Efficient Neutralization of Antibody-Resistant Forms of Anthrax Toxin by a Soluble Receptor Decoy Inhibitor

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A soluble receptor decoy inhibitor (RDI), comprised of the extracellular I domain of ANTXR2, is a candidate anthrax therapeutic. Here we show that RDI can effectively neutralize altered forms of the protective antigen (PA) toxin subunit that are resistant to 14B7 monoclonal antibody neutralization. These data highlight the potential of RDI to act as an adjunct to existing antibody-based therapies and indicate that inhibitors based on RDI might be useful as a stand-alone treatment against specifically engineered strains of Bacillus anthracis.

Bacillus anthracis, the causative agent of anthrax, is a spore-forming gram-positive bacterium that is one of the most dangerous Centers for Disease Control and Prevention category A agents. Of the two major virulence factors expressed by this bacterium, the bipartite anthrax toxin is believed to be responsible for the majority of disease symptoms. The toxin is comprised of a cellular receptor-binding moiety, a protective antigen (PA), and the following two enzymatic moieties: lethal factor (LF), a zinc-dependent metalloprotease that cleaves multiple mitogen-activated protein kinase kinases (7, 24), and edema factor (EF), a calcium- and calmodulin-dependent adenylate cyclase (11). LF and EF combine with PA to form lethal toxin (LeTx) and edema toxin (EdTx), respectively.

The toxin action begins with the binding of the 83-kDa monomeric form of PA (PA83) to either of the two cellular receptors, ANTXR1 or ANTXR2 (5, 22). Both receptors contain an extracellular domain, similar to the I domains of integrins, which is involved in PA binding. Receptor-bound PA is cleaved by a cell surface furin-like enzyme to generate a 63-kDa form (PA63) which spontaneously oligomerizes to form a heptamer prepore (PA63)*7, bound by up to seven copies of the receptor (10). Alternatively, (PA63)*7 that is formed in the bloodstream of an infected host can bind directly to the receptor (8, 15). By binding to PA, the ANTXR2 I domain serves as an effective molecular clamp that prevents prepore-to-pore conversion until the toxin-receptor complex is trafficked to an acidic endosomal compartment (10, 16, 19, 21, 27). Each PA heptamer can bind up to three moieties of LF and/or EF (12, 13). The toxin-receptor complexes are internalized primarily by clathrin-mediated endocytosis (2, 4) and trafficked to an acidic endosomal compartment. Under the influence of acidic pH, conformational changes occur, leading to the formation of the (PA63)*7 pore in either endosomal or intraluminal vesicle membranes (1). Consequently, LF and EF are delivered into the cytosol where they disrupt cellular processes (28).

Various strategies are being pursued to combat anthrax, the most advanced of which are monoclonal antibodies directed against toxin components (3, 17, 28). Although these reagents are expected to be effective against most strains of B. anthracis, they may not be effective against weaponized strains of bacteria that have been specifically engineered to express fully functional, antibody-resistant forms of PA. Cysteine-scanning mutagenesis of PA83 has already shown PA to be a highly malleable protein that can accommodate numerous independent amino acid substitutions (14). Furthermore, antibody-resistant forms of PA that are fully competent to mediate intoxication can be easily generated (18). PA is the major immunogen within the currently licensed vaccine, AVA, and several reports have suggested that modified, vaccine-resistant strains of B. anthracis were generated previously as part of biowarfare programs (3, 9).

Previously, we showed that PA could be effectively neutralized by a soluble receptor decoy inhibitor (RDI) consisting of the ANTXR2 I domain (23). In this report, we tested whether RDI could also efficiently neutralize four independent variants of PA that are resistant to neutralization by the 14B7 monoclonal antibody, which acts by blocking PA receptor binding. These studies evaluate the potential of using soluble receptor decoys as candidate therapeutics against engineered strains of B. anthracis.

To test the ability of RDI to neutralize antibody-resistant forms of PA, four amino acid substitutions were engineered into PA83 (K684A, L685A, L687A, and Y688A) (Fig. 1). These substitutions were previously shown to interfere with 14B7 monoclonal antibody neutralization without significantly compromising the function of this toxin subunit (18). QuikChange mutagenesis (Stratagene) was performed on the previously described PA-pET22b construct (25) to generate genes encoding the PAK684A, PAL685A, PAL687A, and PAY688A variants. The open reading frames of all constructs were confirmed by DNA sequencing. Wild-type PA and mutant PA forms were isolated from the periplasm of Escherichia coli BL21 cells (25) and purified as previously described (20). The soluble ANTXR2 I domain (RDI) was purified as previously described (26). Concentrations of the proteins were determined using the bicinchoninic acid protein assay kit (Thermo Scientific, IL). The purity of each sample was judged using densitometric analysis (Fluorchem; Alpha Innotech Corporation, CA). fol-
lowing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. Confirming the results of a previous study (18), we found that the mutant PA proteins could efficiently mediate LF-dependent intoxication of RAW264.7 cells and, furthermore, that this killing was resistant to 14B7 antibody neuralization (data not shown).

