Passive Protection against Anthrax by Using a High-Affinity Antitoxin Antibody Fragment Lacking an Fc Region

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Passive immunization has been successfully employed for protection against bacterial and viral infections for over 100 years. Immunoglobulin Fc regions play a critical role in the clearance of bacterial pathogens by mediating antibody-dependent and complement-dependent cytotoxicity. Here we show that antibody fragments engineered to recognize the protective antigen component of the *B. anthracis* exotoxin with high affinity and conjugated to polyethylene glycol (PEG) for prolonged circulation half-life confer significant protection against inhalation anthrax despite their lack of Fc regions. The speed and lower manufacturing cost of bacterially expressed PEGylated antibody fragments could provide decisive advantages for anthrax prophylaxis. Importantly, our results suggest that PEGylated antibody fragments may represent a unique approach for mounting a rapid therapeutic response to emerging pathogen infections.

Bacillus anthracis is a spore-forming, gram-positive bacterium that causes anthrax. Upon entry through the skin, ingestion, or inhalation, *B. anthracis* spores germinate into vegetative bacteria. A tripartite exotoxin secreted from the bacteria represents a key virulence factor in anthrax. The protective antigen (PA) component of the exotoxin mediates the host cell entry of the two other components, the lethal factor (LF), a zinc metalloprotease that cleaves several mitogen-activated protein kinase kinases, and the edema factor, a calmodulin-dependent adenylate cyclase. Recently, the structures of all three proteins have been determined (11, 27, 40, 43). In addition, the mechanisms by which the PA-LF complex (lethal toxin [LeTx]) enters the cell have been identified (27) along with the chronology with which these events occur (9, 32). The protective antigen binds to two cell surface receptors, ATR (5) and CMG2 (44). The CMG2 gene is likely to be expressed in most human tissues (14, 44), and, recently, the ATR/TEM8 gene was reported to be highly expressed in epithelial cells (4).

The anthrax attacks of October 2001 heightened awareness concerning the necessity of effective countermeasures for inhalation anthrax exposure. A high anti-PA antibody titer has been correlated with protection in guinea pigs and rabbits immunized with the Anthrax Vaccine Adsorbed vaccine (20, 42). Partial prophylaxis of animals treated with anti-PA rabbit polyclonal antibodies was demonstrated in a guinea pig spore challenge (30). Several murine monoclonal antibodies with mod-

erate affinities towards PA failed to show protection in the same study, although one monoclonal antibody did show a significant increase in the time to death (TTD) of treated animals (30). The detailed mechanism of protection by anti-PA antibodies remains a subject of intense study (51, 52).

Several groups have been pursuing a therapeutic strategy directed against PA (7, 16, 21, 33, 35, 36, 41, 45, 47). We have used the 14B7 antibody (equilibrium dissociation constant [K_D], 4.3 nM) (16) as the starting point for a protein-engineering campaign that led to several variants exhibiting 20-fold to 200-fold greater affinity (16, 33). ETI-204, a humanized immunoglobulin G (IgG) derived from an engineered affinity-enhanced 14B7 variant (31), was recently reported to provide excellent protection against inhalation challenge with spores (100× to 300× 50% lethal dose [LD_{50}]) of the Ames strain of *B. anthracis* in rabbits in both prophylactic and postexposure settings (35). High-affinity anti-PA therapeutic antibodies can serve as effective prophylactics as well as late-stage infection antidotes. Scenarios in which both activities are important can be envisioned, especially in the event of exposure to an antibiotic-resistant anthrax strain (3).

In the adaptive immune response, antibodies are thought to represent the molecular link between recognition of a pathogen and its elimination through phagocytosis (antibody-dependent cytotoxicity) or complement activation (complement-dependent cytotoxicity). In both cases, Fc receptors provide the critical connection between an antibody-antigen binding event and effector functions. Fc receptors recognize and bind either to the hinge region/CH₂ domain interface, or, in some cases, to regions near the CH₂/CH₃ domain interface (8).

