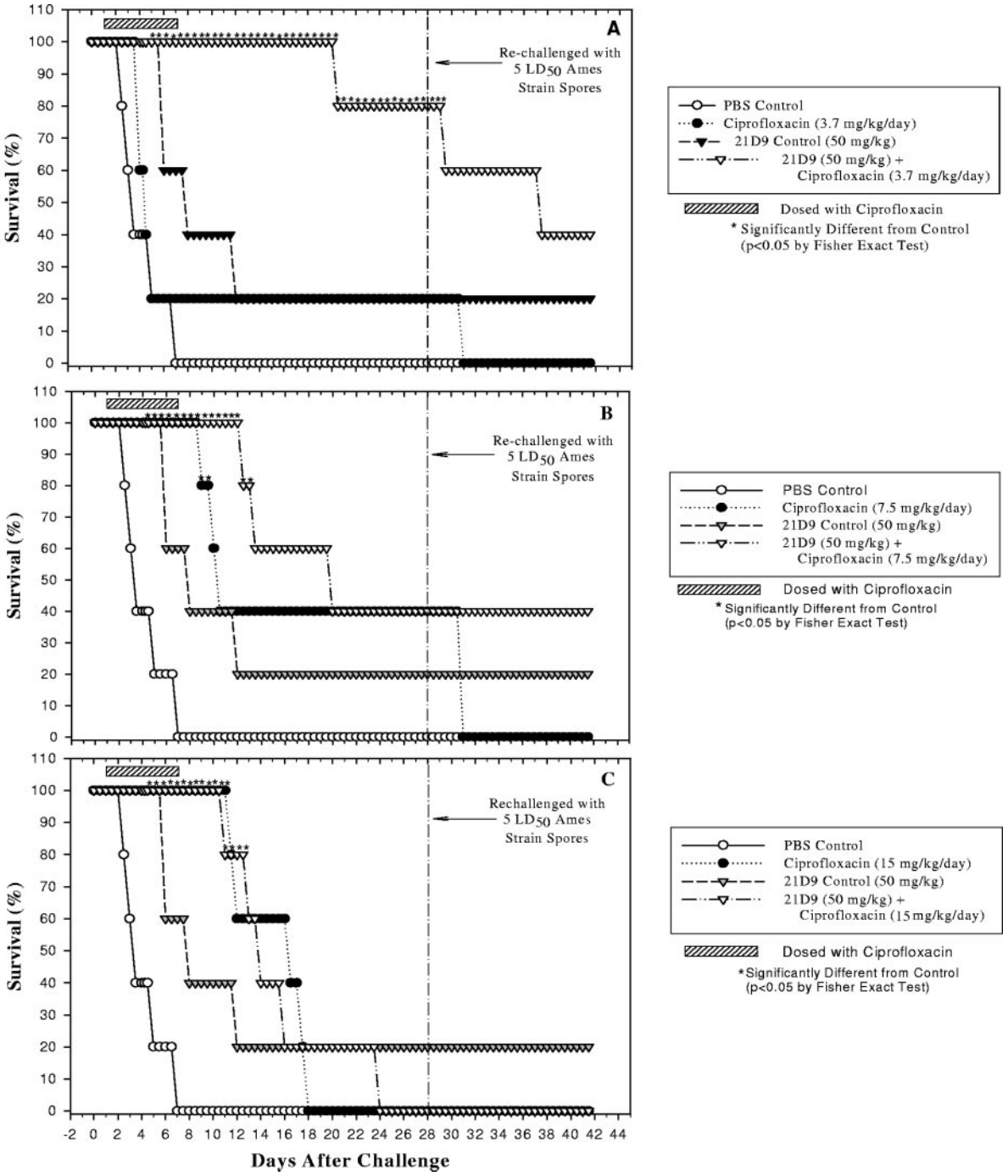

FIG. 3. Protection of Hartley guinea pigs with combinations of AVP-21D9 (50 mg/kg) and various doses of ciprofloxacin: (A) 3.7 mg/kg/6 days; (B) 7.5 mg/kg/6 days; (C) 15 mg/kg/6 days. The number of animals per group was five. The data were analyzed statistically using the Fisher exact test, and a P value of ≤ 0.05 was considered significant.

bits for development of species-specific antibodies to the foreign human monoclonal antibody AVP-21D9. As expected, all animals passively immunized with AVP-21D9 generated serum anti-AVP-21D9 ELISA titers (data not shown). The immune response to AVP-21D9 could have hastened clearance of the foreign human antibody from the circulation prior to secondary challenge and/or contributed to neutralization of AVP-21D9.

