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Detection of Anthrax Toxin in the Serum of Animals Infected with *Bacillus anthracis* by Using Engineered Immunoassays

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Several strategies that target anthrax toxin are being developed as therapies for infection by *Bacillus anthracis*. Although the action of the tripartite anthrax toxin has been extensively studied in vitro, relatively little is known about the presence of toxins during an infection in vivo. We developed a series of sensitive sandwich enzyme-linked immunosorbent assays (ELISAs) for detection of both the protective antigen (PA) and lethal factor (LF) components of the anthrax exotoxin in serum. The assays utilize as capture agents an engineered high-affinity antibody to PA, a soluble form of the extracellular domain of the anthrax toxin receptor (ANTXR2/CMG2), or PA itself. Sandwich immunoassays were used to detect and quantify PA and LF in animals infected with the Ames or Vollum strains of anthrax spores. PA and LF were detected before and after signs of toxemia were observed, with increasing levels reported in the late stages of the infection. These results represent the detection of free PA and LF by ELISA in the systemic circulation of two animal models exposed to either of the two fully virulent strains of anthrax. Simple anthrax toxin detection ELISAs could prove useful in the evaluation of potential therapies and possibly as a clinical diagnostic to complement other strategies for the rapid identification of *B. anthracis* infection.

The gram-positive *Bacillus anthracis* bacterium is the causative agent of anthrax. During inhalational anthrax, spores are inhaled into the lungs, leading to germination followed by the emergence of vegetative anthrax bacteria in circulation. Key anthrax virulence factors include the three components of the anthrax exotoxin, protective antigen (PA), lethal factor (LF), and edema factor (EF), which are secreted into the host's system (11). The PA and LF toxins combined are often referred to as anthrax lethal toxin (LeTx) and operate analogous to other A-B toxin systems (18).

The PA component is secreted as an 83-kDa protein that is cleaved by a host furin-like protease to yield a 63-kDa fragment (5, 24) that readily heptamerizes. The PA_{63} heptamer is bound to the host cell surface receptor ANTXR1 (ATR/TEM8) (45) or ANTXR2 (CMG2) (7) with relatively high affinity (170 pM) (47). The LF component binds to the PA heptamer and gains entry into the cell through clathrin-dependent endocytosis (1). A drop in endosomal pH leads to a conformational change in PA and transport of LF into the host cell cytosol (17, 26). In the cytosol, LF toxin acts as a zinc metalloprotease, cleaving the N termini of mitogen-activated protein kinase kinases (9, 13), leading to a number of signs associated with toxemia and ultimately death (2, 32).

In the rodent model, signs of anthrax infection are rapidly followed by death within a few hours, resulting in a very small, if existent, window for postexposure therapy after observed toxemia. However, in the nonhuman primate model and in human disease, the infection is characterized by delayed death up to 10 days following initial symptom onset (8, 10). It is therefore believed that a window of opportunity for treating anthrax exists during the second phase of human infection.

The 2001 U.S. anthrax attack led to 11 cases of inhalation anthrax resulting in five deaths (23). The mean incubation period ± standard deviation from the time of exposure (when known) to onset of symptoms was 5 ± 1 days. Of the five patients who died, *B. anthracis* infection was confirmed before death in only one patient (4, 23), even though initial visits by three of the patients involved typical cell blood count (CBC) or cerebrospinal fluid analysis which yielded results consistent with bacterial infection (23). However, there was no available test for anthrax toxemia to provide more-specific information about the nature of the infections.

Both small molecule- and protein-based therapeutic approaches are being developed that rely on antitoxin agents intended to provide preexposure prophylaxis and/or postexposure treatment for infection (16, 22, 27, 30, 34–36, 38, 43, 44). The PA toxin is considered a particularly promising therapeutic target because prevention of PA activity would eliminate subsequent LF and EF toxin action as well (29). Additionally, previous studies have shown that antibody-based anti-PA toxin neutralization can result in passive immunity without antibiotic

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treatment (28, 30, 34), whereas anti-LF antibody approaches have thus far failed to provide protective immunity in spore challenges (28).