The *B. anthracis* exotoxin is essential for the survival of the organism in the host (13), so it is possible that the neutralization of PA by long-circulating, ultra-high-affinity antibody fragments may be sufficient to confer passive protection against

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anthrax despite the absence of Fc-mediated immune responses. Benefits of such a strategy could include (i) substantially lower costs of manufacturing antibody fragments, a critical issue since the current prophylactic IgG antibodies require large dosages (4 to 10 mg/kg of body weight) and must be produced by recombinant CHO cells, (ii) elimination of potentially adverse effects associated with Fc, and (iii) rapid production in bacterial cells.

Polyethylene glycol (PEG) has previously been used as a conjugating agent for enhancing pharmacokinetics/bioavailability, improving stability issues, and decreasing immunogenicity among other attributes for therapeutics (22, 28, 29, 48). PEGylation of Fab fragments has provided increased serum half-lives and efficacy for antibody therapy for a variety of applications (23–25). The availability of maleimide-PEG has allowed conjugation to antibodies through reaction with free native or engineered cysteines (12). This chemistry has proven to be suitable not only for Fab but also for single-chain variable fragments (scFvs) (1, 37, 54), immunoliposomes (38), and other conjugates (19).

Here we report the prevention of toxemia from inhalation of *B. anthracis* spores by the very-high-affinity ($K_D = 35$ pM) anti-PA antibody fragment (M18) conjugated to PEG. The M18 antibody fragment was derived from a library of random mutants of 14B7 scFv (31) screened by anchored periplasmic expression (APEX) (16). Intrinsic to the APEX screening strategy is that isolated antibody fragments are highly expressed in bacteria, and M18 is no exception. The conjugation of an M18 antibody fragment with a 40-kDa PEG polymer increased the serum half-life of the construct beyond that of full-length immunoglobulin. Subcutaneous administration of the conjugate resulted in a significant increase in survival and an improved overall mean TTD in a guinea pig model challenged with a relatively high dose of anthrax spores (Vollum 1B). These results reveal that binding to the toxin impairs the ability of *B. anthracis* to colonize the host and demonstrate the feasibility of using bacterially expressed antibody fragments as a prophylactic anthrax treatment. PEGylated antibody fragments can be developed and produced much more rapidly than current whole IgG therapeutics and, thus, could constitute a critical technology for rapid response to emerging bacterial and possibly even viral (e.g., influenza H5N1) pathogenic agents.

MATERIALS AND METHODS

Bacterial cell culture conditions. *B. anthracis* strain Vollum 1B was originally acquired from USAMRIID, and all manipulations of this organism were performed under BSL-3 and BSL-4 biocontainment. A single colony was subcultured in 4 ml of nutrient broth (Difco) at 37°C for 48 h prior to inoculation (1 ml each) into four baffled spinner flasks containing 250 ml of nutrient broth medium. After a 48-h incubation, cells were pelleted by centrifugation at $1,500 \times g$ for 10 min, resuspended into 20 ml G medium (15), and transferred into a 1-liter aerated vessel containing 500 ml G medium. Following 72 h of incubation at 37°C, cells were pelleted as described above, washed briefly with sterile water, resuspended in 20 ml G medium containing 10% glycerol, and stored at -80°C . Aliquots (1 ml) were serially diluted, and one-half of each dilution was subjected to 10 min of heat treatment at 75°C to confirm spore conversion. Both heat-treated and nonheat-treated dilutions were plated in duplicate on sheep blood agar plates to determine spore concentrations.

scAb expression and purification. The M18 scFv gene (16) was cloned via terminal SfiI sites into pMoPac16 (18), a modified version of the pAk4000 vector (26) that carries an scFv gene with the human κ light chain to create a single-chain antibody (scAb) (18, 34). The pMoPac16 plasmid also carries a gene for the coexpression of the *skp* periplasmic chaperone (17, 18). To create a protein suitable for

PEG conjugation, the M18 scAb gene was amplified with a C-terminal primer that incorporated a Cys residue downstream of the C-terminal His₆ purification tag. This latter gene was then ligated into pMoPac16 via NcoI and AscI restriction sites to create the pMoPac16Cys plasmid that contains the M18 scAb-His₆-Cys construct.