Pharmacokinetic studies of AVP-21D9 antibody. Pharmacokinetic parameters were obtained in mice, guinea pigs, and rabbits (Table 1). An initial experiment with mice showed a maximum concentration of antibody in serum (C_{max}) of 692 ± 155 μg/ml that was reached after 5 ± 2 h following a bolus i.p. injection of 50 mg/kg AVP-21D9. The antibody was rapidly eliminated, with an elimination half-life of 2.8 ± 1.4 days. Administration of two doses of 25 mg/kg yielded superimposable terminal elimination curves (data not shown). Similar serum levels (C_{max} = 585 μg/ml) were observed in guinea pigs injected i.p. with 50 mg/kg of AVP-21D9. The time to maxi-

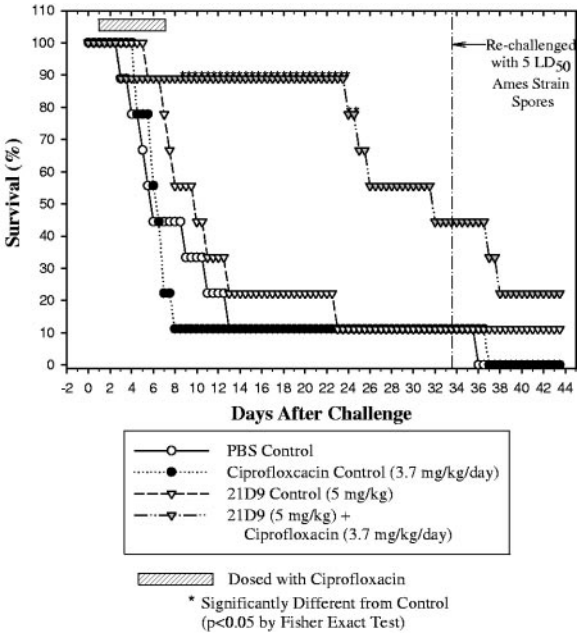

FIG. 4. Protection of Hartley guinea pigs treated with low-dose ciprofloxacin (3.7 mg/kg for 6 days) in combination with a single i.p. dose of AVP-21D9 (5 mg/kg) administered 24 h after challenge. The number of animals per group was nine. The data were analyzed statistically using the Fisher exact test, and a P value of ≤ 0.05 was considered significant.

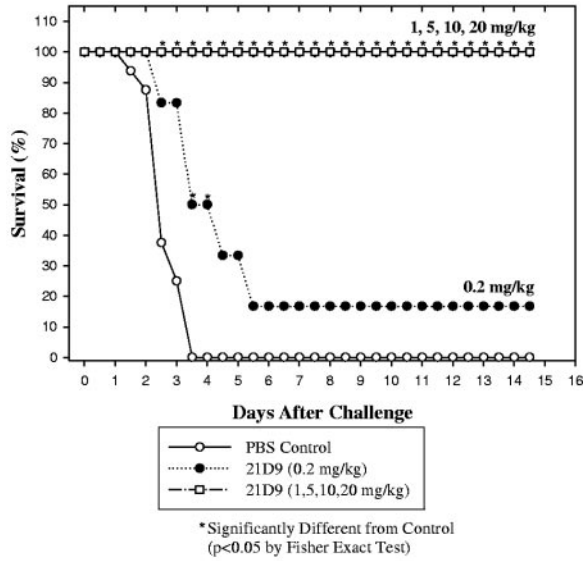

FIG. 5. Survival of Dutch-belted rabbits after challenge intranasally with 100 LD_{50}s (1×10^7 CFU) of *B. anthracis* Ames spores with and without concomitant dosing with 0.2, 1, 5, 10, and 20 mg/kg of AVP-21D9 by the subcutaneous route. The numbers of animals per group were 11 for the PBS control, 6 for the 0.2-mg/kg group, and 7 for the 1-, 5-, 10-, and 20-mg/kg groups. The data were analyzed statistically using the Fisher exact test, and a P value of ≤ 0.05 was considered significant.

