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(54) **Title**: VHH BASED BINDING ANTIBODIES FOR ANTHRAX AND BOTULINUM TOXINS AND METHODS OF MAKING AND USING THEREFOR

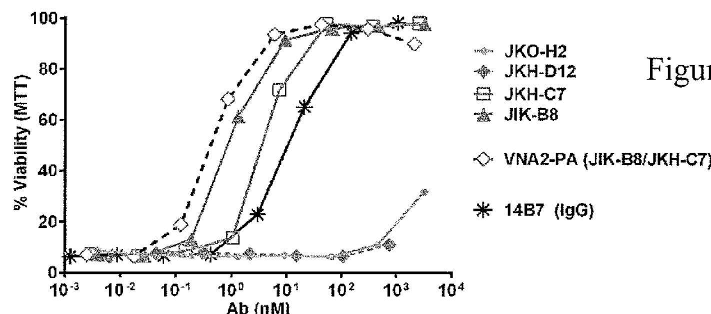

Figure 7C

(57) **Abstract**: Methods, compositions and kits are provided for treating a subject exposed to or at risk for exposure to a disease agent, methods, compositions and kits having a pharmaceutical composition including at least one recombinant binding protein or a source of expression of the binding protein, wherein the binding protein neutralizes at least one or a plurality of disease agents that are toxins, for example at least one of a Botulinum toxin or an Anthrax toxin.

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VHH based binding antibodies for Anthrax and Botulinum toxins and methods of making and using therefor

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Related Application

This application claims priority to U.S. provisional application serial number 62/089,949 filed December 10, 2014 entitled "VHH based neutralizing antibodies for Anthrax and Botulinum toxins", inventor Charles B. Shoemaker, which is incorporated by reference herein in its entirety.

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Technical field

The present invention generally relates to compositions and methods to prevent or treat exposure to Anthrax toxin or to Botulinum toxins by VHH based neutralizing antibodies.

15

Government support

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20 of the NIH, National Institute of Allergy and Infectious Diseases. The government has certain rights in the invention.

Background

The disease, Anthrax is caused by the gram-positive bacterium *Bacillus anthracis* and
25 is a major bioterror concern. Following introduction into a host in spore form and germination of the spore, the bacterium divides and manifests disease and lethality primarily through the action of two toxins, anthrax lethal toxin (LT) and edema toxin (ET). Anthrax toxins have a common receptor-binding component, protective antigen (PA), which is responsible for transport of the lethal factor metalloprotease (LF) or edema factor adenylate
30 cyclase (EF) or both into the host cell cytosol. Injection of the toxins into animals replicates symptoms of anthrax disease.

PA acts as a 'gateway' that allows translocation and action of both LT and ET toxins and hence PA has been the primary target of therapeutics including antibodies developed for treatment of anthrax. PA binds to two cellular receptors as an 83 kDa polypeptide (PA83) and

is rapidly cleaved by cell surface proteases such as furin to a 63 kDa (PA63) form which associates as heptamers or octamers that provide the binding sites for LF or EF. The oligomer bound to one or more molecules of LF/EF is then rapidly translocated. PA63 form of the Anthrax toxin is competent for endocytosis. When PA is cleaved prior to exposure to cells, or produced as PA63, it rapidly oligomerizes and the pre-formed oligomer binds and transports LF/EF into cells. The PA63 oligomer undergoes a conformational change in acidic endosomes to a heat and SDS-stable form, which allows the translocation of LF and EF through a central pore into the cytosol. LF and EF then act on their substrates and manifest toxic effects.

During anthrax infection, the accumulation of anthrax toxins in the blood leads to lethality. Antibodies against PA are considered a primary therapeutic for treatment of the disease. The majority of neutralizing antibodies developed against PA act on the receptor-binding domain to inhibit interaction of the toxin with cells. A few antibodies have been identified which neutralize PA by other mechanisms.

Botulinum toxin is a neurotoxin produced by the bacterium *Clostridium botulinum*. Botulinum toxin is released by *C. botulinum* spores, which are commonly found in soil and water. The *C. botulinum* spores produce botulinum toxin on exposure to low oxygen levels and certain temperatures. Botulinum toxin can cause Botulism, which is a serious and life-threatening paralytic illness in humans and animals. The early symptoms of Botulism are weakness, trouble seeing, feeling tired, and trouble speaking followed by weakness of the arms, chest muscles and legs. Botulinum toxin is an acute lethal toxin with an estimated human median lethal dose (LD-50) of 1.3–2.1 ng/kg intravenously or intramuscularly and 10–13 ng/kg when inhaled. Antibodies against Botulinum toxins are considered a primary therapeutic for treatment of the disease.

A need exists for generating high affinity binding agents that treat both routine incidents of disease and toxicity. The production of antibodies and their storage is a costly and lengthy process. In fact, development of a single antibody therapeutic agent often requires years of clinical study. Yet multiple, different therapeutic antibodies are necessary for the effective treatment of patients exposed to a bio-terrorist assault with a potential weapon such as Anthrax or Botulism. Developing and producing multiple antibodies each of which can bind to a different target (e.g. microbial pathogens, viral pathogens, and toxins) is often a difficult task because it involves separately producing, storing and transporting each of the multiplicity of antibodies of which each is specific for one pathogen or toxin. Production and stockpiling a sufficient amount of antibodies to protect large populations is a

challenge and has not currently been achieved. The shelf life of antibodies is often relatively short (e.g., weeks or months), and accordingly freshly prepared batches of present therapeutic antibodies have to be produced to replace expiring antibodies.

5 Accordingly, there is a need for a cost effective and efficient way to provide alternatives to current therapeutic agents. Further a need exists for alternative therapeutics that are easier to develop and produce, have a longer shelf life, and bind as a single agent to multiple targets on the same disease agent, as well as to different disease agents.

Summary

10 An aspect of invention provides a pharmaceutical composition for treating a subject at risk for exposure to or exposed to at least one disease agent, the pharmaceutical composition including: at least one recombinant binding protein that neutralizes the disease agent and treats the subject for exposure to the disease agent, the binding protein including at least one amino acid sequence selected from the group of: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID 15 NO: 5, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ 20 ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 25 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143.

In some embodiments of the composition, the recombinant binding protein is encoded 30 by at least one nucleotide sequence selected from the group of: SEQ ID NO: 2, SEQ ID NO: 4 SEQ ID NO: 6, SEQ ID NO:8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 SEQ ID NO: 16, SEQ ID NO:18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 SEQ ID NO: 36, SEQ ID NO:38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44 SEQ ID NO: 46, SEQ ID NO:48,

SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54 SEQ ID NO: 56, SEQ ID NO:58, SEQ ID NO: 60, SEQ ID NO:6 2, SEQ ID NO:6 4 SEQ ID NO: 66, SEQ ID NO:68, SEQ ID NO: 70, SEQ ID NO:72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, SEQ ID NO: 128, SEQ ID NO: 130, SEQ ID NO: 132, SEQ ID NO: 134, SEQ ID NO: 136, SEQ ID NO: 138, SEQ ID NO: 140, SEQ ID NO: 142, and SEQ ID NO: 144. In an embodiment of the composition, the binding protein is heteromultimeric and has a plurality of binding regions. In another embodiment of the composition, the binding regions are not identical and each binding region has affinity to specifically bind and neutralize a non-overlapping portion of the disease agent.

In an embodiment of the composition, the binding protein further includes at least one of: a tag epitope that has affinity to bind an antibody; and a linker that separates the binding regions, and the linker including at least one selected from the group of: a peptide, a protein, a sugar, and a nucleic acid. In an embodiment of the composition, the disease agent is a toxin selected from a plant lectin and a bacterial toxin. In some embodiments, the bacterial toxin is at least one selected from a *B. anthracis* toxin, a *C. botulinum* B toxin, and a *C. botulinum* E toxin. In an embodiment of the composition, the bacterial toxin is a *B. anthracis* toxin and the binding protein binds to and neutralizes at least one selected from: an Anthrax protective antigen, an Anthrax lethal toxin, and an Anthrax edema toxin. In some embodiments, the binding protein inhibits or prevents endocytosis of the toxin. In some embodiments, the Anthrax protective antigen is a cell surface generated antigen.

In various embodiments of the composition, the amino acid sequence is substantially identical and has at least 50% identity, at least 60% identity, at least 65% identity, at least 70% identity, at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity, or and at least 95% identity to the amino acid sequence. In some embodiments, the nucleotide sequence is substantially identical and has at least 50% identity, at least 60% identity, at least 65% identity, at least 70% identity, at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity, or and at least 95% identity to the nucleotide sequence.

An aspect of the invention provides a method for treating a subject at risk for exposure to or exposed to at least one disease agent, the method including: administering to

the subject at least one binding protein having at least one binding region including an amino acid sequence selected from: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143, and measuring a decrease in at least one symptom associated with exposure to disease agent.

In an embodiment of the method, measuring the symptom further comprises analyzing an amount of remediation of at least one symptom selected from fever, chills, swelling of neck, soreness of neck glands, sore throat, painful swallowing, hoarseness, nausea, vomiting, bloody vomiting, diarrhea, bloody diarrhea, constipation, headache, flushing, red eyes, stomach pain, fainting, swelling of abdomen, double vision, blurred vision, drooping eyelids, slurred speech, dry mouth, and muscle weakness.

An aspect of the invention provides a method of identifying a therapeutic binding protein for treating a subject at risk for exposure to or exposed to at least one disease agent, the method including: contacting a first sample of a disease agent with a test protein and measuring an amount of binding of the disease agent to the test protein under conditions for the disease agent to interact with the test protein; and comparing the amount of binding to that of a second sample of the disease agent not contacted by the test protein and otherwise identical, such that presence of the therapeutic binding protein is identified by an increase of binding of the disease agent in the first sample compared to the second sample.

In an embodiment of the method, the test protein is a plurality of proteins. In some embodiments, the disease agent is in vitro. In other embodiments, the disease agent is in a cell.

An embodiment of the method further includes contacting the disease agent to a mammalian subject and measuring a decrease in at least one symptom of the disease agent. In some embodiments, the disease agent is a toxin selected from a plant lectin and a bacterial toxin. In some embodiments, the bacterial toxin is at least one selected from a *B. anthracis* toxin, a
5 *C. botulinum* B toxin, and a *C. botulinum* E toxin. In some embodiments, the bacterial toxin is a *B. anthracis* toxin and the binding protein binds to and neutralizes Anthrax protective antigen. In an embodiment, the binding protein inhibits or prevents endocytosis of the toxin. In an embodiment of the method, the Anthrax protective antigen is a cell surface generated antigen.

10 An aspect of the invention provides a method for treating a subject at risk for exposure to or exposed to at least one disease agent, the method including: administering to the subject a source of expression of a binding protein having a nucleotide sequence encoding the binding protein, such that the nucleotide sequence comprises at least one selected from the group consisting of: a naked nucleic acid vector, bacterial vector, and a viral vector, such
15 that the nucleotide sequence includes at least one selected from the group of: SEQ ID NO: 2, SEQ ID NO: 4 SEQ ID NO: 6, SEQ ID NO:8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 SEQ ID NO: 16, SEQ ID NO:18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 SEQ ID NO: 36, SEQ ID NO:38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44 SEQ ID NO: 46,
20 SEQ ID NO:48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54 SEQ ID NO: 56, SEQ ID NO:58, SEQ ID NO: 60, SEQ ID NO:6 2, SEQ ID NO:6 4 SEQ ID NO: 66, SEQ ID NO:68, SEQ ID NO: 70, SEQ ID NO:72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100,
25 SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, SEQ ID NO: 128, SEQ ID NO: 130, SEQ ID NO: 132, SEQ ID NO: 134, SEQ ID NO: 136, SEQ ID NO: 138, SEQ ID NO: 140, SEQ ID NO: 142, and SEQ ID NO: 144.

30 An aspect of the invention provides a kit for treating a subject exposed to or at risk for exposure to a disease agent including: a unit dosage of a pharmaceutical composition for treating a subject at risk for exposure to or exposed to at least one disease agent, the pharmaceutical composition including: at least one recombinant binding protein that neutralizes the disease agent thereby treating the subject for exposure to the disease agent,

such that the binding protein includes at least one amino acid sequence selected from the group of: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143.

In an embodiment of the kit, the recombinant binding protein is encoded by at least one nucleotide sequence selected from the group of: SEQ ID NO: 2, SEQ ID NO: 4 SEQ ID NO: 6, SEQ ID NO:8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 SEQ ID NO: 16, SEQ ID NO:18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 SEQ ID NO: 36, SEQ ID NO:38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44 SEQ ID NO: 46, SEQ ID NO:48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54 SEQ ID NO: 56, SEQ ID NO:58, SEQ ID NO: 60, SEQ ID NO:6 2, SEQ ID NO:6 4 SEQ ID NO: 66, SEQ ID NO:68, SEQ ID NO: 70, SEQ ID NO:72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, SEQ ID NO: 128, SEQ ID NO: 130, SEQ ID NO: 132, SEQ ID NO: 134, SEQ ID NO: 136, SEQ ID NO: 138, SEQ ID NO: 140, SEQ ID NO: 142, and SEQ ID NO: 144.

