Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity

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The tripartite toxin produced by *Bacillus anthracis* is the key determinant in the etiology of anthrax. We have engineered a panel of toxin-neutralizing antibodies, including single-chain variable fragments (scFvs) and scFvs fused to a human constant κ domain (scAbs), that bind to the protective antigen subunit of the toxin with equilibrium dissociation constants (K_d) between 63 nM and 0.25 nM. The entire antibody panel showed high serum, thermal, and denaturant stability. *In vitro*, post-challenge protection of macrophages from the action of the holotoxin correlated with the K_d of the scFv variants. Strong correlations among antibody construct affinity, serum half-life, and protection were also observed in a rat model of toxin challenge. High-affinity toxin-neutralizing antibodies may be of therapeutic value for alleviating the symptoms of anthrax toxin in infected individuals and for medium-term prophylaxis to infection.

Anthrax is a zoonotic soil organism endemic to many parts of the world. Infection by inhalation of the heat-resistant spores of the Gram-positive bacterium *B. anthracis* can result in a mortality rate of up to 80%[1]. The organism was one of the first biological warfare agents to be developed and continues to be a major threat in this regard[2]. Although vaccine strains have been developed, currently there are concerns regarding their efficacy and availability[3]. A passive immunization strategy may be useful in conferring medium-term protection, and can also have benefits for non-immunized patients who seek treatment after the point at which antibiotic therapy alone is effective[4,5].

After inhalation by mammals, *B. anthracis* spores germinate in the alveolar macrophages, then migrate to lymph nodes where they multiply and enter the bloodstream, reaching 10^7–10^8 organisms per milliliter of blood[4] during an initial incubation period of ~11 days[1,6]. The vegetative bacteria excrete the tripartite exotoxin that is responsible for the etiology of the disease. The exotoxin consists of an 83 kDa polypeptide, protective antigen (PA), that binds to a recently identified receptor on the surface of macrophages[7]. After cleavage by a furin-like protease and oligomerization into a heptameric ring, PA facilitates translocation of the two catalytic components of the exotoxin, the lethal factor (LF) and the edema factor (EF), into the cellular cytosol. The LF is a zinc metalloprotease that cleaves several mitogen-activated protein kinase kinases. The EF is a calmodulin-dependent adenylate cyclase that causes local edema and impairs neutrophil function (reviewed in ref. 8).

Preventing PA function is of therapeutic benefit in conjunction with antibiotic therapy because it alleviates the toxin-dependent symptoms of anthrax[4]. Blocking the activity of the toxin has been accomplished by disrupting the function of PA in one of three ways: (i) by using peptides or antibodies to prevent binding of the catalytic subunits[9,10], (ii) by interfering with PA oligomerization using dominant-negative mutants[11,12], or (iii) by blocking the binding of the toxin to its receptor with soluble receptor[7] or anti-PA antibodies[10,13–15].

Passive immunization with antibodies has been used successfully to confer protection against infectious diseases[16], and engineered antibodies have great potential for combating natural diseases and biowarfare agents[17]. For example, humanized neutralizing antibodies are being used clinically as prophylaxis against respiratory syncytial virus[18]. Administration of polyclonal antisera against PA prevents spore infection in guinea pigs[14,15]. In vaccination studies, elicitation of polyclonal antibodies to PA correlates with protection[19,20] and, in fact, PA constitutes the primary immunogenic component of the anthrax vaccine approved by the US Food and Drug Administration. Recent studies indicate that altered spore phagocytosis by host macrophages is involved with this protection[21].

We have engineered a panel of anti-PA scFvs and scAbs (Fig. 1) that compete with the cellular receptor for PA binding and have equilibrium dissociation constants (K_d) between 63 nM and 0.25 nM as measured by surface plasmon resonance (SPR). Protection against anthrax toxin challenge in an *in vitro* cell culture assay and in a rat model correlated strongly with affinity, with the highest-affinity antibody, referred to as 1H (K_d = 0.25 nM), conferring the best protection.