Escherichia coli Tuner cells (Novagen, Madison, WI) were transformed with pMoPac16_M18 and pMoPac16Cys_M18 and were grown at 25°C in 2-liter baffled flasks containing 400 ml Terrific Broth medium with 2% glucose and 200 $\mu\text{g/ml}$ ampicillin. Cultures were induced at an optical density at 600 nm of 1.6 with 1 mM IPTG (isopropyl- β -D-thiogalactopyranoside) (Sigma-Aldrich, St. Louis, MO) for 4 h, and then the cells were pelleted by centrifugation (10 min at $8,000 \times g$). Osmotic shock was carried out at 0°C as previously described (18). Cells were resuspended in 20 ml 0.75 M sucrose and 100 mM Tris-HCl (pH 8.0), with the addition of 1.0 ml of 10 mg/ml lysozyme in the same buffer. After shaking for 10 min at 0°C, 40 ml of 1 mM EDTA was added dropwise, followed by 15 min of further incubation at 0°C. A total of 3.0 ml of 0.5 M MgCl_2 was then added dropwise, followed by an additional 15 min of incubation at 0°C. Spheroplasts were pelleted by centrifugation, and the clarified supernatant was mixed with a 1/10 volume of $10\times$ IMAC buffer (100 mM Tris-HCl, 5 M NaCl, 0.2 M imidazole, pH 8.0) and applied to 1.5 ml Ni-nitrilotriacetic acid agarose resin (QIAGEN, Madison, WI). Following washing with 3×10 ml IMAC buffer, scAb protein was eluted with 500 mM imidazole in IMAC buffer and dialyzed against 2×2 liters phosphate-buffered saline (PBS) at 4°C overnight. Native M18 scAb was then applied to a Superdex 200 HR10/30 column (Amersham Biosciences, Piscataway, NJ) on an Δ ktA fast-protein liquid chromatography system (Amersham Pharmacia, Piscataway, NJ). Fractions were isolated and then concentrated with an Amicon ultra centrifugal filter device (molecular weight cutoff, 10,000; Millipore Corp., Bedford, MA). Protein concentrations were quantified using a micro-bicinchoninic acid quantification kit (Pierce, Rockford, IL).

scAb conjugation and purification. The conjugation of M18 scAb with PEG was performed as described previously (1, 37) with the following modifications: a twofold excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Molecular Probes, Eugene, OR) in PBS was added to purified scAb protein (2 mg/ml in PBS) and incubated overnight, with stirring at 4°C. The solution was then brought to room temperature and a twofold excess of maleimide-PEG (20 kDa or 40 kDa) (Nektar Therapeutics, San Carlos, CA) in PBS was added dropwise over 2 to 4 h. The scAb-PEG conjugate was purified by IMAC using Ni-nitrilotriacetic acid agarose. Eluate was then purified by size exclusion chromatography, concentrated, and quantified as mentioned for native scAb.

Biacore analysis. Surface plasmon resonance (SPR) analysis was performed using a Biacore 3000 instrument (Biacore, Piscataway, NJ). Recombinant PA (List Biological Laboratories, Campbell, CA) in 10 mM $\text{Na}_2\text{H}_2\text{O}_2$ (pH 5.0) was immobilized on a CM5 chip using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide *N*-hydroxy-succinimide chemistry. The PA solution was added as a 5 $\mu\text{g/ml}$ solution until an amount equivalent to 500 response units was obtained. scAb and scAb-PEG proteins (2.5 nM to 40 nM) in HBS-N (Biacore) were used at a flow rate of 100 $\mu\text{l/min}$. A solution of 4 M MgCl_2 was used as the regeneration buffer between runs. Data were analyzed using BIAevaluation software (version 3.0). A second flow cell with bovine serum albumin was used for data baseline correction. For some experiments, the scAb with or without PEG protein was immobilized onto the chip at a level corresponding to 120 response units under the same coupling conditions as described above and PA was used as the analyte.

In vitro anthrax toxin challenge. Anthrax LeTx challenges were performed with RAW 264.7 mouse macrophage cells as previously described (33, 50).