An aspect of the invention provides a method for detecting a presence of a toxin in a sample, the method including: contacting and incubating an aliquot of the sample to an amount of at least one binding protein that specifically binds the toxin, such that the binding

protein includes a binding region having an amino acid sequence selected from: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143, such that the toxin is selected from the group of: a *B. anthracis* toxin, a *C. botulinum* B toxin and a *C. botulinum* E toxin, and SEQ ID NO: 1, SEQ ID NO: 3 SEQ ID NO: 5, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, and SEQ ID NO: 105 encode the binding protein that specifically binds the *B. anthracis* toxin, and SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, and SEQ ID NO: 137 encode the binding protein that specifically binds the *B. botulinum* B toxin, and SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 127, SEQ ID NO: 129,

SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143 encode the binding protein that specifically binds the *B. botulinum* E toxin under conditions to form a complex; separating the complex from unbound binding protein; and measuring amount of complex formed.

In an embodiment of the method, the sample is at least one selected from: a medical sample, a food sample, a beverage sample, a water sample, and an environmental sample. In another embodiment of the invention, the medical sample is at least one selected from: blood, plasma, tissue, stool, urine, perspiration, serum, semen, breast milk, cerebrospinal fluid, skin and hair. An embodiment of the method further includes, analyzing the extent of complex formation, such that the extent of complex formation is a function of extent of toxin present in the sample.

Brief description of drawings

Figure 1A - Figure 1C are Meyer-Kaplan survival plots showing percent survival (% survival, ordinate) of subjects as a function of time in days (abscissa) following contact with LT and VHH binding/neutralizing agents as indicated. Balb/cJ mice were injected intravenously with antibody (Ab) at indicated molar ratios (Ab: toxin) 10 min prior to injection with LT (45 μg for each toxin component, IV). Figure 1C shows a single group (POST) received antibody 2 hours after toxin was administered. Control groups were injected with PBS instead of antibody. Heterodimers JKC-5 and VNA1 (JKD-11) contain the VHHs JIK-B8 and JIJ-B8, both with high affinity for PA but only JIK-B8 having anthrax neutralizing properties. VNA2 contains JIK-B8 and JKH-C7, both potent anthrax neutralizing VHHs. Heterodimers VNA1 and VNA2 also contain a carboxyl albumin-binding-peptide (ABP) which prolongs in vivo serum persistence of similar VNAs in mice. Antibody 14B7 is a previously described neutralizing monoclonal antibody, which binds to the same receptor-interacting domain as JIK-B8. Animals were monitored for 10 days post for signs of malaise and survival.

Figure 2A and Figure 2D are Meyer-Kaplan survival plots showing percent survival (% survival, ordinate) of subjects as a function of time in days (abscissa) following contact with A35 Sterne-like toxigenic *B.anthracis* strain spores ($2x10^7$ spores, SC) and VHH binding/neutralizing agents. Figure 2A shows data obtained using C57BL/6J mice treated with VNA2 heterodimeric VNA or 14B7 mAb control (15 μg/injection/IV) at indicated times before or after spore infection. Figure 2B shows data obtained after antibody was administered post-infection at indicated times and doses. Control mice were treated with

PBS at 15 min, 1 hour and 4 hours post infection. Figure 2C contains data from Balb/cJ mice injected intravenously (IV) with antibody at indicated molar ratios (Ab:toxin) 10 minutes prior to injection with LT (45 µg for each toxin component). Control groups received PBS instead of antibody. Animals were monitored for 10 days post treatment for signs of malaise and survival. Figure 2D shows data from C57BL/6J mice (n=5/group, except PBS controls, n=15), treated with heterodimeric VNA2-PA subcutaneously (SC) at indicated times and doses before or after spore infection (2 x 10^7 spores, also SC at a distal site). Control mice were treated with PBS at 15 min, one hour and four hours post infection (n=5) or at five minutes (n=5) and eight hours (n=5) post infection. Neutralizing mAb 14B7 was used as a positive control in these studies. Mice were monitored for survival and signs of malaise for 10 days.

Figure 3 shows amino acid sequences SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 105, SEQ ID NO: 106, SEQ ID NO: 107, SEQ ID NO: 108, SEQ ID NO: 109, SEQ ID NO: 110, SEQ ID NO: 111, SEQ ID NO: 112, SEQ ID NO: 113, SEQ ID NO: 114, and SEQ ID NO: 115 of VHHs selected for binding to anthrax PA. Sequences shown begin within framework 1 at the site of the primer binding employed in coding sequence DNA amplification from the immune alpaca cDNA and continue through the end of framework 4. The expression in parentheses at the right end indicates that the VHH contains a long hinge (lh) or a short hinge (sh). The locations of each of the three complementarity determining regions (CDRs) are indicated at the top end.

Figure 4A and Figure 4B are amino acid sequences of partial translation products of the two VNAs SEQ ID NOs: 103 and 105. The proteins are expressed in *E. coli* and tested as anthrax antitoxins. Proteins VNA1-PA and VNA2-PA respectively, are the same as previously named JKD-11 and JKU-1 respectively in U.S. provisional application serial number 62/089,949 filed December 10, 2014. Both VNAs contain an amino terminal thioredoxin fusion partner and hexahistidine encoded by the pET32b expression vector. The VHH sequences are flanked by E-tag peptides (underlined) and separated by the unstructured spacer ($(GGGGS)_3$) (SEQ ID NO: 145). The 14 amino acid albumin-binding-peptide (ABP), DICLPRWGCLEWED (SEQ ID NO: 146) described in Nguyen A, et al. (2006) Protein engineering, design & selection: PEDS 19: 291–297 is located at the carboxyl end, separated from the second E-tag by a GGGGS (SEQ ID NO: 147) spacer.

Figure 5A- Figure 5D respectively, are Meyer-Kaplan survival plots showing percent survival (% survival, ordinate) after BoNT/B toxin exposure of each of four amounts , respectively, subjects as a function of time in days (abscissa) following treatment with a preparation of BoNT/B neutralizing VHH heterodimers as indicated. In Figure 5A-Figure 5D, an amount of BoNT/B, toxin of 10, 40, 100 and 500 LD50 respectively was administered by intraperitoneal injection to groups of five C57BL/6J mice. The mice receiving the toxin were treated with 2 μg of one of BoNT/B neutralizing VHH heterodimers (SEQ ID NO:131, SEQ ID NO:133, SEQ ID NO:135, or SEQ ID NO:137). Mice were monitored at least five times per day for survival and symptoms of botulism for seven days.

Figure 6 is a table of the VHH names and binding properties. The first eleven VHHs were obtained by panning using PA83 bound to plastic, after which the K_D values for these VHHs were assessed by SPR- Proteon. The second group of nine VHHs were obtained by panning using 14B7-bound PA83, and the K_D values were assessed by SPR-Biacore. The K_D for JIK-B8, obtained as an internal reference for SPR-Biacore group, was observed to be 1 ± 0.7. EC_{50}s were assessed by dilution ELISAs, and IC_{50}s were assessed by toxin neutralization assays on macrophages and competition groups (C-groups) by competition ELISA. N/A refers to antibodies that were observed to not neutralize toxin and thus have no measurable IC_{50}.

Figure 7A-Figure 7C are graphs showing the results of competition ELISAs and neutralization assays. Data in Figure 7A and Figure 7B were obtained using 14B7- or 2D3-captured PA which was bound to plates and an increasing concentration per plate of each VHH was then added and binding was assessed with an HRP-conjugated anti-Hisx6 or E-tag antibody using standard ELISA protocols. Figure 7C shows representative neutralization assays for each of the four VHHs representing the four competition groups, a heteromultimer of two neutralizing VHHs, and mAb 14B7, with viability of the ordinate as a function of antibody concentration. All data were obtained from assays are representative of at least two separate repetitions.

Detailed description

Anthrax is a toxigenic disease, which rapidly progresses to lethality for the host if left untreated. The bioterrorist attacks utilizing *Bacillus anthracis* spores highlighted the need for

cost-effective treatments that could be produced on a large scale if necessary. Almost all the therapeutics developed against the disease focus on the anthrax toxins, which have been demonstrated to be the primary virulence determinants. Examples herein describe a novel recombinant anti-toxin consisting of a heterodimer of two camelid anti-anthrax PA heavy chain VHH binding domains are an efficient therapeutic agents. A number of antibodies have been produced against the PA receptor-binding component of the tripartite toxin, and protection in animal models is demonstrated by data herein. Most antibodies target the same epitope of the toxin, which is the dominant neutralizing antigenic region, and only differ in varying affinities and clearance rates.

Anthrax disease is caused by a complex toxin that contains a protective antigen (PA), a lethal factor (LF) and an edema factor (EF). Recombinant engineered proteins as antibodies against PA are described herein which are protective against the disease. Heavy-chain-only Ab V_H (VHH) domains with affinity for PA were obtained from immunized alpacas and were screened for anthrax neutralizing activity in macrophage toxicity assays.

Two classes of neutralizing VHHs were identified that recognized distinct and non-overlapping epitopes. One class of VHHs recognized were observed that domain 4 of PA at a neutralizing site that blocks PA binding to cells. Another class of VHHs recognized a novel, conformational epitope. A VHH antibody described herein was observed to inhibit conversion of the PA63 oligomer from “pre-pore” conformation to a SDS and heat-resistant “pore” conformation. The antibody described herein was observed to prevent endocytosis of cell surface generated PA63 subunit. The monomer neutralizing VHHs administered at 2:1 molar ratio to PA were observed to be effective in protecting mice from a lethal anthrax toxin challenge. The highest affinity members of different anti-PA VHH classes were expressed as two heterodimeric VHH-based neutralizing agents (VNAs). VNAs were observed to have improved neutralizing potency in cell assays and to have protected mice from anthrax toxin challenge with better efficacy than their corresponding monomer VHHs. The VNA2-PA (JKU-1) which was observed to be most efficient consists of a heterodimer of the novel oligomer-inhibiting VHH (JKH-C7) and a receptor blocking VHH (JIK-B8). This VNA2-PA was observed to protect mice against toxin challenge at 1:1 molar ratio to toxin and increased survival times were observed at submolar ratios. Furthermore, the antibody also provided protection against A35 spore challenge. VNA2-PA (JKU-1) has potential as an anthrax therapeutic, and its simple and stable nature is amenable to administration by genetic delivery or by respiratory routes.

The novel VHH-based VNA described herein consists of two anti-toxin VHHs targeting independent epitopes of PA and inhibiting the action of the toxin at two different functional steps. The VHH based VNA agent described herein is more effective *in vivo* by a factor of at least about 20-50 fold compared to the well-characterized neutralizing antibody 14B7 which acts on the same epitope as the approved human anti-PA antibody, Raxibacumab (Abthrax) in protecting against anthrax toxin challenge and spore infection. The affinity is 0.07 nM in contrast to the 2.78 nM affinity of Abthrax, a commercially available monoclonal antibody, Raxibacumab , that neutralizes toxins produced by *B.anthracis* (Human Genome Sciences, Rockville, MD).

An antitoxin strategy herein uses VNAs consisting of two or more, linked, toxin neutralizing, VHHs recognizing non-overlapping epitopes on PA. An advantage of covalently linking VHHs together is a resulting increased toxin binding affinity and increase in potency of neutralization through targeting of two different steps in the interaction of the toxin with cells. A benefit of the conformational epitope of the VNA, JKH-C7 arm is the extremely low likelihood of easily circumventing the PA-antibody interaction through a small number of mutations in genes encoding PA. The bulk of previously available anti-PA neutralizing antibodies target the same receptor-binding epitope that the JIK-B8 arm of the VNA targets, and the receptor-binding epitope can be destroyed by genetic manipulation of the PA antigen to eliminate reactivity with these neutralizing antibodies. The complex conformational epitope for the JKH-C7 VHH arm of the antibody described herein is unlikely to be easily disrupted without impact on PA function.

The presence of toxins in the circulation causes a wide variety of human and animal illnesses. Antitoxins are therapeutic agents that prevent toxin infection or reduce further development of negative symptoms in patients that have been exposed to a toxin (a process referred to as "intoxication"). Typically, antitoxins are antisera obtained from large animals (e.g., sheep, horse, and pig) that were immunized with inactivated or non-functional toxin. More recently, antitoxin therapies have been developed using combinations of antitoxin monoclonal antibodies including yeast-displayed single-chain variable fragment antibodies generated from vaccinated humans or mice. See Nowakowski et al. 2002. Proc Natl Acad Sci USA 99: 11346-11350; Mukherjee et al. 2002. Infect Immun 70: 612-619; Mohamed et al. 2005 Infect Immun 73: 795-802; Walker, K. 2010 Interscience Conference on Antimicrobial Agents and Chemotherapy - 50th Annual Meeting - Research on Promising New Agents: Part 1. IDrugs 13: 743-745. Antisera and monoclonal antibodies are difficult to produce economically at scale, usually requiring long development times and resulting in problematic

quality control, shelf-life and safety issues. New therapeutic strategies to develop and prepare antitoxins are needed.