Results and discussion

Engineering of anti-PA antibodies with different binding affinities. The heavy-chain (V_H) and light-chain (V_L) genes of four potent toxin-neutralizing monoclonal antibodies (14B7, 3B6, 10E10, and 1G3)[10,13] were isolated by RT-PCR. Overlap extension PCR was used to produce 750 bp scFv gene fragments, with a sequence encoding

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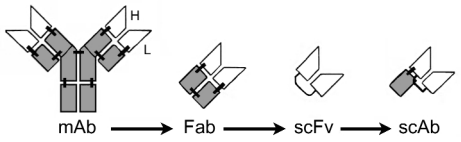

Figure 1. Antibody constructs. mAb, Fab, and scFv consisting of variable heavy (H) and light (L) chains joined by a flexible polypeptide linker; and scAb generated by C-terminal fusions of a human constant κ domain to an scFv. Light gray, variable regions; dark gray, constant regions.

the $(Gly_4Ser)_4$ linker inserted between the C termini of the light-chain sequences and the N termini of the heavy-chain sequences. The scFv genes were fused to the pIII protein in the vector pAK100 for display in filamentous bacteriophage, and clones expressing the active protein were identified by phage enzyme-linked immunosorbent assay (ELISA). After DNA sequencing, expression, and preliminary characterization of the scFv antibodies, 14B7 was chosen for further studies.

Error-prone PCR (predicted error rate, 0.5% nucleotide substitutions per gene[22]) was used to construct a library of 5×10^5 transformants. After five rounds of panning on PA, the affinity-enhanced clone A2E was isolated. The corresponding scFv protein had a threefold slower off-rate (k_{off}) to PA as determined by SPR, and markedly greater stability to both high temperature and chemical denaturation, as compared with the native protein. DNA sequencing revealed that A2E contains three DNA mutations: two silent mutations and a single S56P substitution in the light-chain complementarity-determining region (CDR) L2 (Fig. 2 and Table 1). This mutation was present in all subsequent higher-affinity scFvs. (The mutation S56P was even present in clones selected after backcrossing with the parental 14B7 scFv by DNA shuffling.) Clones isolated from the fifth round of phage panning of the original library were recombined by DNA shuffling[23] with wild-type 14B7. The resulting gene pool was amplified under error-prone PCR conditions and screened using five additional rounds of phage panning. The entire process—shuffling, error-prone mutagenesis, and panning—was repeated, giving rise to clone 1H, which had enhanced antigen affinity (Supplementary Fig. 1 online). DNA sequencing showed that 1H contains, in addition to S56P, the two mutations Q55L, in CDR L2, and K(106A)R, in light-chain framework four. Notably, Gln55 in V_L was identified independently as a residue capable of modulating affinity during an alanine scanning mutagenesis study of 23 residues at the 14B7–PA interface; a Q55A substitution in V_L resulted in a variant with threefold higher affinity (data not shown).

To provide a spectrum of low-affinity, wild-type, and affinity-matured variants, we also constructed an antibody containing the mutation L97A in V_H by site-directed mutagenesis. The L97A substitution results in a fivefold lower affinity than that of the 14B7 scFv and was also identified in the course of alanine scanning mutagenesis studies.

The serum distribution and clearance rates of antibody constructs are affected by molecular size. We therefore generated two monovalent scAbs of larger relative molecular mass (45 kDa) by C-terminal fusion of the 14B7 scFv or the affinity-enhanced variant 1H scFv to a human constant κ domain[24].

Characterization. All the scFvs and scAbs were produced in *Escherichia coli* at comparable levels. Purified yields of 0.25–0.5 μg protein per A_{600} unit or 1–2 mg per liter of culture were obtained, comparable to those of other well-expressed antibody fragments[25,26]. The antibodies were purified by metal affinity chromatography followed by size-exclusion fast-performance liquid chromatography to remove higher-molecular-mass aggregates. Crude scFvs were predominantly (>80%) monomeric, indicating a minimal tendency to dimerize *in vivo* during production in *E. coli* (Supplementary Fig. 2 online), and after repeated chromatography, isolated scFvs remained >95% free of higher-molecular-mass species. Freshly prepared antibody samples were used to determine antigen binding kinetics by SPR and to evaluate efficacy in neutralizing the anthrax toxin.