mum concentration of antibody in serum was reached later (28 ± 14 h), and the elimination half-life was longer (13.8 ± 8.1 days), but there was a lot of variability in this group (the elimination half-lives were 5.4, 14.5, and 21.5 days versus 2.5, 5.2, and 5.7 days in the 5-mg/kg group). The C_{max}, as well as the total amount of AVP-21D9 detected in the serum over the time course of the study (area under the curve [AUC] from time zero to the last measured time point) was reduced approximately 10-fold in guinea pigs given 5 mg/kg AVP-21D9 compared to those that received 50 mg/kg, indicating a linear dose response at these dosing levels (Table 1). An additional study was performed in Dutch-belted rabbits, which were given a single s.c. dose of AVP-21D9 at 1 mg/kg or 10 mg/kg, and serum samples were collected for 14 days. The s.c. route was chosen to eliminate unwanted potential variability of i.p. dosing and to more closely mimic a potential route for human administration. At the 1-mg/kg dose, which completely protected the rabbits from anthrax spore challenge, we observed a C_{max} of 23 μg/ml that was reached after 2 days. Moreover, administration of 10 mg/kg AVP-21D9 yielded approximately 10-fold-higher C_{max} and corresponding AUC values, confirming the dose proportionality seen earlier in guinea pigs. The terminal elimination half-life is comparable to that in guinea pigs (5 to 9 days) and much lower than would be expected for a fully human antibody administered to humans.

DISCUSSION

The pathology of inhalation anthrax in humans and animal models is well characterized and was discussed in prior reports (11, 13, 19, 27, 34, 38). It is clear that *B. anthracis* strains possessing both plasmids pX01 and pX02 are fully virulent in small-animal models, such as the mouse, guinea pig, and rabbit, and that deletion of either or both plasmids reduces virulence (5, 9, 25). It is generally believed that anthrax toxins play a more important role in virulence in humans, nonhuman primates, and rabbits than in mice and guinea pigs during inhalation anthrax (11, 26, 27, 38). The reasons for these differences in the animal models are not known. Despite inconsistencies in the anthrax animal models, each offers advantages for examining new strategies in vaccination and therapy to protect against the disease. In general, therapeutic and prophylactic approaches that prove effective against inhalation anthrax in the mouse and guinea pig models are likely to be highly effective in rabbits and nonhuman primates.

Since *B. anthracis* spores germinate asynchronously and often cause infection long after exposure, a prolonged period of prophylactic treatment with antibiotics is required (2, 10, 12, 33). Because treatment regimens are lengthy, we investigated the protective effect of the human monoclonal antibody AVP-21D9, specific for *B. anthracis* PA, alone and in combination with ciprofloxacin using mouse, guinea pig, and rabbit models of intranasal challenge.

Currently, there are several anti-PA monoclonal antibodies at various stages of development (14, 15, 21, 32, 37), but a direct comparison of the potencies of these antibodies in the same animal model is lacking. Two reports described antibodies that neutralize LeTx in a toxin challenge model (14, 37), but data from a spore challenge model have not yet been reported. Another group showed protection of A/J mice challenged with 30 LD_{50}s of *B. anthracis* Sterne strain spores by 180 μg of a plant-derived version of anti-PA antibodies obtained from IQ6E4 hybridomas (IQ Corporation, The Netherlands) administered 2.5 h before challenge (15). More comparable to the studies described in this report, Beebe et al. (3) reported 100%