Recently, engineered anti-PA monoclonal antibodies (MAbs) have proven to be effective in both prophylactic and postexposure live anthrax spore challenges in the rabbit model (6, 34). We have reported a high-affinity single-chain antibody fragment (scAb) called M18, which was modified by conjugation to polyethylene glycol (30). Like the parent 14B7 murine monoclonal antibody, the engineered M18 scAb targets PA domain IV, the same domain known to bind the cellular receptors ANTXR1 and ANTXR2. The M18 antibody fragment-polyethylene glycol conjugate provided passive immunity against a 250× to 625× 50% lethal dose (LD_{50}) of anthrax spore challenge (Vollum 1b) in the guinea pig model in the absence of Fc immunity-mediated functions (30).

Surprisingly, despite these successes with anthrax antitoxin therapeutics in animal models, the stage of infection in which PA/LF is secreted in vivo after exposure remains relatively unexplored. PA has been previously detected in the serum of infected guinea pigs and rabbits after signs of toxemia are observed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis/Western methods (15, 37), yet without any detection limits reported. More recently, a microarray was engineered to detect unlabeled native toxin (40). Although the reported limit of detection for PA was 1.3 μg/ml, there exists potential in expanding upon immunoassays for toxin detection for infected hosts.

Here, we have engineered sandwich capture enzyme-linked immunosorbent assays (ELISAs) for the detection of both PA and LF. The M18 scAb (19, 30) was utilized as the capture agent in a sensitive ELISA capable of detecting PA (≥1 ng/ml PA) in serum from infected guinea pigs and rabbits. In addition, an LF-specific sandwich ELISA was developed that utilizes the PA heptamer complex as the capture ligand (≥20 ng/ml LF in serum). Preliminary results show that PA is detectable during anthrax infection in the guinea pig model, before and after onset of signs of toxemia. PA and a mutant PA (PA Y688A) were also detected by an ANTXR2/CMG2 assay showing equivalent levels of detection, as expected. In addition, both PA and LF were detected in blood in systemic circulation in the rabbit model at time of death. The application of straightforward, robust immunoassays for diagnosis of anthrax toxemia could prove useful in both research and clinical settings.

MATERIALS AND METHODS

Sandwich ELISA for PA detection. The M18 scFv construct had previously been cloned into pMoPac16 (20), expressed as a scAb, and purified by immobilized metal affinity chromatography (IMAC) via a C-terminal polyhistidine tag and size-exclusion chromatography (30). ANTXR2 was a gift from Robert Liddington's group (The Burnham Institute, La Jolla, Calif.) and was prepared as previously mentioned (41). Rabbit anti-PA polyclonal, native PA, and Y688A PA were produced as previously described (39). Human serum was obtained from a donor at the University of Texas Health Center, Austin. For overnight coating, 50 μl of M18 scAb (4 μg/ml) or ANTXR2 (4 to 30 μg/ml) was incubated overnight in a 96-well ELISA plate (no. 3590; Corning Inc., Corning, NY) at 4°C. The plate was then brought to room temperature and incubated with 2% dried milk (Carnation, Nestle)–phosphate-buffered saline (PBS) or 2% bovine serum albumin (BSA; Sigma)/PBS for 2 h. The plate was washed three times with 1× PBS–0.5% Tween 20 and then once with 1× PBS. For initial assays, native or Y688A PA was diluted into 2% milk–PBS or human serum at 1 μg/ml and serially diluted onto the ELISA plate. For assays detecting PA in blood of infected animals, serum was added to the plate initially diluted 1:1 in 2% milk–PBS and then serially diluted across the plate. Preexposed serum spiked with quantified PA served as the positive control and was serially diluted as well. After a 1-h incubation, the plate was washed as described above. Rabbit anti-PA polyclonal serum was diluted 1:1,000 in 2% milk–PBS and added to the plate for a 1-h incubation. The plate was then washed again, followed by the addition of rabbit anti-goat immunoglobulin G (IgG)-horseradish peroxidase (HRP) conjugate (Bio-Rad) diluted in 2% milk–PBS for 1 h. ELISA reactions were developed with *o*-phenylenediamine (OPD) tablets (Sigma) and quenched by the addition of 50 μl of 4.5 M H_2SO_4. All ELISAs were run in duplicate, averaging each data point with standard deviation. Experimental assay data determining the detection of PA were applied to Prizm 3.03 software (GraphPad Software Inc., San Diego, CA) to calculate the statistical significance for differences between detection methods of each model. In addition, data were curve fitted to a nonlinear regression model to determine "goodness of fit."