The 14B7 scFv had antigen binding kinetics of $k_{on} = 3.0 \pm 0.4 \times 10^5$ M^{-1} s^{-1}, and $k_{off} = 0.0032 \pm 2$ s^{-1}, giving a K_d of 12 nM (these kinetics are identical, within error, to those observed for the 14B7 Fab ($k_{on} = 2.9 \pm 0.5 \times 10^5$ M^{-1} s^{-1} and $k_{off} = 0.0033 \pm 2$ s^{-1}; K_d = 12 nM)). The divalent 14B7 IgG had a K_d of 2.3 nM measured under the same conditions. The A2E and 1H mutants had 4-fold and 50-fold lower K_ds, respectively, primarily as a result of slower off-rates. Notably, the best mutant, 1H, showed a k_{off} of 0.00017 ± 2 s^{-1}, corresponding to an scFv–PA complex half-life of ~100 min. In contrast, the alanine mutant L97 had a K_d over fivefold higher than that of 14B7 scFv (63 nM; Table 1).

Plückthun and coworkers have found that the biodistribution and targeting efficiency of scFv antibodies that recognize the epithelial tumor antigen glycoprotein-2 correlate with thermal and denaturant stability[27]. Although typical scFvs lose activity rapidly in serum at 37°C (refs 28, 29), the family of recombinant antibody fragments reported in this study was found to be highly resistant to deactivation in serum (Table 1), showing no loss of activity after a seven-day incubation at 37°C. The scFvs and the scAbs also showed good stability at elevated temperatures. Notably, the A2E scFv retained about 25% of its binding activity even after a two-hour incubation at 70°C (Supplementary Fig. 3 online). The two scAbs had higher thermal stability than did their respective scFvs, presumably because of the contribution of favorable interactions by the C_κ domain. For all the scFvs, unfolding was detected only at urea concentrations above 5 M, and urea concentrations required for 50% unfolding ($m_{1/2}$ values) were 6.8, 7.7, and 6.5 M for the 14B7, A2E, and 1H scFvs, respectively (Supplementary Fig. 4 online). Typical scFvs have $m_{1/2}$ values of 2.5–4.5 M urea[30,31].

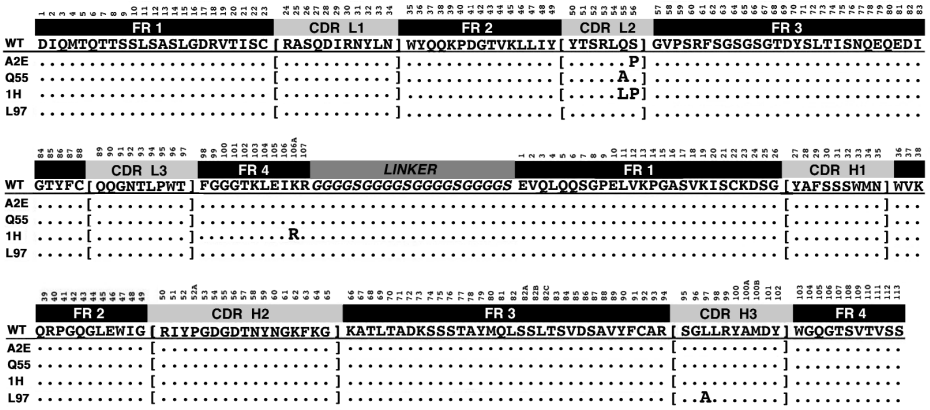

Figure 2. Amino acid sequence alignment of 14B7 scFv and related variants. 14B7 sequence is written in single letter amino acid code. Kabat numbering scheme is indicated alongside; CDRs are bracketed; amino acid changes are indicated.

Table 1. Summary of antibody affinity and stability

Antibody	K_d (nM)	Ab–PA complex half-life (min)	k_{on} ($\times 10^5$ M^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	Stability at: 37°C*	70°C†
14B7 mAb	2.3	12	5.7 ± 1.1	13.5 ± 1.2	100%	15%
14B7 scFv	12	5.2	3.0 ± 0.4	32 ± 2	100%	3.5%
14B7 scAb	11	5.6	2.8 ± 0.3	30 ± 0.8	ND	14%
L97 scFv	63	0.9	3.1 ± 0.7	190 ± 20	100%	10%
A2E scFv	3.0	17	3.2 ± 0.8	10 ± 1.5	100%	28%
1H scFv	0.25	100	6.4 ± 0.8	1.7 ± 0.2	100%	5%
1H scAb	0.26	100	6.1 ± 0.9	1.6 ± 0.4	ND	10%

*Anti-PA activity, as determined by ELISA, after incubation for seven days at 37°C in 90% FBS. †Anti-PA activity after 2 h incubation in PBS at 70°C. ND, not determined.