Antitoxins function through two key mechanisms, neutralization of toxin function and clearance of the toxin from the body. Toxin neutralization occurs through biochemical processes including inhibition of enzymatic activity and prevention of binding to cellular receptors. Antibody mediated serum clearance occurs subsequent to the binding of multiple antibodies to the target antigen (Daeron M. 1997 Annu Rev Immunol 15: 203-234; Davies et al. 2002 Arthritis Rheum 46: 1028-1038; Johansson et al. 1996 Hepatology 24: 169-175; and Lovdal et al. 2000 J Cell Sci 113 (Pt 18): 3255-3266). Multimeric antibody decoration of the target is necessary to permit binding to the Fc receptors which have only low affinity (Davies et al. 2002 Arthritis Rheum 46: 1028-1038 and Lovdal et al. 2000 J Cell Sci 113 (Pt 18): 3255-3266). Without being limited by any particular theory or mechanism of action, it is here envisioned that an ideal antitoxin therapeutic would both promote toxin neutralization to immediately block further toxin activity and would also accelerate toxin clearance to eliminate future pathology if neutralization becomes reversed.

Effective clearance of botulinum neurotoxin (BoNT), a National Institute of Allergy and Infectious Diseases (NIAID) Category A priority pathogen, is believed by some researchers to require three or more antibodies bound to the toxin. Nowakowski et al. 2002. Proc Natl Acad Sci USA 99: 11346-11350 determined that effective protection of mice against high dose challenge of BoNT serotype A (BoNT/A) requires co-administration of three antitoxin monoclonal antibodies, and that all three antibodies presumably promote clearance. Administration of a pool of three or more small binding agents, each produced with a common epitopic tag, reduced serum levels of a toxin when co-administered with an anti-tag monoclonal antibody (Shoemaker et al. U.S. published application 2010/0278830 A1 published November 04, 2010 and Sepulveda et al. 2009 Infect Immun 78: 756-763, each of which is incorporated herein in its entirety). The tagged binding agents directed the binding of anti-tag monoclonal antibody to multiple sites on the toxin, thus indirectly decorating the toxin with antibody Fc domains and leading to clearance of the toxin through the liver.

Pools of scFv domain binding agents with specificity for BoNT/A and each containing a common epitopic tag (E-tag), had been shown to be effective for decorating the botulinum toxin with multiple anti-tag antibodies (Shoemaker et al. U.S. utility patent publication number 2010/0278830 published November 4, 2010 and U.S. continuation-in-part patent publication number 2011/0129474 published June 2, 2011, each of which is incorporated herein by reference in its entirety). Administration of binding agents and

clearance antibodies to subjects resulted in clearance *via* the liver with an efficacy in mouse assays equivalent to conventional polyclonal antitoxin sera. Ibid. and Sepulveda et al. 2009 Infect Immun 78: 756-763. The tagged scFvs toxin targeting agents and the anti-tag monoclonal antibodies were effective to treat subjects at risk for or having been contacted with a disease agent.

The use of small binding agents to direct the decoration of toxin with antibody permits new strategies for the development of agents with improved therapeutic and commercial properties. Examples herein show that a single recombinant heterodimeric binding protein/agent which contains two or more high-affinity BoNT binding agents (camelid heavy-chain-only Ab VH (VHH) domains) and two epitopic tags, co-administered with an anti-tag mAb, protected subjects from negative symptoms and lethality caused by botulism. Further, the binding protein was observed to have antitoxin efficacy equivalent to and greater than conventional BoNT antitoxin serum in two different in vivo assays. Examples herein compare neutralizing or non-neutralizing binding agents administered with or without clearing antibody, and show the relative contributions of toxin neutralization and toxin clearance to antitoxin efficacy. Examples herein show that both toxin neutralization and toxin clearance contribute significantly to antitoxin efficacy in subjects. Toxin neutralization or toxin clearance using heterodimer binding protein antitoxins was observed herein to sufficiently protect subjects from BoNT lethality in a therapeutically relevant, post-intoxication assay. Methods in further Examples herein include an optional clearing antibody for example a monoclonal anti-E-tag antibody.

It was observed in Examples herein that VHH binding agents that neutralized toxin function significantly improved the antitoxin efficacy and even obviated the need for clearing antibody in a clinically relevant post-intoxication BoNT/A assay.

Pharmaceutical compositions

An aspect of the present invention provides pharmaceutical compositions, wherein these compositions comprise an antigen from a toxin of *B. anthracis* or *C. botulinum* peptide or protein, and optionally further include an adjuvant, and optionally further include a pharmaceutically acceptable carrier. In various embodiments, the compositions include at least one atoxic protein or a source of expression of the protein, such that the protein elicits an immune response specific for a *B. anthracis* or *C. botulinum* toxin.

In certain embodiments, these compositions optionally further comprise one or more additional therapeutic agents. In certain embodiments, the additional therapeutic agent or

agents are selected from the group consisting of antibiotics particularly antibacterial compounds, anti-viral compounds, anti-fungals, and include one or more of growth factors, anti-inflammatory agents, vasopressor agents, collagenase inhibitors, topical steroids, matrix metalloproteinase inhibitors, ascorbates, angiotensin II, angiotensin III, calreticulin, tetracyclines, fibronectin, collagen, thrombospondin, transforming growth factors (TGF), keratinocyte growth factor (KGF), fibroblast growth factor (FGF), insulin-like growth factors (IGF), epidermal growth factor (EGF), platelet derived growth factor (PDGF), neu differentiation factor (NDF), hepatocyte growth factor (HGF), and hyaluronic acid.

As used herein, the term "pharmaceutically acceptable carrier" includes any and all solvents, diluents, or other liquid vehicle, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. *Remington's The Science and Practice of Pharmacy Ed.* by LWW 21st EQ. PA, 2005 discloses various carriers used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Carriers are selected to prolong dwell time for example following any route of administration, including IP, IV, subcutaneous, mucosal, sublingual, inhalation or other form of intranasal administration, or other route of administration.

Some examples of materials that can serve as pharmaceutically acceptable carriers include, but are not limited to, sugars such as glucose, and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol, and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator.

In yet another aspect, according to the methods of treatment of the present invention, the immunization is promoted by contacting the subject with a pharmaceutical composition, as described herein. Thus, the invention provides methods for immunization comprising administering a therapeutically effective amount of a pharmaceutical composition comprising

active agents that include an immunogenic toxin protein of *B. anthracis* or *C. botulinum* to a subject in need thereof, in such amounts and for such time as is necessary to achieve the desired result. It will be appreciated that this encompasses administering an inventive vaccine as described herein, as a preventive or therapeutic measure to promote immunity to
5 infection by *B. anthracis or C. botulinum*, to minimize complications associated with the slow development of immunity (especially in compromised patients such as those who are nutritionally challenged, or at risk patients such as the elderly or infants).

In certain embodiments of the present invention a "therapeutically effective amount" of the pharmaceutical composition is that amount effective for promoting production of
10 antibodies and activity in serum specific for the toxins of *B. anthracis* or *C. botulinum*, or disappearance of disease symptoms, such as amount of antigen or toxin or bacterial cells in feces or in bodily fluids or in other secreted products. The compositions, according to the method of the present invention, may be administered using any amount and any route of administration effective for generating an antibody response. Thus, the expression "amount
15 effective for promoting immunity", as used herein, refers to a sufficient amount of composition to result in antibody production or remediation of a disease symptom characteristic of infection by *B. anthracis* or *C. botulinum*.

The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active
20 agent(s) or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state; contact to infectious agent in the past or potential future contact; age, weight and gender of the patient; diet, time and frequency of administration; drug combinations; reaction sensitivities; and tolerance/response to therapy. Long acting pharmaceutical compositions might be administered every three to four days,
25 every week, or once every two weeks depending on half-life and clearance rate of the particular composition.

The active agents of the invention are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. The expression "dosage unit form" as used herein refers to a physically discrete unit of active agent appropriate for one dose to be administered
30 to the patient to be treated. It will be understood, however, that the total daily usage of the compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. For any active agent, the therapeutically effective dose can be estimated initially either in cell culture assays or in animal models, usually mice, rabbits, dogs, or pigs or piglets or other suitable animals. The animal models described

herein including that of chronic or recurring infection by *B. anthracis* or *C. botulinum* is also used to achieve a desirable concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

5 A therapeutically effective dose refers to that amount of active agent which ameliorates at least one symptom or condition. Therapeutic efficacy and toxicity of active agents can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, ED50 (the dose is therapeutically effective in 50% of the population) and LD50 (the dose is lethal to 50% of the population). The dose ratio of toxic to 10 therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and from animal studies are used in formulating a range of dosage for human use.

The therapeutic dose shown in examples herein is at least about 1 μg per kg, at least 15 about 5, 10, 50, 100, 500 μg per kg, at least about 1 mg/kg, 5, 10, 50 or 100 mg/kg body weight of the purified toxin vaccine per body weight of the subject, although the doses may be more or less depending on age, health status, history of prior infection, and immune status of the subject as would be known by one of skill in the art of immunization. Doses may be divided or unitary per day and may be administered once or repeated at appropriate intervals.

20

Administration of pharmaceutical compositions

After formulation with an appropriate pharmaceutically acceptable carrier in a desired dosage, the pharmaceutical compositions of this invention can be administered to humans and other mammals topically (as by powders, ointments, or drops), orally, rectally, mucosally, 25 sublingually, parenterally, intracisternally, intravaginally, intraperitoneally, bucally, sublingually, ocularly, or intranasally, depending on preventive or therapeutic objectives and the severity and nature of a pre-existing infection.

In various embodiments of the invention herein, it was observed that high titers of antibodies, sufficient for protection against a lethal dose of *B. anthracis* or *C. botulinum* 30 toxin, were produced after administration of the engineered atoxic toxin proteins provided herein. Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active agent(s), the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents

and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and
5 mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Dosage forms for topical or transdermal administration of an inventive pharmaceutical composition include ointments, pastes, creams, lotions, gels, powders,
10 solutions, sprays, inhalants, or patches. The active agent is admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required. Administration may be therapeutic or it may be prophylactic.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or
15 wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or
20 suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables. The injectable formulations can be sterilized prior to addition of spores, for example, by filtration through a bacterial-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or
25 dispersed in sterile water or other sterile injectable medium prior to use. In order to prolong the effect of an active agent, it is often desirable to slow the absorption of the agent from subcutaneous or intramuscular injection. Delayed absorption of a parenterally administered active agent may be accomplished by dissolving or suspending the agent in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the agent in
30 biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of active agent to polymer and the nature of the particular polymer employed, the rate of active agent release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the agent in liposomes or microemulsions which are compatible with body tissues.

Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the active agent(s) of this invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the
5 rectum or vaginal cavity and release the active agent(s).

Solid dosage forms for oral, mucosal or sublingual administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active agent is mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, sucrose, glucose,
10 mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as,
15 for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as milk sugar as well as high molecular weight
20 polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings, release controlling coatings and other coatings well known in the pharmaceutical formulating art. In such solid dosage forms the active agent(s) may be admixed with at least one inert diluent such as sucrose or starch. Such dosage forms may also comprise, as is normal practice,
25 additional substances other than inert diluents, *e.g.*, tableting lubricants and other tableting aids such a magnesium stearate and microcrystalline cellulose. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents. They may optionally contain opacifying agents and can also be of a composition that they release the active agent(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed
30 manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Substantially identical amino acid and nucleotide sequences for VHHs

There is a large body of information in the literature supporting the fact that closely related antibody (Ab) sequences are capable of performing the same binding and therapeutic functions such that this is now generally accepted by those with ordinary skill in immunological sciences and is even a dogma. The creation of Abs with small numbers of amino acid sequence variations occurs naturally within mammals and other some other animal species during the process of 'affinity maturation' in which cells producing Abs that bind a newly encountered antigen (Ag) are expanded such that progeny cells contain random mutations within portions of the Ab coding DNA that results in new, related Ab sequences. The cells expressing Abs that have gained improved binding properties for the new Ag are then selected and expanded, increasing the amount of the improved antibody in the animal. This process continues through multiple generations of mutation and selection until Abs with greatly improved binding properties result, thus providing, for example, better immunity against pathogens possessing the new Ag. This process of Ab affinity maturation is widely accepted in the literature and clearly demonstrates that related Ab amino acid sequences can possess similar target binding properties and perform similar therapeutic functions in vivo.

In examples herein, there are numerous examples of related Ab sequences performing similar functions and providing similar therapeutic benefits. The Abs described herein are mostly heavy-chain only Abs (HcAbs) from Camelids. The V_H region from the DNA is isolated encoding these Abs and expressed as single-domain Abs called VHHs. Alpacas are immunized with a selected Ag multiple times to permit the animal to undergo affinity maturation of the HcAbs they produce recognizing this Ag. The HcAbs are then isolated and the DNA encoding the VHH regions are closed for expression of soluble VHHs that bind the Ag and have potential therapeutic or diagnostic properties. During this process, many examples of closely related VHHs are isolated presumably different which are intermediates resulting from the alpacas affinity maturation process. These related VHHs are screened and most promising members of each homology group is identified, and becomes a lead candidate for further development.