Pharmacokinetic studies. Female Hartley guinea pigs (225 to 305 g) (Charles River Laboratories, Wilmington, MA) were given dorsal, subcutaneous injections of 1.2 ml of M18 scAb-PEG protein (20 or 40 kDa) or 14B7 IgG to a dose of 10 mg/kg or PBS as a control. Following sedation with ketamine (80 mg/kg) and xylazine (10 mg/kg), animals were bled when time reached 0 for 15 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, and 72 h at the femoral artery. The serum was collected and centrifuged as mentioned above. The construct concentrations were determined by enzyme-linked immunosorbent assay as follows: PA (2.5 $\mu\text{g/ml}$) in PBS was coated onto CoStar 96-well plates (Corning Inc., Corning, NY) and incubated overnight at 4°C. The wells were then blocked with the addition of 2% milk-PBS for 3 h at room temperature, and diluted serum (1:10) was added in 2% milk-PBS. scAb-PEG constructs were detected by using goat anti- κ light chain horseradish peroxidase (Sigma-Aldrich, St. Louis, MO). 14B7 murine IgG was detected with goat anti-mouse IgG horseradish peroxidase (Bio-Rad, CA). Data were modeled using WinNonlin software (Pharsight, Mountain View, CA), formatted to be consistent with a one-compartment, bolus, first-order elimination model.

Inhalation challenge with *B. anthracis* spores. Animals were housed individually in a One Cage 2100 AllerZone interchangeable microisolator high density housing system (Lab Products Inc., Seaford, DE) in the BSL-4 safety facility at

FIG. 1. M18 scAb–40-kDa PEG (lane A) and scAbCys (lane B) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (4 to 20%), under reducing conditions, stained with Coomassie blue for protein identification.

the Southwest Foundation for Biomedical Research (San Antonio, TX). Anthrax spore inocula were prepared on the day of challenge and diluted to the desired concentration in PBS. Female Hartley guinea pigs (225 to 305 g) were sedated with ketamine (80 mg/kg) and xylazine (10 mg/kg) during all bleeds, injections, and inhalation instillations. Animals were injected subcutaneously with 3.0 ml of PBS containing M18 scAb–40-kDa PEG to a dosage of 40 mg/kg or 80 mg/kg of body weight. Control animals were injected with 3.0 ml of either PBS, unconjugated 40-kDa PEG–maleimide (40 mg/kg), or native M18 scAb (40 mg/kg). Four hours later, the animals were challenged with anthrax spores by unilateral instillation of $250 \times$ to $625 \times$ LD₅₀ (1×10^7 to 2.5×10^7) of *B. anthracis* Vollum 1B (2) spore inocula (100 μ l total volume) in the nares of animals. Animals were monitored for a 2-week period and euthanized by cardiac injection of sodium pentobarbital when considered moribund or at project end.

Lungs and spleens were removed from the guinea pigs after euthanasia. A 100-mg section was excised and homogenized with 0.4 ml of sterile PBS in a sterile tissue grinder. The homogenates were serially diluted in sterile PBS and then plated in duplicate on sheep blood agar plates. After 24 h of incubation at 37°C, the colonies were enumerated to determine CFU/100 mg of tissue.

RESULTS

Conjugate preparation and characterization. The scAb form of M18 was fused to a His₆ tag followed by a terminal Cys residue for the conjugation of PEG–maleimide (1, 37, 54). We have found that the scAb form of antibodies, comprised of an scFv fragment fused to a human C κ domain (34), has superior expression, stability, and serum half-lives compared to scFv fragments (33).

Expression from a *lac* promoter in *E. coli* Tuner cells, followed by purification by IMAC, produced a mixture of a monomer and a disulfide-linked dimer with an average yield of 8 mg/liter (1.3 mg/liter optical density at 600 nm) in shake flask culture. Reduction of the inter-scAb disulfide by using TCEP produced pure monomeric M18 scAb, which was conjugated to either 20-kDa or 40-kDa PEG–maleimide (Fig. 1). Following gel filtration fast-protein liquid chromatography, the purified protein conjugates were found to contain <2 ng lipopolysaccharide/mg of protein by using the QCL-1000 *Limulus* amoebocyte lysate assay kit (Bio-Whittaker, MD).

The antigen binding kinetics of PEGylated and free M18 scAb antibody fragments was evaluated by SPR on a Biacore 3000 instrument using two complementary assay formats. The measured dissociation rates (k_d) of the antigen-antibody complex were not affected by the conjugation of PEG, regardless of whether PA or the scAb was immobilized on the chip. On the other hand, the association rates (k_a) did depend on the assay format. Specifically, binding of M18-scAb constructs in the mobile phase to PA immobilized on the Biacore chip resulted in a reduction in the rate of complex formation as the attached PEG size increased comparable to that observed in earlier studies (54) (Table 1). However, when the M18-scAb antibody constructs were attached to the Biacore chip and binding was measured with free PA in the mobile phase, the observed association rates were no longer affected by the presence or the size of the PEG chain, and, thus, similar overall equilibrium dissociation constants were measured for all constructs.