Table 2. Serum half-lives of proteins

Protein	Size (kDa)	$t_{1/2}\alpha$ (min)[a]	s.d. (min)	No. of rats
14B7 scFv	28	5.4[b,c]	0.3	5
1H scFv	28	5.2[b,c]	0.7	4
14B7 scAb	45	10.6[b,c]	1.1	5
1H scFv	45	10.4[b,c]	1.2	5
PA	83	35[c]	14	4

Serum half-lives of different antibody fragments and PA after intravenous injection were determined in rats (see Experimental Protocol). [a]Significant differences were calculated with a two-tailed Student's *t*-test. [b]$P < 0.0005$ when 14B7 scFv or 1H scFv were compared with 14B7 scAb or 1H scAb. [c]$P < 0.01$ when PA was compared with the antibody fragments. s.d., standard deviation.

We measured serum clearance rates, and as expected[27,32], antibody fragments are cleared rapidly at rates that correlate with size for scFv and scAb constructs ($t_{1/2}\alpha$ values of ~5 min and 10 min, respectively; Table 2). The amino acid differences between 14B7 and 1H did not alter clearance rates. PA, an 83 kDa molecule, is cleared considerably more slowly than either recombinant antibody ($t_{1/2}\alpha$ of ~35 min; Table 2).

Toxin neutralization *in vitro*. We comprehensively evaluated protection of RAW 264.7 mouse macrophages by antibody constructs administered at different times before or after toxin challenge (1.2 nM PA, 0.6 nM LF). At all time points, protection correlated strongly with the affinity of all scFvs, with higher affinity conferring greater protection (Fig. 3A). The 14B7 scFv and 14B7 scAb antibody fragments showed comparable protection, whereas the 14B7 IgG monoclonal antibody (mAb) gave somewhat greater protection, presumably because it is bivalent (Fig. 3B). Notably, the high affinity 1H scAb was significantly more effective even when compared with the 14B7 IgG, resulting in lower IC_{50} dosage (Fig. 3B). For example, at a 3 nM dose, 1H conferred significant protection even 20 min after challenge (protecting ~20% of cells), whereas administration of the parental 14B7 scFv and the 14B7 IgG mAb resulted in less than 3% survival. These results indicate that the antibody-mediated inhibition of PA action *in vitro* correlates with affinity and that significantly better protection is achieved when the antibody–PA affinity is comparable to or higher than that of PA binding to its macrophage receptor (K_d = 1 nM)[33].

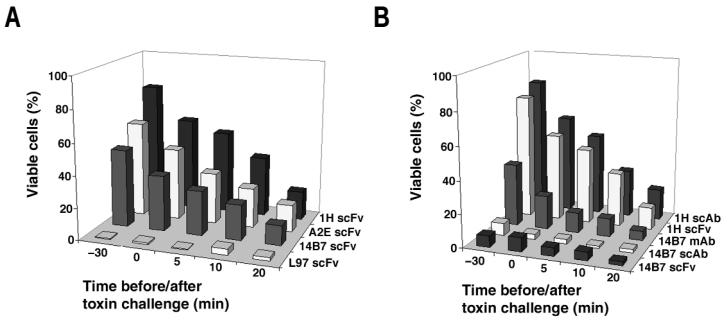

Figure 3. *In vitro* protection of RAW 264.7 mouse macrophages by antibodies. Survival of RAW 264.7 mouse macrophage-like cells after administration of antibodies at specified times after challenge with toxin (100 ng/ml PA, 50 ng/ml LF). Concentrations were chosen from a titration curve to show the largest dynamic range. (A) Comparison of scFv antibody fragments with varying affinity at 9.5 nM scFv. (B) Comparison of different antibody constructs at 3 nM antibody. Antibodies were either pre-incubated with toxin for 30 min or added to cells 0, 5, 10, or 20 min after toxin challenge. The percentage of cells surviving toxin challenge at a specified antibody dose, as compared with sham-treated cells, was measured by an MTT assay (Experimental Protocol). The average of triplicate measurements is reported. Trends were similar for replicate experiments.