VHHs, like all mammalian antibodies, consist of four well-conserved 'framework' regions (FRs) which are important to form the antibody structure. Between the FRs (FR1, FR2, FR3 and FR4) are three much less well-conserved 'complementarity determining regions' or CDRs which form the interactions with the Ags. These binding regions must bind to widely varying structures (epitopes) on different Ags, therefore, the CDRs must also vary widely so as to interact and bind to these Ags. The third CDR, CDR3, is generally the longest and most diverse of the CDRs within VHHs, both in size and sequence. CDR3 in VHHs can

range in size from about 7 to about 28 amino acid residues [1]. The CDR3 regions of VHHs from the same alpacas selected for their binding to a common target Ag, prove to be very similar in their size and have many amino acid identities; the chance that this occurred by random chance are astronomical. Therefore, these VHHs resulted from affinity maturation of a common precursor VHH within the animal and are classified as being a 'homology group'. The individual VHHs within a homology group are classified for binding to a target the members of the VHH homology group 'compete' with each other for binding, thus demonstrating that they bind to the same region on the target.

Since the FRs are critical for sustaining the structure of the VHH and the positioning of the CDRs for binding to their target Ag, the FRs must not vary too much in sequence. Some variation, particular when replacement amino acids are related in properties, is permissible and these changes can often be found naturally within VHHs that have undergone affinity maturation in an animal. In addition to the FRs, the CDRs also must not vary too much in sequence or their Ag binding affinity will be compromised. An excellent way to estimate how much amino acid sequence variation is tolerated within VHHs without compromising their Ag binding character is to observe the variation that occurs naturally within affinity matured homology groups of VHHs isolated from the same animals and shown to bind to the same Ag.

An example of VHH sequence relatedness necessary to retain common Ag binding properties is described in U.S. patent number 8,349,326, issued January 8, 2014 and represented in Figure 5. In this example, the substantial identity of five different VHH sequences shown in the patent, JDO-E9, JDQ-B2, JDQ-B5, JDQ-C5, and JDQ-F9 is represented as a phylogenic tree. These sequences are substantially different from each other and form a clear homology group when their sequences are compared to the sequences of seven random VHHs. All five members of this homology group had been selected for their binding to Botulinum neurotoxin serotype A (BoNT/A), all had clearly related CDR3 regions, and all were found to compete with each other for binding to BoNT/A. Therefore, these sequences had a common binding site. Despite their common clonal origin and common Ag binding sites, these VHHs of 108 amino acid length contained as many as 26 amino acid differences. This implied that VHHs containing up to 24% amino acid sequence variation had retained their ability to bind to the same region of BoNT/A.

Another example that describes acceptable amount of VHH sequence variation within related VHHs having the same Ag binding character is described in Tremblay et al., 2013 Infect Immun 81: 4592-4603. Proteins in large homology group are described containing 11

VHH sequences, Stx-A3, A4, A5, D4, F1, G6, H3, H5, H9, H10, and H12 with closely related CDR3 sequences of identical size, and the unusual property of cross-specific binding to two different Shiga toxins, Stx1 and Stx2. Two of the more distantly related members of this homology group, VHHs Stx-A4, Stx-A5 are characterized as having common Ag binding character. These two related VHHs have 32 amino acid changes in their full 120 or 121 residue VHH sequence. Therefore, 26% amino acid variation in sequence does not result in the loss of their common Ag binding property.

A portion of the data herein was published as follows, "Prolonged prophylactic protection from botulism with a single adenovirus treatment promoting serum expression of a VHH-based antitoxin protein" by co-authors Mukherjee J, Dmitriev I, Debatis M, Tremblay JM, Beamer G, Kashentseva EA, Curiel DT, Shoemaker CB, in the journal PLoS ONE 9(8): e106422 2014 Aug. doi:10.1371/journal.pone.0106422; "Adenovirus vector expressing Stx1/2-neutralizing agent protects piglets infected with E. coli O157:H7 against fatal systemic intoxication" by co-authors Sheoran AS, Dmitriev IP, Kashentseva EA, Cohen O, Mukherjee J, Debatis M, Shearer J, Tremblay JM, Beamer G, Curiel DT, Shoemaker CB, Tzipori S, in the journal Infect Immun. 2014 Nov 3. pii: IAI.02360-14; and "A heterodimer of a VHH (variable domains of camelid heavy chain-only) antibody that inhibits anthrax toxin cell binding linked to a VHH antibody that blocks oligomer formation is highly protective in an anthrax spore challenge model" by co-authors Moayeri M, Leysath CE, Tremblay JM, Vrentas C, Crown D, Leppla SH, Shoemaker CB, in the journal J Biol Chem. 2015 Mar 6;290(10):6584-95 which appeared online January 6, 2015. These papers are hereby incorporated in their entireties herein.

The invention now having been fully described, it is further exemplified by the following claims.

Examples

Example 1: Toxins and spores

Endotoxin-free mutant PA proteins, including wild type PA83, PA63, and LF were purified from *B. anthracis* as described in Park, S., et al., 2000 Protein expression and purification 18, 293-302. The PAΔΔ is a mutant from which amino acid residues at positions 162-167 and 304-317 of the amino acid sequences have been genetically deleted, such that the protein cannot be cleaved by furin and accumulates on the cell surface. PAdFF is a mutant in which phenylalanine residues at positions 313 and 314 have been deleted thereby making the protein unable to translocate LF and EF (Singh, Y., et al., 1994 The Journal of

biological chemistry 269, 29039-29046). Concentrations of LT correspond to the concentration of each toxin component (i.e. 1 µg/mL LT is 1 µg/mL PA + 1 µg/mL LF). Spores of the non-encapsulated, toxigenic Sterne-like strain A35 (Pomerantsev, A. P., et al., 2006 Infection and immunity 74, 682-693) used to infect mice were prepared as described in
5 Moayeri, M., et al., 2010 PLoS pathogens 6, e1001222.

Example 2: Reagents

Rabbit anti-PA83 polyclonal serum #5308 and neutralizing anti-PA mouse monoclonal antibody (mAb) 14B7, which blocks binding of PA (both PA83 and PA63) to its
10 cellular receptors was manufactured as described in Rosovitz, M. J., et al., 2003 The Journal of biological chemistry 278, 30936-30944. Antibodies against the N-terminus of MEK1 (Calbiochem-EMD Biosciences, San Diego, CA), horse radish peroxidase (HRP)-conjugated and non-conjugated anti-E-tag polyclonal antibodies (Bethyl Labs, Montgomery, TX) and various IR-dye tagged secondary antibodies (Rockland Labs, Boyertown, PA) were
15 purchased. The dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO).

Example 3: VHH-display library preparation from genes expressed in immunized alpacas

Three alpacas were immunized with PA83 (100 µg) by five successive multi-site
20 subcutaneous (SC) injections at three week intervals. For the first immunization, the adjuvant was alum/CpG and subsequent immunizations used alum. All alpacas achieved ELISA anti-PA titers of 1:1,000,000. Blood was obtained from the alpacas for lymphocyte preparation seven days after the fifth immunization, and RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). Two VHH-display phage libraries were prepared as described in
25 Maass, D. R., et al., 2007 International journal for parasitology 37, 953-962 and Tremblay, J. M., et al., 2010 Toxicon 56, 990-998. The forward and reverse primers used to amplify the VHH coding region repertoire contained Not1 and Asc1 sites, which were used to ligate the JSC vector for gene III phage display. The first library (JIG-2) was constructed using RNA obtained from peripheral blood lymphocytes (PBLs) of one immunized alpaca and contained
30 about 1×10^7 independent clones, and the second library (JKF-1) was generated from RNA obtained from a pool of PBLs of the other two alpacas, and contained about 3×10^7 independent clones.

Example 4: ELISAs

Purified VHH preparations were serially diluted onto ELISA plates coated with 1 μg/ml of each of the different PA proteins, incubated for one hour at room temperature, washed and then incubated for one hour with HRP-anti-E-tag. Bound HRP was detected
5 using 3,3',5,5'-tetramethylbenzidine (Sigma) and values were plotted as a function of the input VHH concentration. EC_{50} values were calculated for the VHH concentration that secreted in a signal equal to 50% of the maximum signal.

Example 5: Anti-PA VHH identification and preparation

10 Phage library panning, phage recovery and clone fingerprinting were performed as described in Mukherjee, J., et al., 2012 PLoS ONE 7, e29941, Maass, D. R., et al., 2007 International journal for parasitology 37, 953-962 and Tremblay, J. M., et al., 2010 Toxicon 56, 990-998, as follows. The first panning process utilized the JIG-2 VHH-display library and employed purified PA83 or PA63 coated onto Nunc Immunotubes at 10 μg/ml for the first
15 low stringency pan and 1 μg/ml for the second high stringency pan. After two panning cycles, 70% of random clones selected on each target produced a signal two fold greater than background. The clones that produced strongest 'bug supernatant' ELISA (Tremblay, J. M., et al., 2013 Infection and immunity 81, 4592-4603) signals on plates coated with 0.5 μg/ml PA83 were fingerprinted. The VHHs that had been panned on PA83 or PA63 were observed
20 to recognize both PA83 and PA63. VHH coding sequences were determined for 24 clones displaying clear unique fingerprints (Tremblay, J. M., et al., 2013 Infection and immunity 81, 4592-4603). Sequence alignments showed 11 distinct homology groups. Amino acid sequences of clones representing each group are shown in Figure 3. A second panning process using the JKF-1 library was performed similar to the first panning process and PA83
25 was used as the target. About 300 colonies were picked randomly and screened by bug supernatant ELISA on replica plates coated with either 0.5 μg/ml PA, or 3 μg/ml 14B7 mAb (Little, S. F., et al.,1988 Infection and immunity 56, 1807-1813) followed by 1 μg/ml PA83. Screening on 14B7-captured PA83 was performed to block binding of VHHs recognizing the dominant epitope (C-group 1, Figure 6) that was identified following the screening of the first
30 library. About 70% of clones recognized PA83 and about 20% recognized 14B7-captured PA83. From data obtained by ELISA and DNA fingerprinting, about 70 different VHH coding sequences were obtained. The alignment of these 70 different VHH coding sequences led to the identification of eight new homology groups not previously identified in the first screen (VHHs: JKH-A4, JKH-C7, JKH-D12, JKM-A6, JKO-A4, JKO-B8, JKO-E12, AND

JKO-H12 in Figure 3 and 6). At least one VHH from each homology group was selected for protein expression. Expression and purification of VHHs in *E. coli* as recombinant thioredoxin (Trx) fusion proteins containing hexahistidine was performed as previously described in Tremblay, J. M., et al., 2010 Toxicon 56, 990-998. VHH heterodimers were
5 genetically engineered to be linked by a 15-amino acid flexible spacer ($(GGGGS)_3$) (SEQ ID NO: 145). All VHHs were expressed with a carboxyl-terminal E-tag epitope. Competition ELISA analysis was performed as previously described, with minor modifications (Mukherjee, J., et al., 2012 PLoS ONE 7, e29941).

10 Example 6: Affinity analyses

The kinetic parameters of the VHHs were assessed by performing surface plasmon resonance, using either a ProteOn XPR36 Protein Interaction Array System (Bio-Rad, Hercules, CA; VHHs: JHD-B6, JHE-D9, JIJ-A12, JIJ-B8, JIJ-D3, JIJ-E9, JIJ-F11, JIK-B8, JIK-B10, JIK-B12, and JIK-F4 in Figure 6) or a Biacore 3000 (GE Healthcare; VHHs: JKH-
15 A4, JKH-C7, JKH-D12, JKM-A6, JKO-A4, JKO-B8, JKO-E12, and JKO-H12 in Figure 6). In each assay, the VHH was immobilized to the chip (GLH for ProteOn, CM5 for Biacore) by amine coupling chemistry, involving sequential activation of the chip surface with a mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and sulfo-N-hydoxysuccinimide (sulfo-NHS), injection of PA83 at pH 5 (sodium acetate buffer), and deactivation with an
20 ethanolamine injection.

For the ProteOn data set, a range of PA concentrations was passed over the chip surface at 100 μL/min for 60 s, and dissociation was recorded for 600 s or 1200 s. Running buffer for these assays was 10 mM Hepes, pH 7.4, 150 mM NaCl, 0.005% Tween-20. The surface was regenerated between runs with a 30 s injection of 50 mM HCl at 50 μL/min. Data
25 were evaluated with ProteOn Manager software (version 3.1.0.6) using the Langmuir interaction model to obtain K_D values. Reported values are the mean of at least four replicates.

For the Biacore data set, VHHs were passed over the PA immobilized on the chip surface at 100 nM and 100 μl/min for 60 s, and dissociation was recorded for 600 s or 1200 s.
30 Running buffer for these assays was 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% Tween-20. The surface was regenerated between runs with a 30 s injection of 10 mM glycine (pH 3) at 50 μl/min. Dissociation and association phases of each curve were fit separately using BIAevaluation software (GE) using the 1:1 Langmuir model to obtain K_D values. Reported values are the mean of three replicates. A series of four replicates at 100 nM through 2 μM

JKO-B8 resulted in comparable K_D values at each concentration. A negative control VHH (anti-EF) did not exhibit any binding to the PA-coated chip. JIK-B8 was run at the beginning and end of the series to provide a point of comparison to the ProteOn data set.