Sandwich ELISAs for LF detection. Recombinant PA_{83} and PA_{63} were purchased from List Laboratories (New Jersey). PA_{63} is a cleavage product that is capable of binding LF (45). For the sandwich ELISA, 50 μl of PA at 63 kDa and 83 kDa (6 μg/ml) was applied to a 96-well plate and blocked with 2% milk–PBS as described above. For initial assays, LF was diluted in PBS or human serum at 5 μg/ml. For assays detecting LF in infected animals, serum was added to the plate initially diluted 1:1 in 2% milk–PBS and then serially diluted across the plate in duplicate. After a 1-h incubation, the plate was washed as described above. Goat anti-LF polyclonal serum (List Labs) was diluted 1:1,000 in 2% milk–PBS and added to the plate for a 1-h incubation in duplicate. The plate was then washed, followed by the addition of goat anti-rabbit IgG-HRP conjugate (Bio-Rad) diluted in 2% milk–PBS for 1 h. ELISA reactions were developed with OPD tablets (Sigma) and quenched by the addition of 50 μl of 4.5 M H_2SO_4. Observed concentrations were calculated from positive control. Data were applied to Prizm 3.03 (GraphPad Software Inc., San Diego, CA) software for statistical analysis.

Animal spore exposure and histology. Female Hartley guinea pigs (225 to 305 g) (Charles River Laboratories, Massachusetts) were housed individually in a One Cage 2100 AllerZone Interchangeable Micro-Isolator high-density housing system (Lab Products Inc., Seaford, DE), and New Zealand rabbits (2.0 to 2.5 kg) (Charles River Laboratories, Wilmington, MA) were housed individually in ventilated rabbit cage racks (model no. RBV243116US6-2X; Allentown Caging Equipment Company, Inc., Allentown, NJ). Animals were housed in a biosafety level 4 facility at The Southwest Foundation for Biomedical Research (San Antonio, TX) for spore challenge. Anthrax spore inocula, both the Ames and Vollum strains, were prepared on the day of challenge and diluted to the desired concentration in PBS. Both guinea pigs and rabbits were sedated with ketamine (80 mg/kg of body weight) and xylazine (10 mg/kg) during all bleeds, injections, and inhalation instillations, according to the approved IACUC protocol. Guinea pigs were exposed to *B. anthracis* Vollum 1B (3) spore inoculum of 500× LD_{50} (2.0×10^7) (100-μl total volume), and rabbits were exposed to a 20× LD_{50} (2.1×10^6) (48) of *B. anthracis* Ames spores via intranasal inoculation in both cases. Blood samples were taken from the femoral artery at various times. The guinea pigs were monitored for signs of toxemia and euthanized by cardiac injection of sodium pentobarbital when considered moribund. Symptoms of toxemia include labored breathing, curling of digits, edema, abnormal color in nose/feet/tail, and immobility. For the rabbit studies, euthanasia was not used and blood samples were taken only at the time of death. Serum was obtained by centrifugation and separation using StatSampler columns (StatSpin, Norwood, MA).

For histology, lungs and spleens were removed from the guinea pigs postmortem. 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 (SBA) plates. After 24 h of incubation at 37°C, the colonies were enumerated to determine the number of CFU/100 mg of tissue.

RESULTS

PA detection ELISAs. A highly sensitive sandwich ELISA for PA capture was developed based on the engineered M18 antibody. Briefly, the M18 murine scFv (19) was isolated from an error-prone library derived from the anti-PA 14B7 murine monoclonal antibody (29) using a new bacterial display technology referred to as anchored periplasmic expression (APEx) (19). The M18 scFv was cloned into the pMoPac 16 vector (20)