Toxin neutralization *in vivo*. To evaluate the effect of antibody neutralization *in vivo*, we examined protection against toxin challenge in the Fisher 344 rat[34]. In earlier studies, protection against anthrax toxin was evaluated by mixing the neutralizing agent with toxin before injection into the animal model[10,11]. As a more stringent test, the antibody preparations in our study were administered five minutes before injection of toxin to allow for distribution in the animal. Rats were challenged with a 10× minimum lethal dose (MLD) amount of PA and LF (40 μg and 8 μg, respectively)[35]. Survival was monitored for five hours after toxin injection. (A five-hour end point was mandated by the Southwest Foundation for Biomedical Research Institutional Animal Care and Use Committee.) Control rats receiving only PBS as treatment all died at about 90 min after injection (Fig. 4A).

A correlation between scFv affinity, delayed time to death, and number of survivors was observed in the rats receiving the four scFv antibody constructs ($P < 0.001$ based on Spearman rank correlation corrected for tied ranks; Fig. 4A). Specifically, animals treated with the low-affinity variant L97 scFv (K_d = 63 nM; 2.0 nmol dose) were not protected, whereas those receiving 14B7 scFvs (12 nM; 2.0 nmol) showed a small increase in the time to death compared with the PBS control rats. On the other hand, a significant increase in time to death and number of survivors was observed with the affinity-enhanced A2E (K_d = 3 nM; 2 nmol) and 1H (K_d = 0.25 nM; 2 nmol) scFvs ($P < 0.01$, Mann-Whitney *U*-test).

Conversion of the highest-affinity 1H scFv into the larger scAb antibody fragment format afforded complete protection against animal intoxication at a 2.0 nmol dose over the five hours of the experiment (Fig. 4B). As seen with the scFvs, the protection afforded by the 1H scAb was significantly greater (P = 0.005) than that provided by the 14B7 scAb. Enhanced protection by the scAb fragments as compared with the scFv antibodies is most likely a consequence of greater serum half-life (Table 2). However, the possibility that the human C_κ chain of scAb antibodies somehow enhances neutralization, although unlikely, cannot be completely ruled out. The strong *in vivo* protection conferred by the 1H binding site is particularly notable, considering that the serum half-lives of scFvs and scAbs are considerably shorter than that of PA (Table 2). Thus, protection by the 1H scFv or scAb likely depends on the antibody fragment remaining bound to PA until the PA–antibody complex is cleared from the serum. Such a scenario is made plausible by the relatively long PA–1H antibody complex half-life for dissociation (100 min; Table 1). It should be noted that the overall high structural stability of the engineered antibody variants, revealed in the serum, thermal, and denaturant stability studies, probably contributes to efficacy *in vivo* as well[27].