5 Example 7: Toxicity and neutralization assays

RAW264.7 mouse macrophages were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 10 mM HEPES, and 50 µg/mL gentamicin (all purchased from Life Technologies, Grand Island, NY). For neutralization assays PA83 and LF (250 ng/ml) in serum-free Dulbecco's Modified Eagle Medium were
10 incubated with each of various dilutions of antibody in 96-well plates for one hour prior to addition to RAW264.7 macrophages. Viability was assessed by MTT staining as described in Chen, Z., et al., 2009 Infection and immunity 77, 3902-3908, at a time point when greater than 90% of toxin-treated controls were observed to be lysed by assessment by light microscopy. In certain experiments PA83 or PA63 (1 µg/ml) were pre-bound to antibodies or
15 were added to cells at 37^0C or 4^0C followed after one hour by washing with serum-free DMEM at the same temperature and addition of medium containing LF or antibodies prepared in LF (1 µg/ml). Cells were then incubated at 37^0C for 12-16 hours. Viability was then assessed by MTT staining relative to untreated cell controls.

20 Example 8: Mouse studies

For toxin challenge, Balb/cJ mice (female, 8 weeks old, Jackson Laboratories, Bar Harbor, ME) were treated with antibody agents by the IV route at the doses (molar ratios relative to PA) and times described in brief description of the figures. Mice were challenged with LT (45 µg, IV) and monitored for 10 days for survival. For spore challenges, C57BL/6J
25 mice (8 weeks old, female, Jackson Laboratories) were challenged with the lethal dose of 2 x 10^7 spores (SC, 200 µl) before or after antibody administration (SC) at various doses and times as noted in brief description of the figures.

Example 9: Ethics statement

30 All examples were performed under protocols approved by Tufts University and National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Use Committees. Work with alpacas was performed at Tufts under approved protocol Tuskegee University School of Veterinary Medicine (TUSVM) and Institutional Animal Care and Use

Committee (IACUC) Protocol #G2011-08. Mouse studies were performed at NIAID under approved protocols LPD8E and LPD9E.

Example 10: Anthrax PA-binding VHHs

5 VHH-display phage libraries were prepared from genetic material obtained from three alpacas, which had been immunized with purified anthrax PA83. Two separate libraries were selected for clones binding to PA83 or, to PA83 immobilized on mAb 14B7. The mAb 14B7 is a well-characterized neutralizing mAb that binds to an immunodominant epitope through which PA binds to its receptor.

10 Of total clones obtained and sequenced, 19 VHHs with apparently unrelated sequences were identified (Figure 3). Competition assays between the various VHHs, and with 14B7 and 2D3 mAbs, which bind distinct immunodominant regions of PA83 (Little, S. F., et al.,1988 Infection and immunity 56, 1807-1813) showed that the identified VHHs fall into four distinct competition groups (identified as 1, 2, 3, and 4 in Figure 6) and thus likely 15 each protein in a group binds to one of four non-overlapping epitopes on PA. Ten of the 11VHHs selected by binding to PA83-coated tubes (VHHs: JHD-B6, JHE-D9, JIJ-A12, JIJ-B8, JIJ-D3, JIJ-E9, JIJ-F11, JIK-B8, JIK-B10, JIK-B12, and JIK-F4 in Figure 6) competed with 14B7. Eight unique PA-binding VHHs, including six that bind PA83 at sites different than 14B7 (Figure 7A and Figure 7B) were subsequently selected by binding to 14B7-20 immobilized (and thereby blocked) PA (VHHs: JKH-A4, JKH-C7, JKH-D12, JKM-A6, JKO-A4, JKO-B8, JKO-E12, and JKO-H12 in Figure 6). Binding of one of the VHH, clone JIJ-B8 was not blocked by either mAb, and binding of another clone JKO-H2 was inhibited by both mAbs (Figure 7A and Figure 7B). The VHHs were characterized for PA affinity by dilution ELISA (for EC_{50}) and by surface plasmon resonance (for K_D) (Figure 6). A selected VHH 25 representative of each of the four epitope competition groups is illustrated by a shaded portion in Figure 6. These are JIK-B8 (C-group 1), JKH-C7 (C-group 3), JKH-D12 (C-group 2), and JKO-H2 (C-group 4).

Example 11: Anthrax toxin neutralization

30 Cell-based anthrax toxin neutralization assays were performed on each of the 19 unique VHHs, and the data showed potencies ranging from IC_{50} of about 200 pM to no activity in an assay using PA at 1.25 nM (Figure 6; representative assay with antibodies from each competition group shown in Figure 7C). VHHs recognizing the immunodominant PA domain (group 1) differed widely in their ability to neutralize the toxin, with four of 12

showing no neutralizing ability. VHHs JIK-B8 and JKO-E12 of the C-group 1 class displayed the highest affinity and lowest IC_{50} values. One VHH recognized a second epitope (JKH-C7, group 3, Figure 6) showed potent anthrax neutralizing activity (Figure 7C). VHHs that had been characterized as recognizing C-group 2 and C-group 4 showed weak or undetectable toxin neutralizing activity (Figure 7C). VHH JKO-H2 (group 4) displayed no recognition of PA63, suggesting that furin cleavage either removes the epitope or alters it in a manner that it cannot be recognized.

Example 12: Heterodimeric VHH-based neutralizing agents (VNAs) protect against anthrax toxin and spore infection in mice

Linking toxin-neutralizing VHHs into heteromultimeric VNAs has been found to improve toxin affinity and, more importantly, to substantially improve *in vivo* antitoxin efficacy (Mukherjee, J., et al., 2012 PLoS ONE 7, e29941; Tremblay, J. M., et al., 2013 Infection and immunity 81, 4592-460; Vance, D. J., et al., 2013 The Journal of biological chemistry 288, 36538-36547; Yang, Z., et al., 2014 The Journal of infectious diseases 2014 Sep 15;210(6):964-72).

A heterodimeric VNA (VNA2-PA) was prepared to contain the two potent neutralizing VHHs, JIK-B8 and JKH-C7, separated by a short unstructured peptide, was expressed and purified (amino acid sequence shown in Figure 4A). This construct VNA2-PA, was observed to have potent neutralizing toxin activity (Figure 7C). VNA2-PA was compared to monomeric VHHs for the ability to protect mice from anthrax toxin. The toxin dose between 1-2 LD100 (45 μg LT) was administered by IV route for the Balb/cJ strain. Treatment doses were selected to test efficacy at various molar ratios of agent to toxin. Heterodimeric VNAs each bind at two separate sites on each toxin, so a dose that can fully occupy both binding sites must be present at a 2:1 molar ratio agent:toxin. Each single monomer VHH was observed as not able to protect mice or provide any beneficial effect at a 1:1 molar ratio, with percent survivals as low as that for mice administered control PBS. The heterodimeric VNA2-PA in contrast was highly protective at 1:1 (Figure 1C) yielding 100% protection for the entire time course. Thus, VNA2-PA was able to shift the time to death significantly even at submolar ratios to toxin. Importantly, the heterodimer offered greater protection against toxin than a pool of the two VHHs used in a 1:1:1 ratio with toxin, providing evidence of the improved in vivo efficacy of the heterodimer form (Figure 1C). VNA2-PA treatment two hour post-toxin administration was also highly protective (Figure 1C). This finding was surprising in light of the fact that the bulk of PA has been shown to be

cleaved to PA63 and removed from circulation by two hours after a bolus administration (Moayeri, M., et al., 2007 Infection and immunity 75, 5175-5184). Thus, it is here envisioned that a significant amount of active PA not measurable in circulation (plasma) may remain accessible to antibody at crucial tissue sites. A second VHH heterodimer engineered by the
5 methods herein, VNA1-PA, incorporating as component monomers the neutralizing JIK-B8 VHH with a non-neutralizing VHH (JIJ-B8) was observed to fail to provide any protection if administered 1:1, but was fully protective in this assay if administered at a two-fold molar excess (Figure 4B and Figure 2C).

VNA2-PA was tested with 14B7 mAb control for protection of C57BL/6J mice
10 against infection with a single LD100 dose of the A35 Sterne-like toxigenic *B. anthracis* strain. Antibody provided 15 min prior to subcutaneous spore infection or at three sequential times of dosing, at 15, 60 and 240 min post-infection, was also fully protective (Figure 2A-Figure 2D).

A single administration of the VNA2-PA antibody at the lower dose of 30 μg at four
15 hours post infection resulted in survival of 2/5 mice. Mice treated with this dose of 14B7 died during the time course, likely because only one third the number of antibody molecules were present compared to VNA2-PA. Increasing the time gap between spore infection and antibody administration to eight hours resulted in a complete loss of protection unless antibody was increased to a much higher dose of 250 μg, at which dose a surprising full
20 protection of the entire mouse group was observed (Figure 2A- Figure 2D).

Example 13: Heterodimeric VHH-based neutralizing agents (VNAs) protect against BoNT/B toxin in mice

BoNT/B neutralizing heterodimer VHHs were tested for the ability to protect mice
25 from BoNT/B lethality. An amount of BoNT/B toxin of 10, 40, 100 and 500 LD50 respectively was administered by intraperitoneal injection to groups of five C57BL/6J mice. The mice receiving the toxin were treated with 2 μg of one of BoNT/B neutralizing VHH heterodimers (SEQ ID NO:131, SEQ ID NO:133, SEQ ID NO:135, or SEQ ID NO:137). Mice were monitored at least five times per day for survival and symptoms of botulism for
30 seven days.

Mice contacted with BoNT/B toxin of 10, 40 and 100 LD50 respectively by intraperitoneal injection were treated with 2 μg of one of BoNT/B neutralizing VHH heterodimers SEQ ID NO:131, SEQ ID NO:133, SEQ ID NO:135, or SEQ ID NO:137. It was observed that the treated mice were fully protected, having a survival rate of 100%. In

contrast, control mice untreated with VHH heterodimers died within 24 hours as shown in Figure 5A- Figure 5C.

At the even greater BoNT/B toxin concentration of 500 LD50, VHH heterodimers SEQ ID NO: 131, SEQ ID NO:133 and SEQ ID NO:135 provided protection for two days and VHH heterodimer SEQ ID NO: 137 showed 100% survival rate till day 7 and 80% survival rate thereafter (Figure 5D).

What is claimed is:

1. A pharmaceutical composition for treating a subject at risk for exposure to or exposed to at least one disease agent, the pharmaceutical composition comprising: at least one recombinant binding protein that neutralizes the disease agent and treats the subject for exposure to the disease agent, the binding protein comprising at least one amino acid sequence selected from the group of: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO: 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143.

2. The pharmaceutical composition according to claim 1, wherein the recombinant binding protein is encoded by at least one nucleotide sequence selected from the group of: SEQ ID NO: 2, SEQ ID NO: 4 SEQ ID NO: 6, SEQ ID NO:8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 SEQ ID NO: 16, SEQ ID NO:18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 SEQ ID NO: 36, SEQ ID NO:38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44 SEQ ID NO: 46, SEQ ID NO:48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54 SEQ ID NO: 56, SEQ ID NO:58, SEQ ID NO: 60, SEQ ID NO:6 2, SEQ ID NO:6 4 SEQ ID NO: 66, SEQ ID NO:68, SEQ ID NO: 70, SEQ ID NO:72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO: 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ

ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, SEQ ID NO: 128, SEQ ID NO: 130, SEQ ID NO: 132, SEQ ID NO: 134, SEQ ID NO: 136, SEQ ID NO: 138, SEQ ID NO: 140, SEQ ID NO: 142, and SEQ ID NO: 144.

5 3. The pharmaceutical composition according to claim 1, wherein the binding protein is heteromultimeric and has a plurality of binding regions.

4. The pharmaceutical composition according to claim 3, wherein the binding regions are not identical and each binding region has affinity to specifically bind and neutralize a
10 non-overlapping portion of the disease agent.

5. The pharmaceutical composition according to claim 3, wherein the binding protein further comprises at least one of: a tag epitope that has affinity to bind an antibody; and a linker that separates the binding regions, and the linker comprising at least one selected from
15 the group of: a peptide, a protein, a sugar, and a nucleic acid.

6. The pharmaceutical composition according to claim 1, wherein the disease agent is a toxin selected from a plant lectin and a bacterial toxin.

20 7. The pharmaceutical composition according to claim 5, wherein the bacterial toxin is at least one selected from a *B. anthracis* toxin, a *C. botulinum* B toxin, and a *C. botulinum* E toxin.

8. The pharmaceutical composition according to claim 7, wherein the bacterial toxin is a
25 *B. anthracis* toxin and the binding protein binds to and neutralizes at least one selected from: an Anthrax protective antigen, an Anthrax lethal toxin, and an Anthrax edema toxin.

9. The pharmaceutical composition according to claim 8, wherein the binding protein inhibits or prevents endocytosis of the toxin.

30

10. The pharmaceutical composition according to claim 8, wherein the Anthrax protective antigen is a cell surface generated antigen.