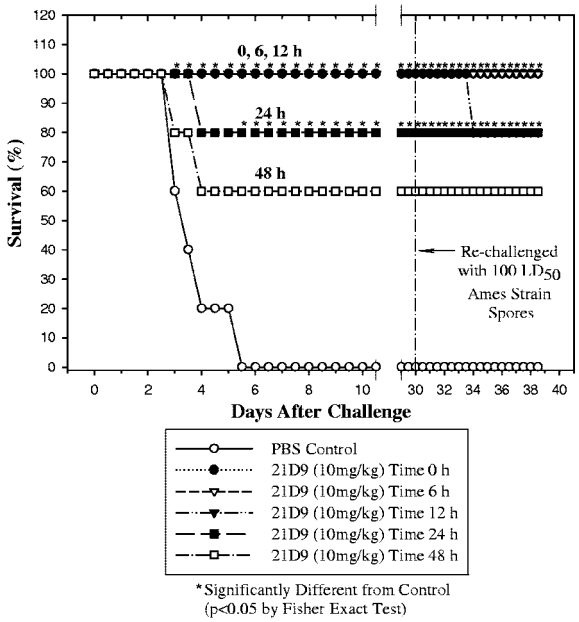

FIG. 6. Survival of Dutch-belted rabbits after challenge intranasally with 100 LD_{50}s (1×10^7 CFU) of *B. anthracis* Ames spores and dosing with 10 mg/kg AVP-21D9 by the subcutaneous route at 0, 6, 12, 24, and 48 h postchallenge. The number of animals per group was five. The data were analyzed statistically using the Fisher exact test, and a *P* value of ≤0.05 was considered significant.

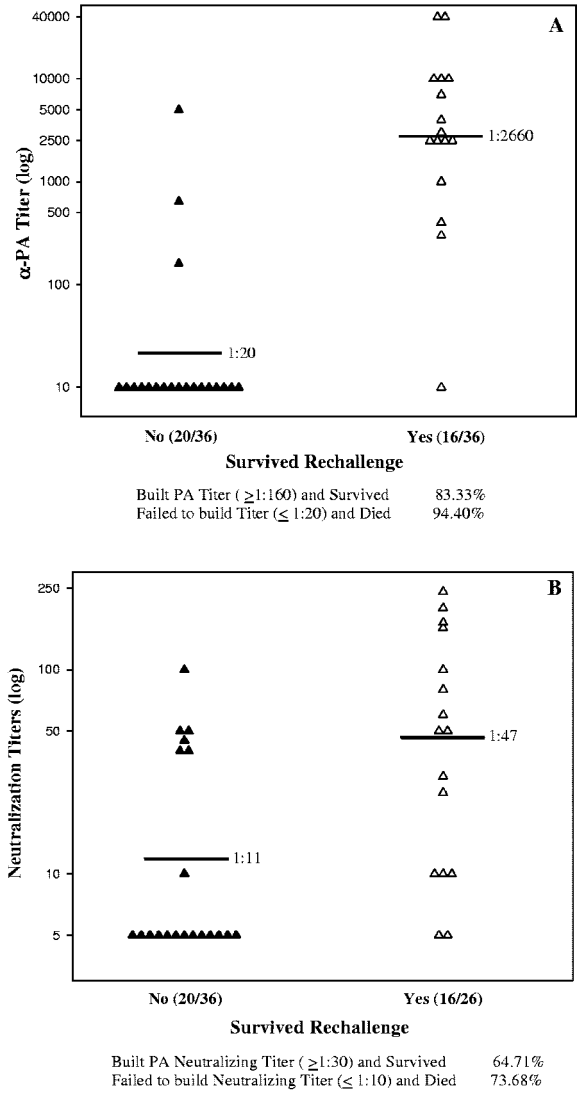

FIG. 7. Correlation of guinea pig anti-PA titers with survival following rechallenge with *B. anthracis* Ames spores. Blood samples from guinea pigs surviving initial challenge were collected at 30 days, and their sera were analyzed by guinea pig-specific ELISA for anti-PA (A) or -LeTx (B) neutralization assay in RAW 264.7 cells.

protection of New Zealand White (NZW) rabbits challenged by the aerosol route with 100 LD_{50}s of Ames spores when 40 mg/kg of human PA monoclonal antibody (5H3; ABthrax) was administered intravenously at the time of challenge. Cui et al. showed that a PA monoclonal antibody significantly improved the outcome of a lethal toxin infusion rat model when the antibody was administered at 10-fold molar excess to PA up to 6 h after initial exposure to LeTx (8). This human antibody was found to be safe and well tolerated when administered as a single intramuscular or intravenous dose in a human phase I trial at doses ranging from 0.3 to 40 mg/kg (32).