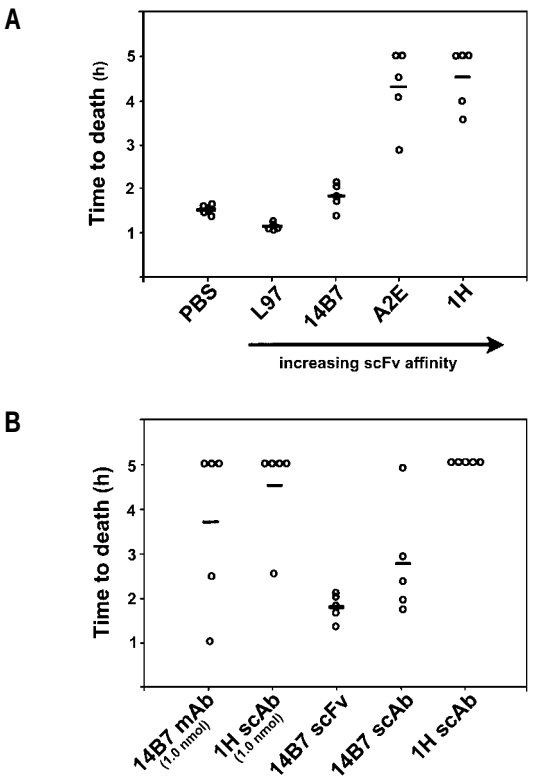

Figure 4. *In vivo* protection data. Rats received either PBS or antibodies in a 200 µl volume by penile vein injection 5 min before receiving a 10× MLD of anthrax toxin, also by penile vein injection, and time to death (TTD) was determined. Individual TTD is indicated by ○ (survivors counted at five hours) and the average TTD for a treatment group by —. All doses at 2.0 nmol, unless indicated. (A) Comparison of PBS control and increasing affinity of 14B7-derived scFvs. (B) Comparison of different antibody constructs and doses.

This study presents a comprehensive analysis of the effects of affinity, stability, and molecular weight on toxin neutralization. We have shown that the highest-affinity 1H antibody provides the best protection against anthrax intoxication both in an *in vitro* assay and in the rat model. The higher protection afforded by the 1H scAb relative to the scFv indicates that the benefit of increased binding affinity becomes even more pronounced with antibody fragments of higher molecular mass. On the basis of these considerations, we are currently generating humanized IgG antibodies incorporating the 1H binding site for testing in a non-human primate model. In conjunction with antibiotics, these antibodies are likely to prove valuable for alleviating the toxin-related symptoms of infected individuals. In addition, high-affinity, recombinant anti-PA antibodies may confer medium-term protection against the progression of anthrax infection[19,20].

Experimental protocol

Cloning from hybridomas. The heavy- and light-chain variable regions were cloned from anti-PA hybridomas[10,13] into the scFv phage display vector pAK100 exactly as described by Krebber *et al.*[36]. Monoclonal phage ELISA was used to identify PA-reactive clones.

Antibody affinity maturation. Error-prone libraries of the 14B7 wild-type scFv gene were constructed using standard protocols[22]. DNA shuffling was performed as described[23]. The library construction and screening strategies and the lineage of affinity-improved clones are described in Supplementary Figure 2 online. Panning was performed by coating immunotubes (Nunc, Rochester, NY) or high-binding ELISA wells (Costar, Corning, NY) with decreasing concentrations of PA (0.5 µg/ml to 0.03 ng/ml) overnight, blocking with 5% milk–PBS, adding 10^{11}–10^{12} plaque-forming units of phage for one hour, and then adding soluble PA to bind low-affinity phage (60 nM) and incubating tubes for two hours. After washing (20× with PBS containing 0.1% Tween-20 followed by 20× with PBS), phage were eluted with 1 ml 0.1 M ethanolamine for 10 min, transferred to new tubes, and neutralized with 500 µl 1M Tris-HCl, pH 7.5. Eluted phage were titered and used to infect exponentially growing TG1 cells for the next round of panning. Each library was panned for five rounds before being screened for affinity-matured variants.

Antibody expression. scFvs were subcloned from the phage-display vector pAK100 via *Sfi*I-*Sfi*I into the expression vectors (i) pAK300, for scFv expression, or (ii) pMoPac16, a pAK400 derivative (scFv with a C-terminal human constant κ–domain fusion) co-expressing the periplasmic chaperone *skp*, for scAb expression (A. Hayhurst, B.L.I., and G.G., unpublished results). Proteins were produced in the periplasm of *E. coli* strain BL21, and purified by osmotic shock and immobilized metal affinity chromatography as reported previously[37]. Monomeric and dimeric scFv and scAb proteins were resolved by size-exclusion chromatography (Superdex 200, Amersham Pharmacia, Piscataway, NJ) with PBS as eluant (Supplementary Fig. 1 online). Levels of contaminating endotoxin were measured by the *Limulus* amebocyte lysate test (Associates of Cape Cod, Falmouth, MA) and were found to be <10 ng/ml for all preparations. SDS–PAGE with Coomassie staining verified the homogeneity and purity of the protein preparations; protein concentrations were measured by micro-bicinchoninic acid assay (Pierce, Rockford, IL).