11. The pharmaceutical composition according to claim 1, wherein the amino acid sequence is substantially identical and has at least 50% identity, at least 60% identity, at least 65% identity, at least 70% identity, at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity, or and at least 95% identity to the amino acid sequence.

5

12. The pharmaceutical composition according to claim 2, wherein the nucleotide sequence is substantially identical and has at least 50% identity, at least 60% identity, at least 65% identity, at least 70% identity, at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity, or and at least 95% identity to the nucleotide sequence.

10

13. A method for treating a subject at risk for exposure to or exposed to at least one disease agent, the method comprising:

administering to the subject at least one binding protein having at least one binding region comprising an amino acid sequence selected from: SEQ ID NO: 1, SEQ ID NO: 3,
15 SEQ ID NO: 5, SEQ ID NO:7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, SEQ ID NO: 45, SEQ ID NO: 47, SEQ ID NO: 49, SEQ ID NO: 51, SEQ ID NO: 53, SEQ ID NO: 55, SEQ ID
20 NO: 57, SEQ ID NO: 59, SEQ ID NO: 61, SEQ ID NO: 63, SEQ ID NO: 65, SEQ ID NO: 67, SEQ ID NO: 69, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 75, SEQ ID NO: 77, SEQ ID NO:79, SEQ ID NO: 81, SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87, SEQ ID NO: 89, SEQ ID NO: 91, SEQ ID NO: 93, SEQ ID NO: 95, SEQ ID NO: 97, SEQ ID NO: 99, SEQ ID NO: 101, SEQ ID NO: 103, SEQ ID NO: 105, SEQ ID NO: 107, SEQ ID NO:
25 109, SEQ ID NO: 111, SEQ ID NO: 113, SEQ ID NO: 115, SEQ ID NO: 117, SEQ ID NO: 119, SEQ ID NO: 121, SEQ ID NO: 123, SEQ ID NO: 125, SEQ ID NO: 127, SEQ ID NO: 129, SEQ ID NO: 131, SEQ ID NO: 133, SEQ ID NO: 135, SEQ ID NO: 137, SEQ ID NO: 139, SEQ ID NO: 141, and SEQ ID NO: 143, and

measuring a decrease in at least one symptom associated with exposure to disease
30 agent.

14. The method according to claim 13, wherein measuring the symptom further comprises analyzing an amount of remediation of at least one symptom selected from fever, chills, swelling of neck, soreness of neck glands, sore throat, painful swallowing, hoarseness,

nausea, vomiting, bloody vomiting, diarrhea, bloody diarrhea, constipation, headache, flushing, red eyes, stomach pain, fainting, swelling of abdomen, double vision, blurred vision, drooping eyelids, slurred speech, dry mouth, and muscle weakness.

5 15. A method of identifying a therapeutic binding protein for treating a subject at risk for exposure to or exposed to at least one disease agent, the method comprising:

contacting a first sample of a disease agent with a test protein and measuring an amount of binding of the disease agent to the test protein under conditions for the disease agent to interact with the test protein; and

10 comparing the amount of binding to that of a second sample of the disease agent not contacted by the test protein and otherwise identical, wherein presence of the therapeutic binding protein is identified by an increase of binding of the disease agent in the first sample compared to the second sample.

15 16. The method according to claim 15, wherein the test protein is a plurality of proteins.

17. The method according to claim 15, wherein the disease agent is in vitro.

18. The method according to claim 15, wherein the disease agent is in a cell.

20

19. The method according to claim 15, further comprising contacting the disease agent to a mammalian subject and measuring a decrease in at least one symptom of the disease agent.

20. The method according to claim 15, wherein the disease agent is a toxin selected from
25 a plant lectin and a bacterial toxin.

21. The method according to claim 20, wherein the bacterial toxin is at least one selected from a *B. anthracis* toxin, a *C. botulinum* B toxin, and a *C. botulinum* E toxin.

30 22 The method according to claim 21, wherein the bacterial toxin is a *B. anthracis* toxin and the binding protein binds to and neutralizes Anthrax protective antigen.

23. The method according to claim 22, wherein the binding protein inhibits or prevents endocytosis of the toxin.

24. The method according to claim 22, wherein the Anthrax protective antigen is a cell surface generated antigen.

5 25. A method for treating a subject at risk for exposure to or exposed to at least one disease agent, the method comprising:

administering to the subject a source of expression of a binding protein having a nucleotide sequence encoding the binding protein, wherein the nucleotide sequence comprises at least one selected from the group consisting of: a naked nucleic acid vector,
10 bacterial vector, and a viral vector, wherein the nucleotide sequence comprises at least one selected from the group of: SEQ ID NO: 2, SEQ ID NO: 4 SEQ ID NO: 6, SEQ ID NO:8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14 SEQ ID NO: 16, SEQ ID NO:18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24 SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 SEQ ID NO: 36, SEQ ID NO:38, SEQ ID NO: 40, SEQ ID
15 NO: 42, SEQ ID NO: 44 SEQ ID NO: 46, SEQ ID NO:48, SEQ ID NO: 50, SEQ ID NO: 52, SEQ ID NO: 54 SEQ ID NO: 56, SEQ ID NO:58, SEQ ID NO: 60, SEQ ID NO:6 2, SEQ ID NO:6 4 SEQ ID NO: 66, SEQ ID NO:68, SEQ ID NO: 70, SEQ ID NO:72, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 78, SEQ ID NO: 80, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88, SEQ ID NO: 90, SEQ ID NO: 92, SEQ ID NO: 94, SEQ ID NO:
20 96, SEQ ID NO: 98, SEQ ID NO: 100, SEQ ID NO: 102, SEQ ID NO: 104, SEQ ID NO: 106, SEQ ID NO: 108, SEQ ID NO: 110, SEQ ID NO: 112, SEQ ID NO: 114, SEQ ID NO: 116, SEQ ID NO: 118, SEQ ID NO: 120, SEQ ID NO: 122, SEQ ID NO: 124, SEQ ID NO: 126, SEQ ID NO: 128, SEQ ID NO: 130, SEQ ID NO: 132, SEQ ID NO: 134, SEQ ID NO: 136, SEQ ID NO: 138, SEQ ID NO: 140, SEQ ID NO: 142, and SEQ ID NO: 144.