The in vitro toxin neutralization activity of the M18 scAb–20-kDa PEG and M18 scAb–40-kDa PEG conjugates were analyzed using the mouse macrophage assay (33, 50) (Fig. 2). RAW 264.7 mouse macrophage cells (ATCC TIB-71) were treated with M18 scAb, M18 scAb–20-kDa PEG, and M18 scAb–40-kDa PEG serially diluted from 10 nM to 0.31 nM. LeTx (100 ng/ml PA, 80 ng/ml LF) was added, and cell viability was assayed. The unconjugated M18 scAb, as well as both PEG conjugates, displayed complete protection at concentrations above 5 nM. These results verify that the presence of the PEG chain does not significantly influence neutralization of anthrax LeTx, consistent with the Biacore results.

To evaluate the serum persistence of the M18 scAb-PEG antibody fragment, Hartley guinea pigs were injected subcutaneously with 10 mg/kg of M18 scAb–20-kDa PEG, M18 scAb–20-kDa PEG, or 14B7 murine monoclonal IgG. Serum concentrations of the constructs were determined by enzyme-

TABLE 1. Kinetic analysis of anti-PA scAb with or without PEG binding using SPR

Biacore format ^a	Construct	Analysis value for:		
		k_a (1/Ms)	k_d (1/Ms)	K_D (M)
PA down; antibody analyte	M18 scAb	1.4×10^6	6.3×10^{-5}	4.4×10^{-11}
	M18 scAb–20-kDa PEG	1.0×10^5	7.0×10^{-5}	6.9×10^{-10}
	M18 scAb–40-kDa PEG	2.7×10^4	7.2×10^{-5}	2.6×10^{-9}
Antibody down; PA analyte	M18 scAb	9.3×10^5	8.7×10^{-5}	9.5×10^{-11}
	M18 scAb–20-kDa PEG	7.8×10^5	2.0×10^{-5}	2.5×10^{-11}
	M18 scAb–40-kDa PEG	8.7×10^5	3.8×10^{-5}	4.3×10^{-11}

^a Two Biacore formats were used for measuring binding affinities. In the first format, with PA immobilized as the ligand, an apparent decrease in k_a was observed with PEGylation and decreases with increasing PEG molecular size. The second format used the scAb with or without PEG constructs as ligands and PA as the analyte. The decrease in k_a seen in the previous format was not observed.

FIG. 2. In vitro toxin challenge. RAW 264.7 mouse macrophage cells were grown on microtiter wells, incubated with 0.31 to 10 nM scAb or PEGylated scAb as shown, and challenged 5 min later with lethal toxin (100 ng/ml PA, 80 ng/ml LF). The percentage of cells surviving toxin challenge at a specific antibody dose, compared to that of the negative control, untreated cells, was measured by the viability assay. The average of triplicate experiments is reported.

linked immunosorbent assay, and the data were fit to a one-compartment, bolus, first-order elimination model (Fig. 3). As expected, conjugation with PEG dramatically extended half-life in a manner dependent on the molecular mass of the PEG chain. Notably, the M18 scAb–40-kDa PEG antibody conjugate displayed a serum half-life of 108 h, slightly longer than that of murine IgG (96 h).

Inhalation challenge experiments. Prophylactic treatment with M18 scAb PEG conjugates was tested in female Hartley guinea pigs challenged with *B. anthracis* Vollum 1B spores via the inhalation route. Guinea pigs were exposed to 250 $\times$ to 625 $\times$ LD₅₀ spores of the Vollum 1B strain 3 h after subcutaneous injection of 40 mg/kg (five animals) and 80 mg/kg (six animals) of M18

FIG. 3. Pharmacokinetic analysis of Hartley guinea pigs injected subcutaneously with 14B7 IgG (black), M18 scAb conjugated to 20-kDa PEG (red), or M18 scAb conjugated to 40-kDa PEG (green). scAb–20-kDa PEG displayed a half-life of 23.2 h, 14B7 displayed a half-life of 96 h, and scAb–40-kDa PEG displayed a half-life of 108 h. Solid lines were calculated from a subcutaneous, bolus, first-order elimination model using WinNonLin software.