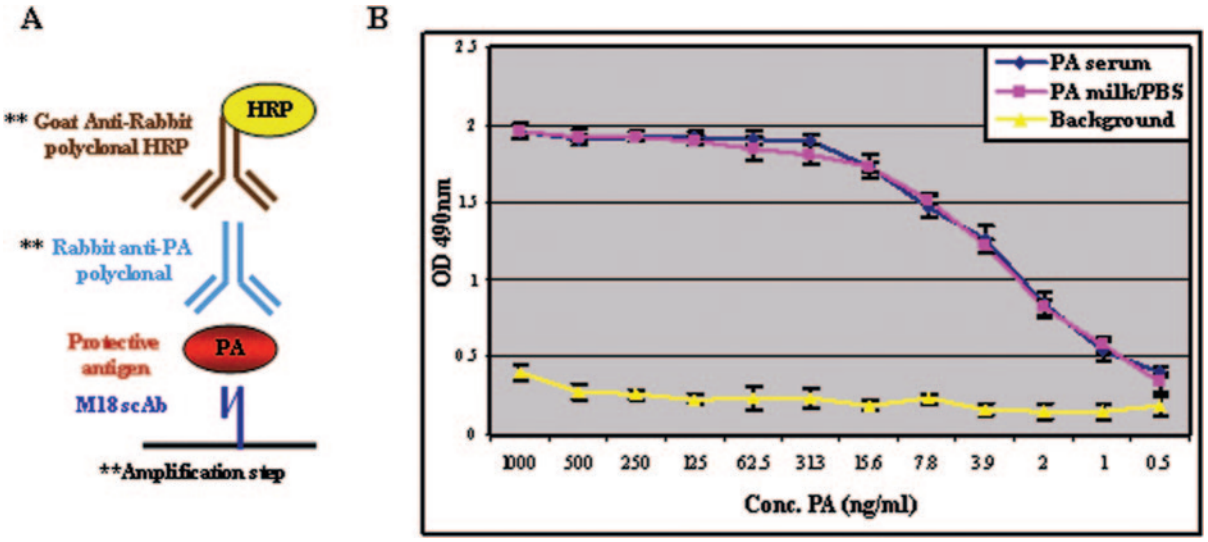

FIG. 1. PA detection immunoassay. (A) Schematic for PA detection assay with M18 scAb. (B) For experimental detection, PA was detected at 1 μg/ml in milk-PBS (purple squares) or human serum (blue diamonds) and serially diluted in the plate assay. After probing with the primary and secondary antibodies as described, results showed that PA is detectable in milk-PBS and serum at very similar levels, with an overall lower detection limit of ≈1 ng/ml.

to produce a fusion between a human kappa chain and the heavy-chain variable domain of the scFv. The resulting construct is referred to as a scAb (21). The purified M18 scAb fragment binds PA with an equilibrium dissociation constant (K_D) of 35 pM (19).

Figure 1A shows a schematic of the anti-PA detection ELISA. The well bottoms of a 96-well plate were coated with the M18 scAb to serve as the capture agent. PA was serially diluted in 2% milk–PBS and human serum and then incubated for 1 h on the plate. After washing, rabbit anti-PA polyclonal serum was diluted in 2% milk–PBS and added to the plate. After washing, goat anti-rabbit polyclonal IgG-HRP conjugate was added for detection of the bound rabbit IgG. Note that having two polyclonal detection antibodies adds potential signal amplification steps. The results from these serial dilution assays revealed that PA can be detected in 2% milk–PBS and human serum with an observed lower limit of detection of ≈1 ng/ml (Fig. 1B) based upon the concentration of PA at which a threefold-higher signal over background or negative control was seen. The significance between the curves was calculated as a two-way analysis of variance (ANOVA) model using Prism 3.03 software (GraphPad Software, San Diego, CA), resulting in an overall P value of <0.0001. "Goodness of fit" for each curve was determined via a nonlinear regression model using the same software to reveal R^2 values of 0.990 (serum) and 0.994 (milk) for PA detection.

Engineered ELISA for PA variant. The 14B7 antibody was originally identified as a neutralizing antibody and shown to act by blocking binding of PA to cells (29). It has been shown to bind to the PA domain 4 small loop region involved in receptor binding (39, 41). Domain IV is responsible for the majority of binding to the ANTXR2 (CMG2) (42) and ANTXR1 (ATR/TEM8) (7) receptors, although a contribution from PA domain II was recently identified by X-ray crystallographic analysis of the complex (41).