Similar results were reported for ETI-204, a chimeric anti-PA antibody with an equilibrium dissociation constant of 0.33 nM, which provided 90 to 100% protection when given at 10 mg/kg to NZW rabbits prior to challenge with 100 to 200 LD_{50}s of aerosolized *B. anthracis* Ames spores (21). As shown in this report, AVP-21D9 provided full protection to rabbits at 1 mg/kg (i.e., a 40-fold-lower dose than the above-mentioned PA monoclonal antibody 5H3), and it provided complete protection to rabbits even when administered subcutaneously 12 h post-spore challenge, with peak levels of serum antibody being reached 40 h later. A potency similar to that of AVP-21D9, i.e., protection at 1 mg/kg, has been claimed only for MDX-1303 (Valortim), but the data have not been published other than in a press release and therefore could not be independently confirmed. We anticipate that AVP-21D9 will have a safety profile in humans similar to those of the above-mentioned PA monoclonal antibodies and will potentially require lower doses of antibody, which are expected to be achievable by various routes of administration in humans. These qualities should qualify AVP-21D9 as a top candidate for further development for use in humans, either for preexposure/postexposure prophylaxis or for therapeutic treatment of individuals exposed to anthrax spores.

The affinity measurements from eight different lots of AVP-21D9 antibody showed an average equilibrium dissociation constant of 0.05 ± 0.03 nM. Further characterization of the antibody determined that it inhibited PA heptamer formation, a prerequisite for toxin entry into the cell (35). In this report, the pharmacokinetic results in uninfected mice, guinea pigs, and rabbits revealed the presence of peak levels of AVP-21D9 antibody in blood during the critical period (2 to 5 days) when deaths from inhalation anthrax occurred with the highest frequency (Table 1). The elimination half-life and serum levels in Dutch-belted rabbits reported here are comparable to the values reported for other anti-PA antibodies in NZW rabbits (3, 18), and it is expected that the half-life of the fully human AVP-21D9 will be significantly increased in humans (32). How-

TABLE 1. Pharmacokinetic analysis of AVP-21D9 in Dutch-belted rabbits, Hartley guinea pigs, and Swiss-Webster mice

Parameter[a]	Value[b]				
	Rabbit		Guinea pig		Mouse
No. of animals	4	3	3	3	5
Dose (mg/kg)	1	10	5	50	50
C_{max} (μg/ml)	23 ± 6	218 ± 43	64 ± 4	585 ± 34	692 ± 155
T_{max} (h)	48 ± 0	40 ± 14	20 ± 7	28 ± 14	5 ± 2
E (days)	5.0 ± 2.7	8.9 ± 2.7	4.5 ± 1.6	13.8 ± 8.1	2.8 ± 1.4
AUC_{0-t} (μg-day/ml)	193 ± 35	1,816 ± 219	324 ± 32	3,606 ± 169	2,715 ± 485

[a] E, elimination half-life; AUC_{0-t}, AUC from time zero to the last measured time point; T_{max}, time when concentration was maximum.
[b] Mice were dosed with AVP-21D9 using a single i.p. injection of 50 mg/kg. Guinea pigs were dosed by i.p. injection with either 5 mg/kg or 15 mg/kg of AVP-21D9. Rabbits were dosed with AVP-21D9 using a single s.c. injection of either 1.0 mg/kg or 10 mg/kg, and blood samples were collected at various intervals. Blood samples were collected from all of the animals at various times after the animals were dosed with AVP-21D9, and the levels of human IgG were determined. Study durations were as follows: rabbit, 14 days; guinea pig, 9 days; mouse, 10 days.

ever, the serum levels alone are not predictive of protection in all of the animal models. For example, the C_{max} of AVP-21D9 in Dutch-belted rabbits was only 23 ± 3 μg/ml in the fully protected group treated with 1 mg/kg AVP-21D9, while guinea pigs treated with 50 mg/kg AVP-21D9 reached a C_{max} of 580 ± 16 μg/ml but were not fully protected by antibody alone.