25

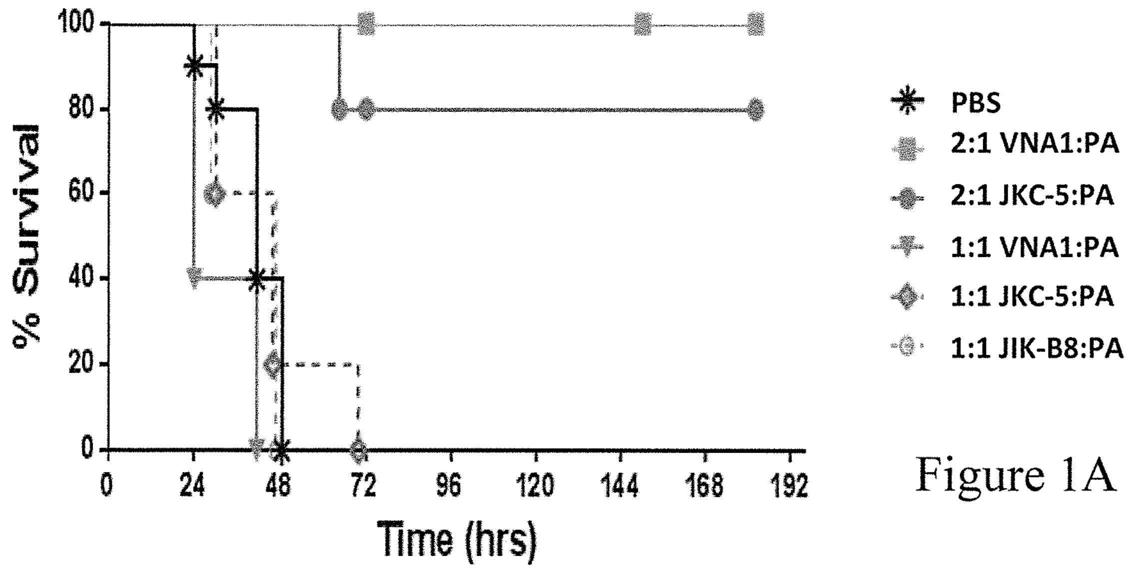


Figure 1A

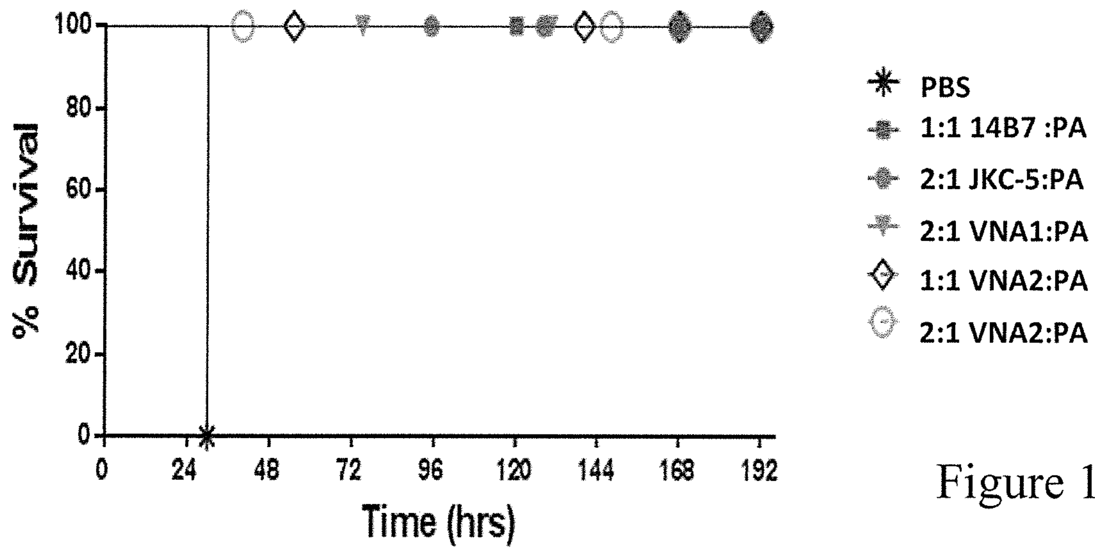


Figure 1B

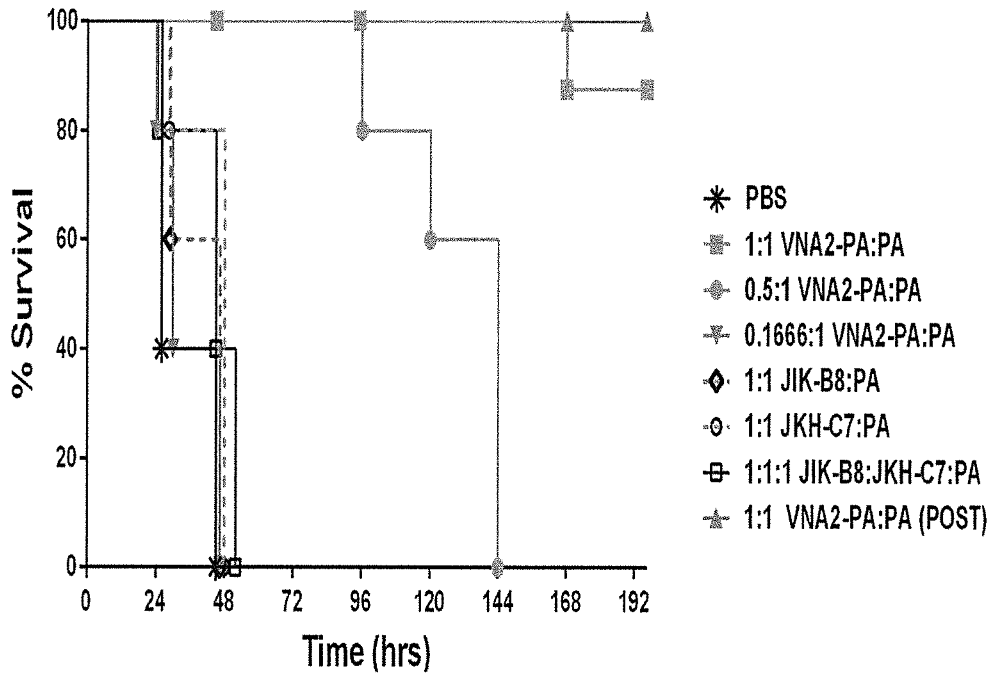


Figure 1C

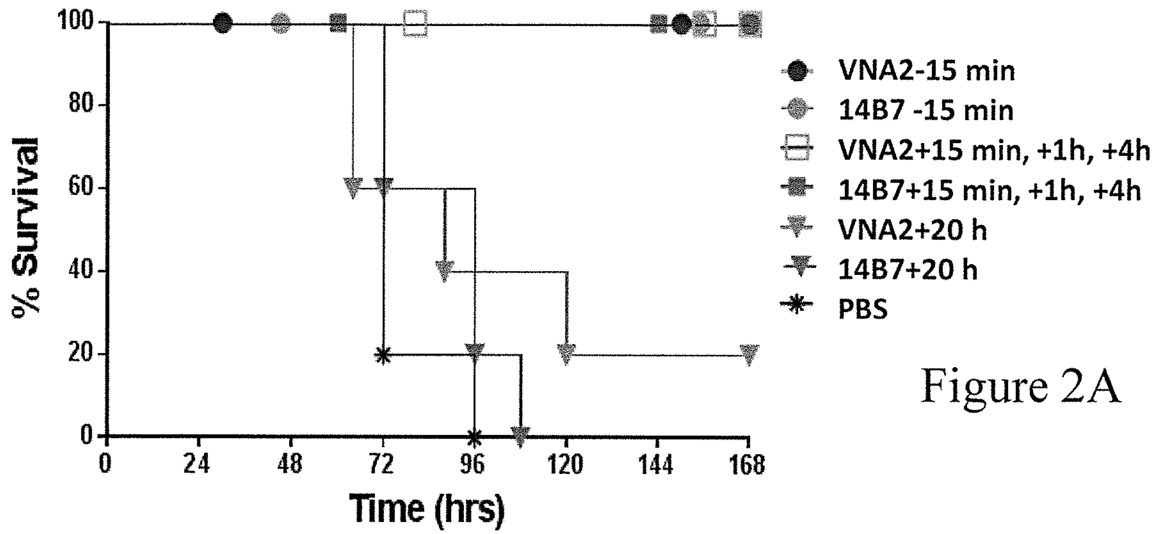


Figure 2A

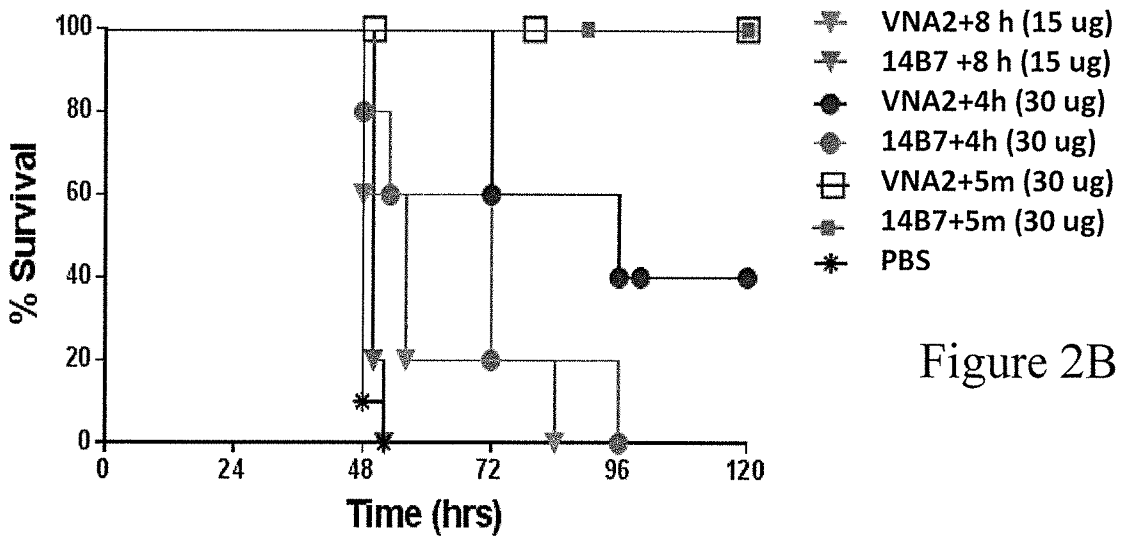


Figure 2B

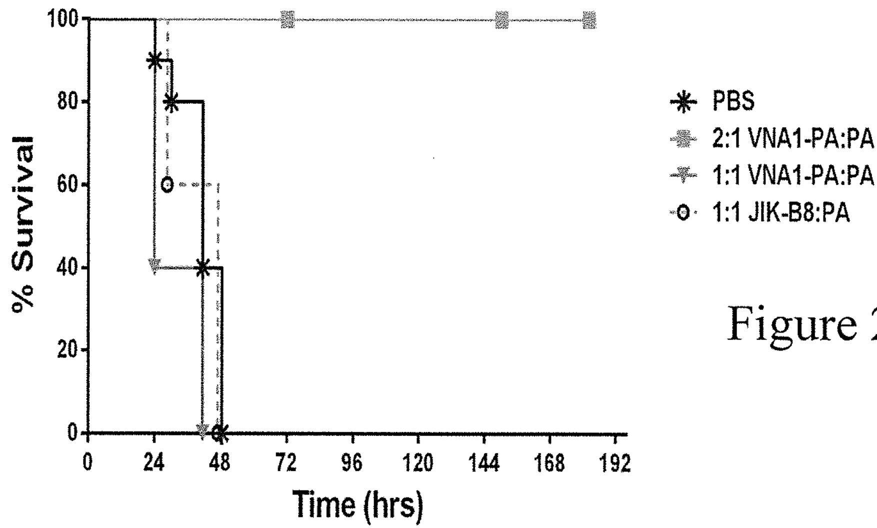


Figure 2C

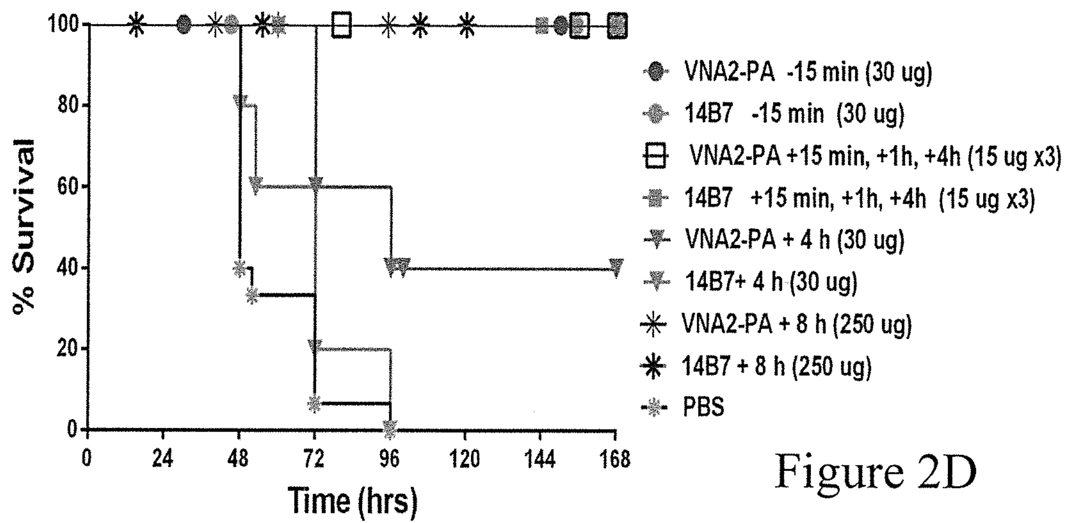


Figure 2D

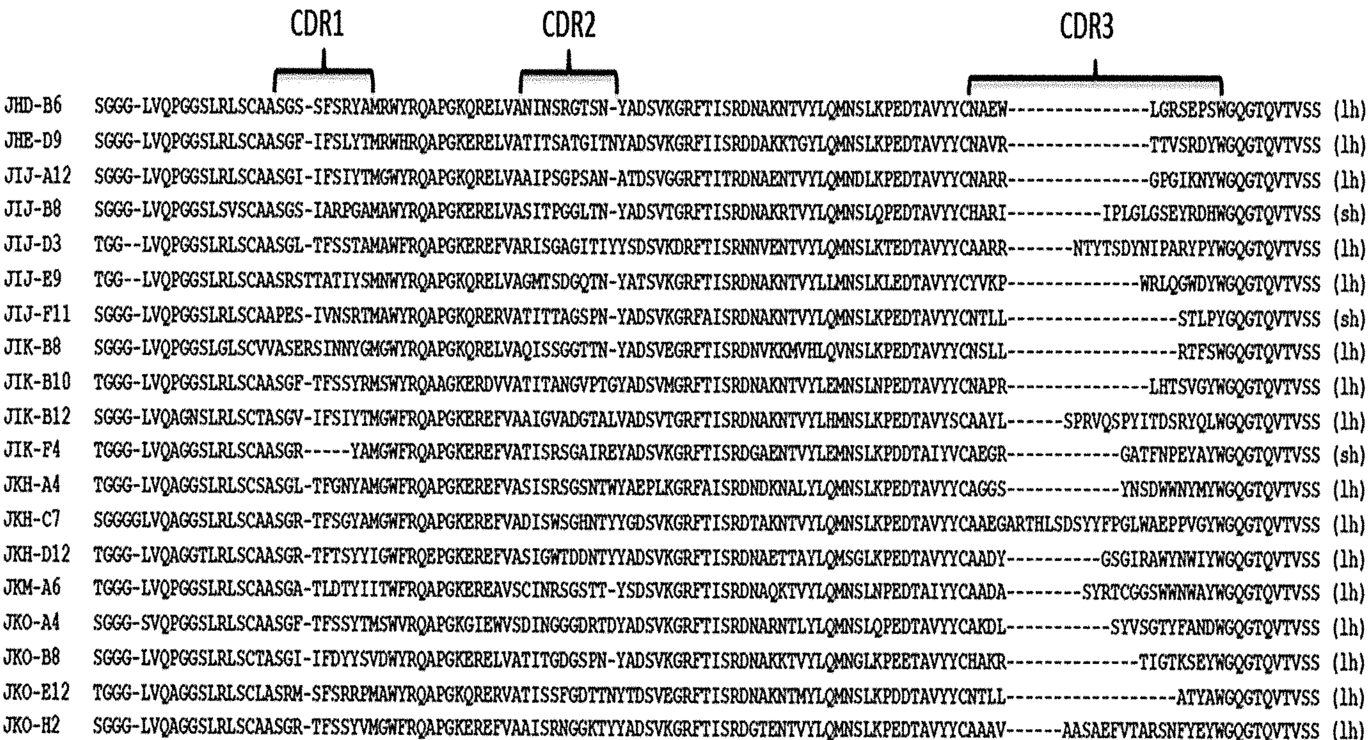


Figure 3

Figure 4A

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDA
NLAGSGSGHMHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMAISDPNS///GAPVPYPDPLEPR///AAAQVQLAESGGGLVQPGGSLGLSCV
VASERSINNYGMGWYRQAPGKQRELVAQISSGGTTNYADSVEGRFTISRDNVKKMVHLQVNSLKPEDTAVYYCNSLLRTFSWGQGTQVTVSSEPKTPKPQAIA///
GGGGSGGGGSGGGGS///LQGQVQLVESGGGLVQPGGSLSVSCAASGSIARPGAMAWYRQAPGKERELVASITPGGLTNYADSVTGRFTISRDNAKRTVYLQMNSL
QPEDTAVYYCHARIIPLGLGSEYRDHWGQGTQVTVSSAHHSEDPSARQ///GAPVPYPDPLEPR///GGGS///DICLPRWGCLWED*

Figure 4B

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDA
NLAGSGSGHMHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMAISDPNS///GAPVPYPDPLEPR///AAAQVQLAESGGGLVQPGGSLGLSCV
VASERSINNYGMGWYRQAPGKQRELVAQISSGGTTNYADSVEGRFTISRDNVKKMVHLQVNSLKPEDTAVYYCNSLLRTFSWGQGTQVTVSSEPKTPKPQAIA///
GGGGSGGGGSGGGGS///LQGQVQLAESGGGGLVQAGGSLRLSCAASGRTFSGYAMGWFRQAPGKEREFVADISWSGHNTYYGDSVKGRFTISRDTAKNTVYLQMN
SLKPEDTAVYYCAAEGARTHLSDSYYFPGLWAEPPVGYWGQGTQVTVSSEPKTPKPQPARQ///GAPVPYPDPLEPR///GGGS///DICLPRWGCLWED*