The ability to genetically modify a potential biowarfare agent to evade current and future therapeutics must be taken into consideration. Rosovitz and colleagues showed that a PA mutant having the Y688A substitution in domain IV retains full toxicity (39), even though binding by some anti-PA antibodies was disrupted. We sought to develop an assay capable of detecting anthrax strains containing a genetically modified PA component that retains receptor binding, and thus virulence, but might evade any single capture ELISA antibody. The ANTXR2 receptor was chosen as a capture agent due its reported high affinity to PA (7). The soluble component of the ANTXR2 receptor was substituted into the capture ELISA described above in place of the M18 scAb construct. The ELISA was then performed corresponding to the schematic shown in Fig. 2A. Plates containing either the ANTXR2 soluble receptor or the M18 scAb were analyzed for detection of serially diluted native PA and PA Y688A. Results from the assays are shown in Fig. 2B. Assays using ANTXR2 as the capture agent revealed very similar curves for both native PA ($R^2 = 0.9935$) and PA Y688A ($R^2 = 0.9979$), with a reported P value of <0.0001 using the two-way ANOVA model. On the other hand, the M18-based assay showed a marked difference in sensitivity between the two PA samples, with the assay for PA Y688A ($R^2 = 0.9851$) being close to an order of magnitude less sensitive than M18 binding native PA ($R^2 = 0.9986$). Interestingly, despite a relatively high affinity reported for this receptor binding to PA, the ANTXR2-based assay was considerably less sensitive than the ELISA based on M18, even in the case of PA Y688A detection. The discrepancy could involve the way in which the ANTXR2 receptor binds to the plate, possibly leading to lower overall assay sensitivity by precluding unobstructed access to the PA toxin binding site.

LF detection ELISA. The PA 63-kDa fragment was purchased from List Labs (New Jersey) and applied as the capture ligand for LF detection in a sandwich ELISA (Fig. 3A). Full-length PA_{83} was used as a negative control since this fragment cannot bind LF, whereas PA_{63} was presumed to be in hep-