We observed that AVP-21D9 anti-PA alone delayed the deaths of mice and guinea pigs from anthrax and, when combined with low doses of ciprofloxacin, conferred additive or synergistic protection in both of the models. We surmised that the lower levels of antibiotic allowed some replication of the bacteria, which in turn served as a potent source of PA and other antigens that elicited an adaptive immune response. The synergy observed between ciprofloxacin and the human anti-PA antibody offers promise for future improvements in prophylaxis and treatment against inhalation anthrax.

We determined that mice, guinea pigs, and rabbits formed a specific antibody response to AVP-21D9 (human IgG). We do not anticipate that this will occur in humans dosed with AVP-21D9, since it is a wholly human monoclonal antibody. Passive immunization with human monoclonal antibodies to PA offers an opportunity to protect or treat patients exposed to *B. anthracis* spores. It is unlikely that unvaccinated patients at risk of contracting anthrax would be treated using an effective antibody alone, simply because of the gravity of the infection, the fulminating course of the clinical disease, the dangerously high mortality of the infection for the affected patient, and ethical issues related to the use of effective antibiotics. Consequently, evaluation of new treatment regimens in combination with antibiotics is a rational medical practice. Fortunately, human-to-human secondary transmission of inhalation anthrax has not been reported (36).

We observed that AVP-21D9, with or without ciprofloxacin, did not provide long-term protection to mice. Likewise, few mice developed a species-specific anti-PA response, but it is unclear whether this observation is related to the limited duration of resistance. In contrast, we observed that if guinea pigs were kept alive for 36 days, with ciprofloxacin and/or human monoclonal antibody to PA following initial challenge with *B. anthracis* spores, 50% (18/36) of them developed a geometric mean guinea pig-specific anti-PA ELISA titer of >1:300, which provided them with an 80% chance of surviving rechallenge. That one-half of the animals failed to develop an anti-PA antibody response could have been due to the inhibitory effect of the anthrax toxins on T cells formed during the primary infection (1, 31). Exceptions to this generalization were three dead animals that had anti-PA ELISA titers greater than 1:160 and the one survivor of rechallenge with an undetectable PA antibody response. The low LeTx neutralizing antibody titers possibly reflect a low survival rate after rechallenge. Based on study results, we could have predicted with reasonable accuracy survival following rechallenge, based on a minimum anti-PA ELISA titer of >1:300 (80% survival), but would have been unable to predict survivors based on the low neutralization titers, as neutralization titers of >1:30 yielded only a 50% probability of an animal surviving rechallenge. These findings do not imply that neutralization titers are unimportant in evaluating vaccines against anthrax, as the development of serological responses to PA, following experimental infection, was likely reduced by the immunosuppressive effects of LeTx on dendritic cells and lymphocytes (1, 24, 31).

Analysis of rabbit sera 3 weeks after dosing with AVP-21D9 and challenge with 100 LD_{50}s of *B. anthracis* spores revealed significant levels of AVP-21D9 remaining, which likely contributed to their protection against secondary challenge. These animals were also protected by rising titers of rabbit anti-PA antibody formed in response to PA and possibly other antigens presented following initial challenge. Taken together, these data indicated that development of anti-PA, and possibly antibodies to other *B. anthracis* antigens, by the host immune response was primarily responsible for providing protection to the animals after subsequent challenge with *B. anthracis* spores.

In conclusion, protection of both guinea pigs and mice was significantly enhanced by concomitant treatment with ciprofloxacin and a potent human monoclonal antibody to PA (AVP-21D9), but there were substantial differences in the two models, with the mouse being the most difficult to protect with antibodies to PA alone. In rabbits, AVP-21D9 alone was highly effective in protecting against lethal infection. The importance and strength of this study are that we evaluated the efficacy of anti-PA monoclonal antibody in three small-animal models. Our data support the further evaluation of AVP-21D9 in non-human primate challenge models and in humans for safety as a potential candidate for use in protecting humans exposed to anthrax spores.

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