FIG. 4. In vivo inhalation anthrax spore challenge. Female Hartley guinea pigs (250 to 310g) were exposed to 250 $\times$ to 625 $\times$ LD₅₀ anthrax spores via intranasal inoculation 4 h after administration of scAb–40 kDa PEG at 40 mg/kg (yellow triangles) or 80 mg/kg (pink squares), PBS (blue diamond), unconjugated PEG–maleimide (green square), or native scAb (purple square). Animals were monitored for at least 14 days after spore exposure.

scAb–40-kDa PEG or controls using PBS (five animals) or unconjugated PEG (three animals) (Fig. 4). The relatively high load of spores was intended to simulate a possible bioterrorism anthrax attack.

Within 72 h, all animals treated with PBS alone or with unconjugated PEG died at times corresponding to dosage (2) with symptoms characteristic of inhalation anthrax toxemia (Fig. 4). Culture of tissues harvested at necropsy revealed high levels of *B. anthracis* in both the lungs (3.2×10^7 to 1.6×10^9 CFU/100 mg tissue) and spleen (1.3×10^7 to 7.2×10^9 CFU/100 mg tissue). In contrast, all 11 animals treated with a subcutaneous injection of M18 scAb–40-kDa PEG exhibited significantly prolonged TTD, with 60% (3/5) and 50% (3/6) surviving the 14-day time course of the experiment at the 40 mg/kg and 80 mg/kg doses (P , <0.001; Holm-Sidak test), respectively (Fig. 4). Necropsy of the surviving animals that were euthanized at the project's end revealed healthy organ appearance, and no bacteria or spores were detected in the lungs or spleens in culture or upon microscopic examination of tissue samples. Analysis results of animals that perished after treatment with the 40 mg/kg dose displayed lymph depletion, edema, minimal hemorrhage, and low levels ($\sim 10^2$ to 10^5 CFU/100 mg tissue) of anthrax bacteria in the lungs and spleens. Analysis of a guinea pig that succumbed in the 80 mg/kg antibody conjugate group showed a very small amount of hemorrhage and lymph depletion, yet no bacteria could be seen in a microscopic inspection of lung or spleen tissues.

DISCUSSION

In this study, we investigated the ability of high-affinity anti-PA antibody fragments conjugated to PEG to confer prophylactic protection against challenge with inhalation anthrax spores. Previously, random mutagenesis and flow cytometric screening of a library derived from 14B7 scFv using APEX (16) led to the isolation of the M18 antibody fragment that contains

10 amino acid substitutions and exhibits a 200-fold increase in affinity compared to that of 14B7 (31). The introduction of a single C-terminal Cys into the M18 scAb permitted conjugation to PEG-maleimide in a high yield without the formation of undesirable by-products such as antibody multimers or multiple PEG-conjugated species. As with other antibodies isolated by flow cytometric screening of microbial libraries (6, 10), the M18 scAb was well expressed. An average yield of 8 mg/liter of shake flask culture was obtained using the moderately strong *lac* promoter. A satisfactory expression level is critical for the preparative production of antibody therapeutics.

Conjugation of PEG to M18 scAb had no effect on the dissociation rate constant, K_D , while the association rate constant, K_A , was found to be dependent on the Biacore assay format. When the PA was immobilized on the Biacore chip and the scAb constructs were in the mobile phase, a decrease in K_A with increasing PEG size was seen. No such trend was observed when the M18 scAb constructs were immobilized on the chip. Although the biologically relevant value of K_A is not clear at this point, it should be noted that the therapeutic potency of anti-PA antibody fragments can be influenced by the dissociation rate constant, K_D , (33) which, in the case of the M18 scAb, is clearly not affected by PEGylation. Consistent with this assertion, PEGylation did not affect the activity of the antibody in protecting RAW 264.7 macrophages from toxin challenge in vitro. Importantly, the PEG modification significantly increased serum persistence to the point that following subcutaneous administration, the M18 scAb-40-kDa PEG conjugate exhibited a serum half-life beyond that of the 14B7 whole IgG.