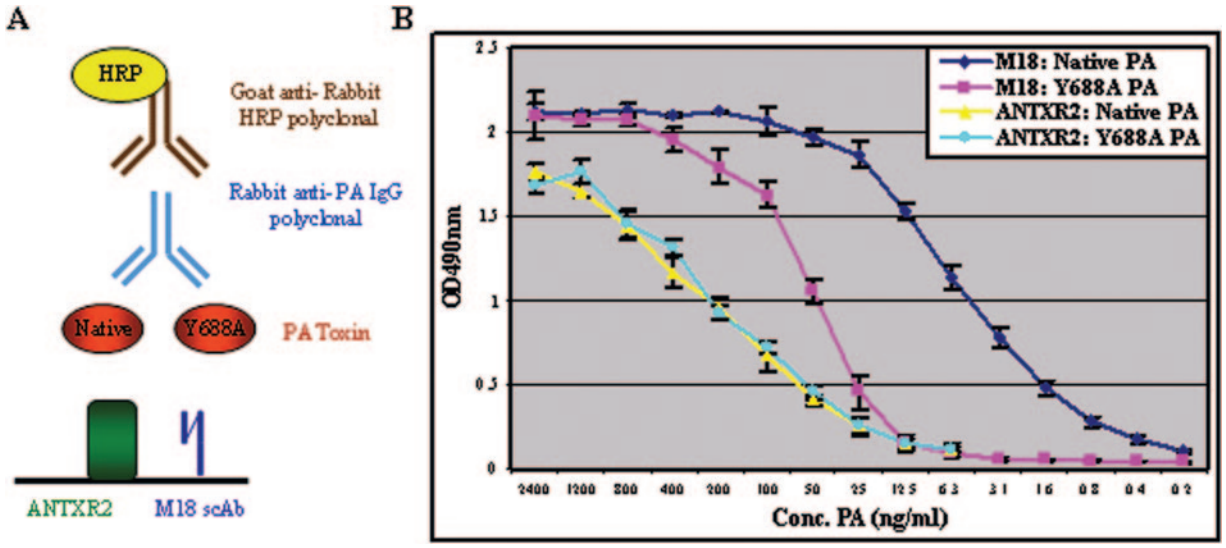

FIG. 2. Sandwich detection ELISA for native PA and mutant PA Y688A. (A) The soluble anthrax receptor, ANTXR2, and M18 scAb were capture ligands for detection of native PA and the mutant PA Y688A in domain IV. (B) Experimental results show that ANTXR2 bound to both native and mutant PAs with similar affinities. There was an order-of-magnitude decrease in sensitivity for M18 to the mutant PA compared to native toxin; however, overall the M18 assay was still more sensitive in both cases.

tameric form and possess binding sites for LF (33) due to solution conditions from the supplier. As for our PA ELISA, detection of captured LF was accomplished by primary polyclonal anti-LF followed by HRP-conjugated secondary antibodies. As expected, using PA_{63}, but not PA_{83}, as the capture agent produced an ELISA signal. "Goodness of fit" resulted in R^2 values of 0.9904 for serum and 0.9899 for milk PA detection. Two-way ANOVA calculated a P value of <0.0001 for comparison between the two detection methods. A lower detection limit of ≈20 ng/ml LF (Fig. 3B) was determined for the PA_{63}-based assay in serial dilution experiments, which may correspond to the binding affinity (low nM) of LF to PA (14).

Diagnostic detection of PA from exposed guinea pigs. To examine the ability of the assay to detect PA produced during infection, female Hartley guinea pigs (225 to 300 g) were exposed to a 500× LD_{50} (2×10^7) of Vollum anthrax spores (3) and then bled twice daily until euthanasia or death. PA was detected in blood in systemic circulation using the M18 capture

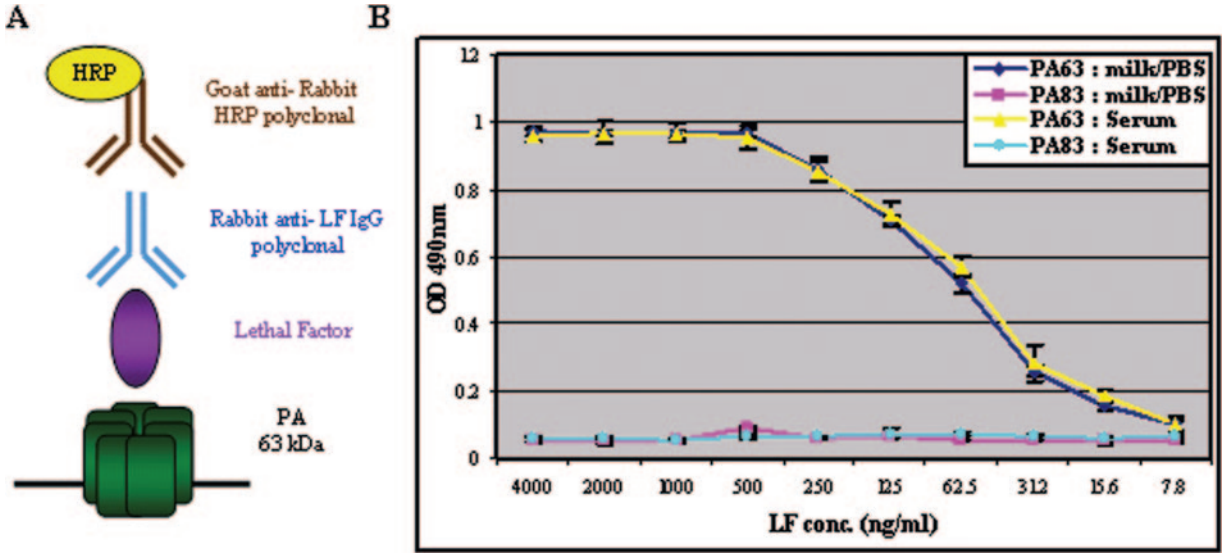

FIG. 3. Sandwich detection ELISA for LF. (A) Schematic shows the PA 63-kDa fragment as the capture ligand in the ELISA for LF. (B) For experimental detection, the PA 63-kDa or 83-kDa (control) fragment was applied as the capture ligand for LF detection. LF was detected at 4 μg/ml in milk-PBS (blue diamonds) or human serum (yellow triangles). The results showed that LF is detectable in milk-PBS and serum at very similar levels, with an overall lower detection limit of ≈20 ng/ml when the PA 63-kDa fragment was used as the capture ligand. There was no detection of LF when the PA 83-kDa fragment was the capture ligand. The lower detection limit of the assay was calculated as a signal threefold higher than the PA 83-kDa negative control (purple squares and turquoise circles).