An important feature of a candidate receptor decoy-based anthrax therapeutic is that it forms a stable complex with PA and prevents cellular receptor binding. Monomeric RDI–wild-type PA (WT-PA) complexes display a very high binding affinity (a dissociation constant [\(K_d\] of 170 pM) due in large part to a very slow off-rate with the lifetime of the complex estimated at around 30 h (26). To determine if the mutations that render PA resistant to 14B7 antibody neutralization significantly alter this off-rate, a capture enzyme-linked immunosorbent assay (ELISA) was performed.

The WT-PA and mutant PA proteins were immobilized overnight at 4°C by adding each protein at 100 \(\mu\)g/well in phosphate-buffered saline (PBS) to the wells of a 96-well plate. The wells were blocked with 3% nonfat milk in Tris-buffered saline (TBS), pH 7.0, for 1 h at room temperature. RDI was then added to each well at 50 ng/well, diluted in 1% nonfat milk in TBS, pH 7.0, with 0.05% Tween 20 (mTBST) and incubated for 1 h at room temperature. RDI dissociation was then followed over a 24-h period using chicken anti-ANTXR2 antibody (diluted 1:200 in mTBST) (20), followed by rabbit anti-chicken antibody coupled to horseradish peroxidase (HRP; Zymed; diluted 1:30,000 in mTBST). A 1-step Ultra TMB ELISA (Pierce) was used as the HRP substrate, and the reactions were stopped using 2 M \(\text{H}_2\text{SO}_4\) and monitored at 450 nm. All of the incubation steps were performed in a volume of 100 \(\mu\)l per well, except for the dissociation step for which 300 \(\mu\)l/well was used. The wells were washed five times with mTBST after each incubation step. The experiment was set up so that all of the samples were processed at the same time with the antibodies and the HRP substrate. These studies revealed that there was no significant difference in the rate of RDI loss from the altered PA proteins compared to that of WT-PA as judged by one-way analysis of variance (Prism; GraphPad Software, Inc., San Diego, CA), indicating a similar off-rate (Fig. 2). These results demonstrate that RDI forms stable complexes with the antibody-resistant forms of PA.

Previously, we showed that RDI was capable of efficiently neutralizing the anthrax toxin in cultured cells, and in vivo using a rat model system, when it was added at between 1:1 and 2:1 stoichiometric concentrations relative to those of PA (23). To test its efficacy against antibody-resistant forms of PA, the RDI was titrated against fixed amounts of LF (5 \(\times\) 10\(^{-9}\) M) and WT-PA or mutant PA proteins. The amounts of each PA protein used were those that gave rise to maximal killing of RAW264.7 cells, i.e., 0.5 \(\times\) 10\(^{-7}\) M of WT-PA, PA\(_{K684A}\), PA\(_{L685A}\), and PA\(_{Y688A}\) and 1 \(\times\) 10\(^{-7}\) M of PA\(_{L687A}\). The toxin-RDI samples (in a total volume of 100 \(\mu\)l) were then added to cultures of 10\(^4\) RAW264.7 cells that were seeded overnight in a 96-well plate. Following an overnight incubation, cell viability was then assessed using CellTiter-Glo (Promega, WI). The results clearly demonstrate that RDI is as effective
against antibody-resistant forms of PA as it is against WT-PA (neutralizing each when added at a 2:1 ratio) (Fig. 3).

We next tested the ability of RDI to neutralize LeTx containing an antibody-resistant form of PA (PA\textsuperscript{L685A}) in a rat model system. Male Harlan Sprague-Dawley rats (180 to 200 g; Harlan, Indianapolis, IN) were anesthetized with isoflurane and inoculated with 500 μL LeTx mixture by intravenous injection in the tail vein. The anthrax LeTx was prepared for each group by mixing 12 μg LF (List Biological Laboratories, Campbell, CA) with 40 μg PA (List Biological Laboratories, Campbell, CA) or with 40 μg PA\textsuperscript{L685A} in 250 μL PBS vehicle per rat. RDI was prepared in PBS vehicle with a total volume of 250 μL per rat so that it would be at a 2:1 molar ratio relative to PA when combined with the LeTx samples immediately prior to injection. The LeTx sample (250 μL per rat) was mixed with the RDI sample (250 μL per rat) for groups that received the inhibitor or with PBS (250 μL per rat) for groups that did not receive the inhibitor. These samples were immediately coinjected into rats. Control rats injected with 500 μL of PBS per rat were also included in the analysis. Rats recovered from anesthesia within 5 min and were monitored for symptoms of intoxication and death as determined by cessation of respiration. The group of rats that received LF and either form of PA died with similar kinetics in the absence of RDI (Table 1). Importantly, RDI protected all rats against intoxication even when the antibody-resistant form of PA was used (Table 1). These results highlight the potential for the future development of RDI-based therapeutics as candidates for the treatment of disease caused by both wild-type and engineered strains of \textit{B. anthracis}.

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### REFERENCES