Prophylactic administration of the M18 scAb-40-kDa PEG construct resulted in a significant increase in TTD and survival of between 50 to 60% of the animal group for the 14-day duration of the experiments compared to a 3-day mean TTD for the group of animals immunized with PBS alone, unconjugated PEG, or with native scAb. No statistically significant increase in protection was observed with the 80 mg/kg dose of M18 scAb-40-kDa PEG compared to the 40 mg/kg dose, indicating that the latter or perhaps an even smaller dose may be adequate to confer the same level of protection.

In the group that was treated with 40 mg/kg, two of the six animals died on days 12 and 13, raising the possibility that perhaps the percentage survival might have been lower if the experiments had been continued for longer times. This is probably not the case, however, since histological inspection of the surviving animals did not reveal any bacteria in the lungs or the spleens, suggesting that the infection had been eradicated.

It is not yet entirely clear how a high anti-PA antibody serum titer, elicited either through active immunization or through passive immunization with anti-PA antibodies (30, 35), prevents inhaled spores from causing anthrax. The data presented here indicate that an ultra-high-affinity, anti-PA antibody fragment lacking an Fc region is sufficient to confer prophylactic protection against heavy challenge with inhalation anthrax spores. The prolonged TTD and low or nonexistent amount of live bacteria in all treated animals suggests that a higher degree of prophylaxis could be possible upon repeated antibody fragment administration.

To our knowledge, the results presented here represent the first report of immunological protection against pathogen in-

fection by antibody fragments lacking Fc domains and administered directly to the animal prior to challenge. In the absence of Fc, the mechanism of protection is unlikely to involve either antibody-dependent cytotoxicity or complement-dependent cytotoxicity. Thus, it appears that the interaction of the high-affinity antibody fragment with toxin is sufficient to prevent the establishment of infection by preventing spore germination (52), by preventing the dissemination of vegetative bacteria from lymphoid organs, or by perhaps some other mechanism.

Regardless of the mechanism, the extent of protection conferred by M18 scAb-40-kDa PEG is roughly comparable to that observed with guinea pigs treated with rabbit polyclonal anti-PA IgG in a previous study (30). We note, however, in the earlier study by Little et al. (30), the guinea pigs were exposed to a substantially lower dose of spores relative to the present experiments ($40 \times \text{LD}_{50}$ versus $250 \times$ to $600 \times \text{LD}_{50}$) and a different strain (Ames versus Vollum 1B), so caution must be used when making any direct comparison. Nevertheless, it is reasonable to suggest that the protection observed with M18 scAb-40-kDa PEG is significantly better than that reported for the parent 14B7 IgG monoclonal antibody (30). A reasonable conclusion is that the very high affinity of the engineered M18 antibody fragment is responsible for the increased protective activity. As support for this hypothesis, we note that the interaction between PA and the CMG2 receptor is relatively strong; K_D was 170 pM (53), meaning that M18 ($K_D = 35$ pM) can effectively compete with this interaction, while 14B7 ($K_D = 4.3$ nM) cannot.

Since the anti-PA M18 scAb-40-kDa PEG lacking an Fc region is able to confer prophylactic protection against heavy challenge with inhalation anthrax spores, it is reasonable to assume that other means of inactivating anthrax toxins should also hold promise as potential therapeutics for anthrax. These include multivalent peptides that bind to PA (36), small molecule inhibitors of LF (39, 46) and dominant negative PA mutants (45).

From a therapeutic development perspective, the key advantages of PEGylated antibody fragments include the ease of isolation of scFv or scAb from combinatorial libraries and the facile low-cost production by well-established techniques using *E. coli*. Combined with the ease of subcutaneous injection, the approach presented here represents a highly practical strategy for a large-scale prophylactic response to anthrax exposure, including antibiotic-resistant strains (3). Studies are under way to determine whether PEGylated, high-affinity antibody fragments can be employed for protection or therapy against other bacterial agents where pathogenicity is intimately associated with toxin production, e.g., Shiga toxin-producing hemolytic *E. coli* (49), other microbial pathogens, and, finally, viral infections. If that proves to be the case, PEGylated antibodies will likely represent a practical and rapidly deployable therapeutic avenue for combating emerging infections.

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