TABLE 1. Detection of PA in sera from animals exposed to *B. anthracis* spores[a]

Animal	PA concn in μg/ml (sample time [h])	Time to death (h)	Mode of death
25535	0	96	Euthanized
25536	1.7 ± 0.3 (72)	72	Euthanized
25537	0.1 ± 0.03 (58)	60–70	Expired
25538	0.4 ± 0.01 (72)	72	Euthanized
25539	0.4 ± 0.06 (72), 24 ± 5 (81)	81	Expired

[a] Male guinea pigs (225 to 300 g) (Charles River) were exposed to a 500× LD_{50} of anthrax spores. Blood samples were taken twice daily for analysis via ELISA. The results indicate that unbound PA was detected in the sera of 80% of exposed animals in the late stages of infection.

ELISA in 4 of the 5 animals before death (Table 1). As mandated by the approved IACUC protocol, animals displaying any observed signs of toxemia were immediately euthanized. However, two animals expired from anthrax in spite of constant monitoring for signs, and their serum samples were taken postmortem. For the three animals euthanized, two showed detectable levels of circulating PA at the time of euthanasia. Both of the animals that died from anthrax possessed detectable serum levels of PA prior to onset of toxemic signs as well as after death. One animal (25539) was found to have a relatively high serum PA level (24 ± 5 μg/ml) at the time of death, compared to the level of 0.4 ± 0.06 μg/ml detected in the same animal 9 h earlier.

Taken together, our results demonstrate that PA can be detected in the late stages of guinea pig infection, especially within 12 h of death. Tissue samples from all five animals were taken for histology tests and revealed that animals considered moribund, and thus euthanized, possessed equivalent CFU ($\approx 10^7$ to 10^9) of bacteria compared to those that expired due to infection (data not shown).

Toxin detection from rabbits exposed to Ames spores. As a preliminary investigation into the generality of the approach to PA detection and to evaluate the effectiveness of the LF assay, another animal model, New Zealand White rabbits, was investigated using the other fully virulent laboratory strain of anthrax, the Ames strain. Two rabbits were exposed to a 20× LD_{50} of Ames spores via intranasal inoculation. Upon death (mean, ≈48 h), serum samples were taken for detection of PA and LF components of the toxin. Table 2 summarizes the ELISA results. The two animals expired with a mean time to death (MTTD) of ≈48 h, an MTTD very similar to those of previous reports of exposure to a 150× LD_{50} (34, 48). Upon death, both animals possessed detectable levels of PA and LF in the systemic circulation. PA was found to be present at concentrations of 80 ± 7 μg/ml and 100 ± 13 μg/ml in the two animals, using the M18 antibody capture ELISA. The elevated PA levels compared to those in the guinea pigs might indicate that toxin secretions differ among animal models and/or between different strains (Ames versus Vollum 1b). The two animals expired with an MTTD of ≈48 h, an MTTD very similar to those of previous reports of exposure to a 150× LD_{50} (34, 48). At time of death, blood samples were taken and PA values of 80 to 100 μg/ml were detected. The elevated PA levels compared to those of the guinea pigs might indicate that toxin secretions differ among animal models and/or between different strains (Ames versus Vollum 1b).