Preparation of monoclonal antibody and Fab fragments. Ascites fluid was prepared from mouse IgG1 14B7 hybridomas (Rockland Immunochemicals, Gilbertsville, PA). IgG and Fab were purified as described[38] and applied to a size-exclusion column (Superdex-200, Amersham Pharmacia) as a final step. The absence of contaminating Fc and intact IgG in the Fab fraction was confirmed by ELISA and SDS–PAGE.

Antibody–antigen binding and stability analysis. The analysis of antigen binding kinetics by SPR was performed essentially as described[39]. Briefly, antigen (PA or BSA control) was immobilized on a CM5 chip (Biacore International, Uppsala, Sweden) at a level of approximately 1,000 response units (RU). To diminish rebinding effects, samples were run at high flow rate (60 µl/min) in HBS buffer (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.005% P20 surfactant, pH 7.4). On-rates were determined using at least five concentrations of antibody, ranging between 25 nM and 300 nM.

Antibody stability at 37°C, 70°C, and 4°C (as a control) was determined by incubating quadruplicate samples (16 µg/ml protein in PBS) at the respective temperatures and monitoring the amount of active antibody remaining by ELISA.

Urea denaturation experiments and data analysis were performed as described elsewhere[40,41]. The reversibility of urea denaturation was evaluated, and data are reported only for proteins that showed a fully reversible transition.

Protection of mouse macrophages to toxin challenge. Survival of RAW 264.7 mouse macrophage-like cells (ATCC #TIB-71) after administration of antibodies was determined essentially as described[42] except that antibodies titrated from 300–0.003 nM were either pre-incubated with toxin for 30 min or added 0, 5, 10, or 20 min after toxin challenge (100 ng/ml PA, 50 ng/ml LF). After three hours of exposure to toxin, an indirect viability assay was performed using MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Sigma, St. Louis, MO). Cells were incubated with medium containing 2 mg/ml MTT for 30 min, solubilized with acidic isopropanol (40 mM HCl, 0.5% SDS in 90% isopropyl alcohol) and the absorbance was measured at A_{595}. The percentage of cells surviving toxin challenge at a specified antibody dose, as compared with sham-treated cells, is reported as measured by MTT assay ((average test well – average of eight toxin-only wells) × 100%/average of eight no-toxin wells). Each data point is the average of triplicate measurements; standard deviations were typically ~5%.

Protection to toxin challenge in the rat model. *In vivo* neutralization experiments were performed essentially as described[34]. Fischer 344 rats (225–275 g) were anesthetized by intraperitoneal injection of ketamine (80 mg per kg body mass) and xylazine (10 mg per kg). Antibodies (or sterile PBS) were

administered in a 200 µl volume in a double-blind study, followed after 5 min by a 10× MLD of anthrax toxin (40 µg PA, 8 µg LF)[35] in a 200 µl volume, both via penile vein injection. Five animals were used for each test condition, and were monitored for discomfort and time of death versus survival, as assessed on the basis of cessation of breathing and heartbeat. Rats were maintained under anesthesia for five hours or until death to minimize discomfort; surviving rats were killed by overdose of sodium phenobarbitol given by intraperitoneal injection. The protective ability of antibody preparations was measured as a delayed time to death. All experimental protocols involving animals were reviewed and approved by the Southwest Foundation for Biomedical Research Institutional Animal Care and Use Committee (San Antonio, TX).

Pharmacokinetic measurements. Serum clearance rates for scFv, scAb, and PA proteins were determined essentially as described[43]. Fischer 344 rats (225–275 g) were purchased with indwelling jugular venous cannulae, and anesthetized as during protection experiments. Doses (2 nmol) of scFv, scAb, and PA were administered in 200 µl of sterile saline by penile vein injection. Blood samples (200 µl) were taken before protein administration and 1, 3, 5, 10, 15, 20, 30, 60, 120, and 180 min after protein administration. Antibody and PA serum concentrations were determined by chemiluminescent capture ELISA; clearance rates were determined by nonlinear regression on a graph of percent injected dose over time with a biphasic exponential function. Values for the α clearance phase were calculated from the average of data from four or five rats (Table 2).

Monoclonal antibody sequences. DNA sequences of 3B6, 10E10, and 1G3 variable regions are available upon request.

Note: Supplementary information is available on the Nature Biotechnology *website.*

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Competing interests statement

The authors declare that they have no competing financial interests.

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