TABLE 2. Detection of *B. anthracis* in samples from rabbits exposed to Ames spores

Parameter[a]	Result for:	
	Animal 1	Animal 2
Amt of toxin detected (μg/ml)		
PA	100 ± 13	80 ± 7
LF	15 ± 3	11 ± 2
No. of CFU detected in:		
Lymph nodes	6.1×10^6	1.3×10^8
Liver	2.5×10^{11}	3.8×10^8
Lung	1.6×10^{11}	7.7×10^8
Spleen	9.0×10^{10}	2.0×10^8
Blood	2.0×10^9	9.6×10^{10}

[a] Two New Zealand rabbits were exposed to a 20× LD_{50} of Ames spores and monitored until death at ≈48 h. Serum samples taken at the time of death revealed detectable PA levels between 80 and 100 μg/ml and LF levels between 11 and 15 μg/ml. Numbers of CFU were measured per 100 mg of tissue or per ml of blood. Both animals possessed bacteria in all measured organs as well as in systemic circulation.

DISCUSSION

We have engineered a PA detection ELISA based on single-epitope capture ligands and secondary polyclonal detection antibodies. The assay using the engineered M18 high-affinity anti-PA antibody fragment was able to detect PA with a lower detection limit of ≈1 ng/ml in serum. PA was detected in the sera of four out of five guinea pigs exposed to spores of the Vollum 1b strain of *B. anthracis*. Of these, PA was detected in two of the three animals that were euthanized. In addition, both of the animals that expired due to anthrax displayed levels of circulating PA before initial signs of toxemia. It thus appears that the M18-based ELISA can detect serum PA toxin secretion in animals both before and after external onset of toxemic signs. It must be noted that this assay strategy does not distinguish between PA_{83} and PA_{63} in the serum of the animal (15). However, these assays are capable of detecting PA via the mammalian cell binding domain (PA domain IV), with M18 scAb serving as a detection capture ligand as well as a potential therapeutic for passive immunity (30).

An unambiguous time course of PA levels is difficult to extrapolate from these data. For example, although the administered spore dosages were expected to be uniform among all five guinea pigs, the amounts that reached the lungs may have differed tremendously among the different animals. Such variation could affect incubation/germination periods, growth of nascent vegetative bacteria, toxin secretion, and eventually toxemia onset. Therefore, experiments for time-dependent toxin secretion might be best executed by injection of spores or bacteria to avoid respiratory incubation period differences. Nevertheless, our guinea pig data represent successful in vivo serum PA detection in animals exposed to anthrax spores by utilizing plate assays. Unfortunately, the LF detection assay was not yet available for investigation of LF levels in these guinea pig samples.

Previously, capsular antigen and bacteremia in infected animals were detected well before time of death (25). Therefore, it is somewhat surprising that PA detection occurred only in the last stages of infection in four of the five guinea pigs we examined. However, PA is secreted in an environment

surrounded by tissue in which PA receptors, specifically ANTXR2, are highly expressed. With such a high affinity to ANTXR2 ($K_D \approx 170$ pM) (47), detection might occur only at or near saturation of the vascular system with PA. LF detection, as well, might vary according to the quantity remaining unbound and free in the blood.

We also used the soluble form of the ANTXR2 anthrax receptor for the detection of a Y688A PA variant that was reported to be capable of evading murine monoclonal 14B7 binding yet retaining cell toxicity (39). As expected, the ANTXR2 ELISA was able to detect both native PA and the PA Y688A variant with similar sensitivities, while M18 showed an order-of-magnitude decrease in sensitivity with samples of PA Y688A. Nevertheless, the overall sensitivity of the M18 scAb-based ELISA was far superior to that of the ANTXR2 assay in both cases: 2 orders of magnitude more sensitive in the case of native PA and an order of magnitude more sensitive with the PA Y688A variant. While demonstrating the value of the engineered M18 antibody, these results also indicate that a sandwich ELISA format may be useful as a way to screen potential therapeutic molecules for relative binding to native PA and PA variant molecules.

As a diagnostic, the new sandwich detection ELISAs could provide evidence of toxin secretion in a clinical setting. The incorporation of a toxin or bacterial diagnostic specific to anthrax could prove useful during this time frame, as some patients did not receive the ciprofloxacin or other antibiotic treatment until other antimicrobials failed to improve patient status. Recent anthrax diagnostic tools focus on PCR (31) and bacterial surface antigens (12) for *B. anthracis* confirmation. Our ELISAs can be run in under 2 h, which is comparable to PCR and other antigen assays. In addition, an immunoassay could readily be incorporated into an automated, multiplexed assay platform such as the Luminex bead assay (46).

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