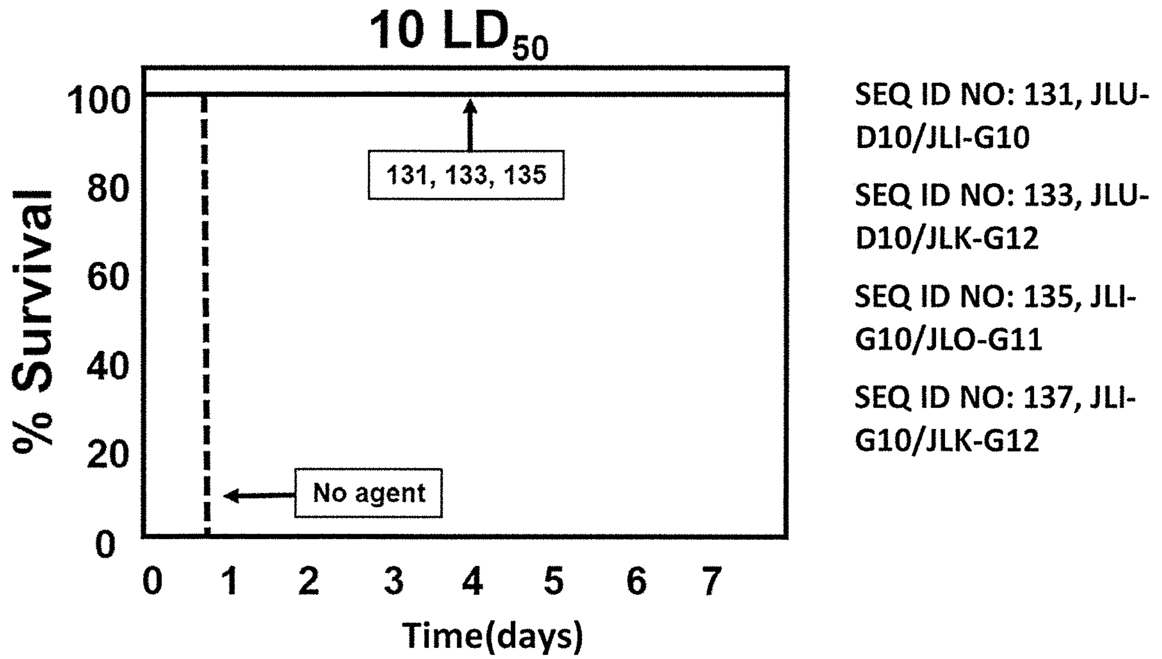


Figure 5A

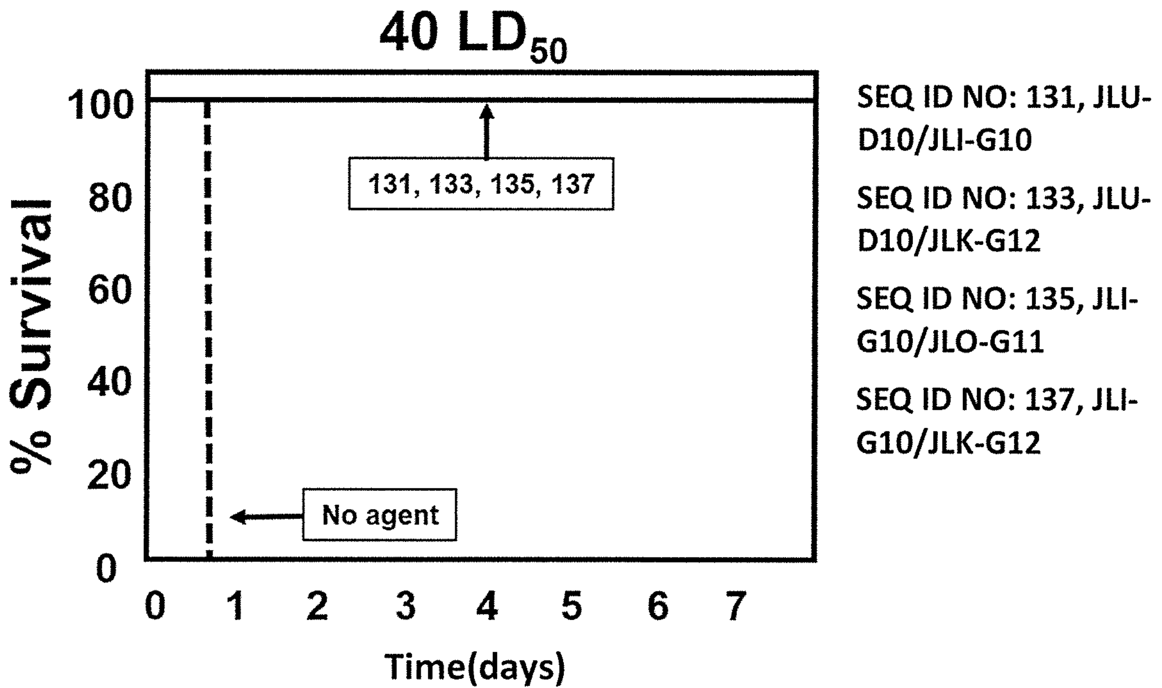
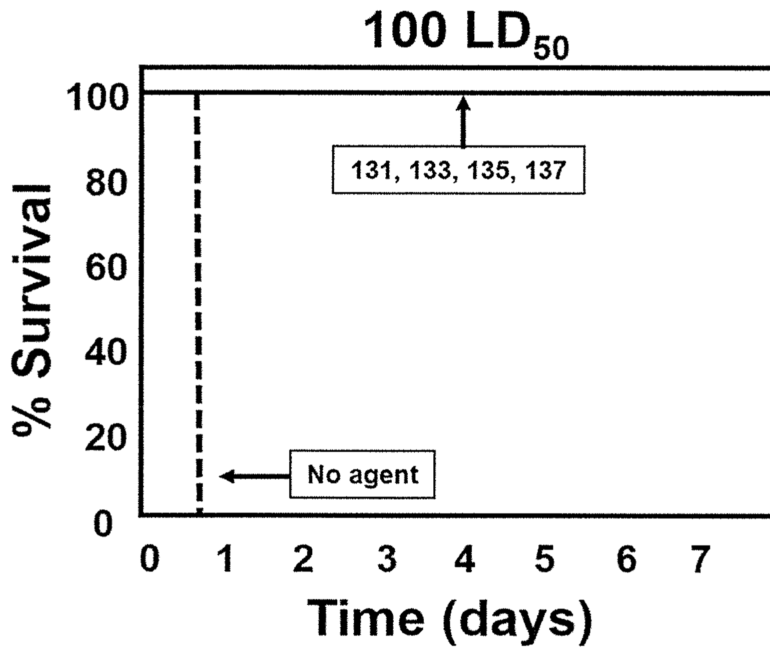


Figure 5B



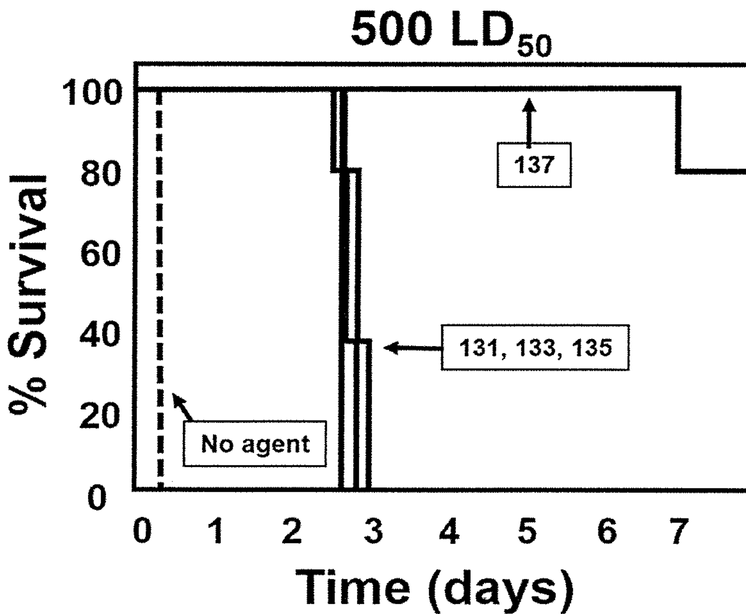
SEQ ID NO: 131, JLU-D10/JLI-G10

SEQ ID NO: 133, JLU-D10/JLK-G12

SEQ ID NO: 135, JLI-G10/JLO-G11

SEQ ID NO: 137, JLI-G10/JLK-G12

Figure 5C



SEQ ID NO: 131, JLU-D10/JLI-G10

SEQ ID NO: 133, JLU-D10/JLK-G12

SEQ ID NO: 135, JLI-G10/JLO-G11

SEQ ID NO: 137, JLI-G10/JLK-G12

Figure 5D

Protein	Clone	K_D (nM)	EC_{50} (nM)	IC_{50} (nM)	C-group	Comments
JHD-B6	JHP-18	66 +/- 2	0.5	10	1	
JHE-D9	JHP-25	13 +/- 1	5	N/A	1	Non-neutralizing
JIJ-A12	JIX-2	45 +/- 8	2	N/A	1	Non-neutralizing
JIJ-B8	JJS-2	8 +/- 8	0.4	N/A	2	Non-neutralizing
JIJ-D3	JIX-7	18 +/- 0.7	0.3	9	1	
JIJ-E9	JIX-10	12 +/- 1	0.4	30	1	
JIJ-F11	JIX-15	200 +/- 20	20	N/A	1	Non-neutralizing
JIK-B8	JIX-20	0.13 +/- 0.06	0.2	1	1	
JIK-B10	JIX-26	9 +/- 1	0.2	8	1	
JIK-B12	JIX-31	12 +/- 3	0.3	6	1	
JIK-F4	JIX-37	7 +/- 1	0.2	3	1	
JKH-A4	JKT-1	9 ± 0.5	0.5	N/A	2	Non-neutralizing
JKH-C7	JKT-3	7 ± 2	2	5	3	
JKH-D12	JKT-5	1 ± 0.4	0.3	N/A	2	Non-neutralizing
JKM-A6	JKT-8	20 ± 5	0.3	N/A	2	Non-neutralizing
JKO-A4	JKT-10	10 ± 2.2	0.3	N/A	2	Non-neutralizing
JKO-B8	JKT-11	200 ± 70	>100	N/A	1	Non-neutralizing
JKO-E12	JKT-13	0.2 ± 0.07	0.1	0.2	1	
JKO-H2	JKT-15	4 ± 1	0.6	N/A	4	Poor binding to PA63
VNA1-PA	JKD-11	ND	ND	0.5		JIK-B8+ JIJ-B8
VNA2-PA	JKU-1	0.07	1.0	0.5		JIK-B8+ JKH-C7

Figure 6

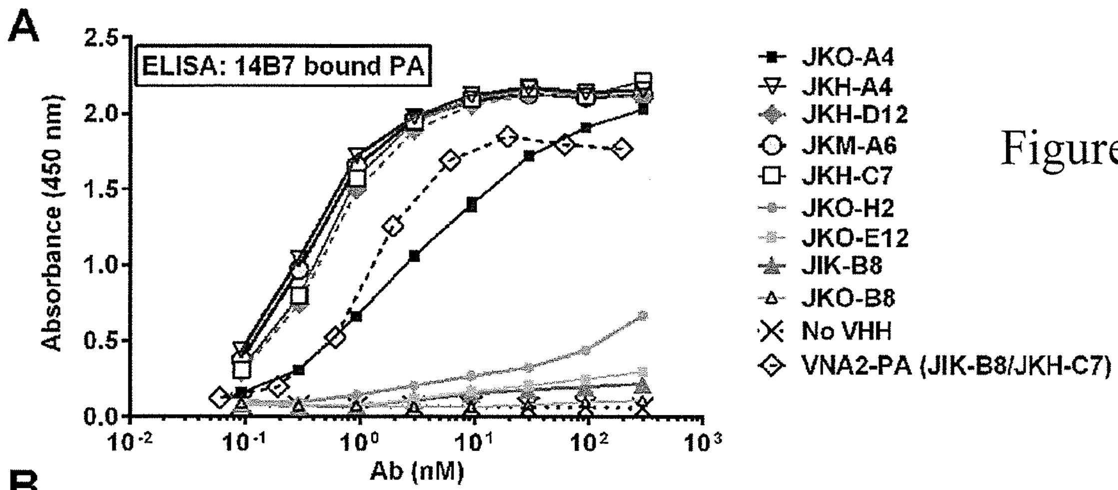


Figure 7A

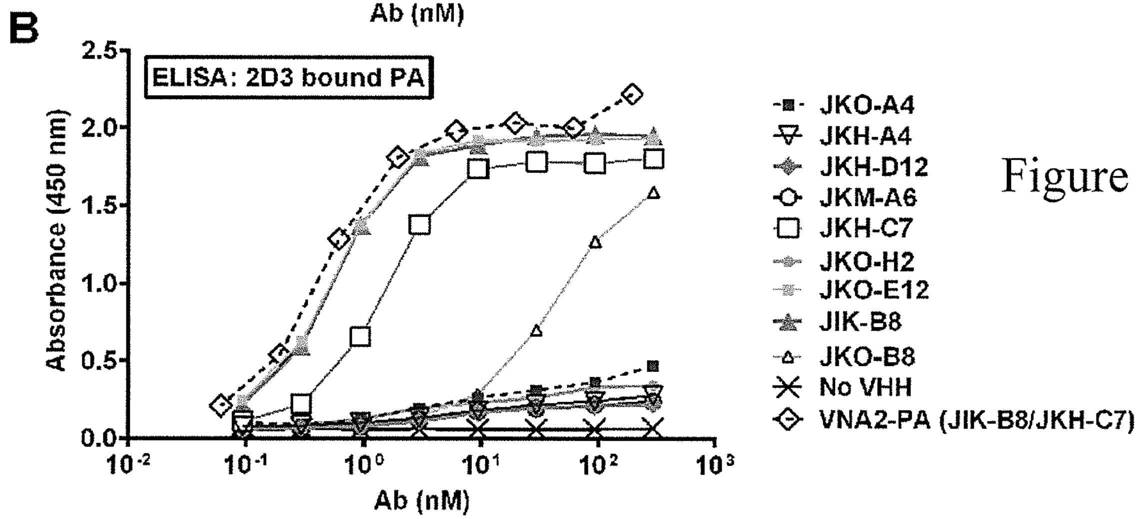


Figure 7B

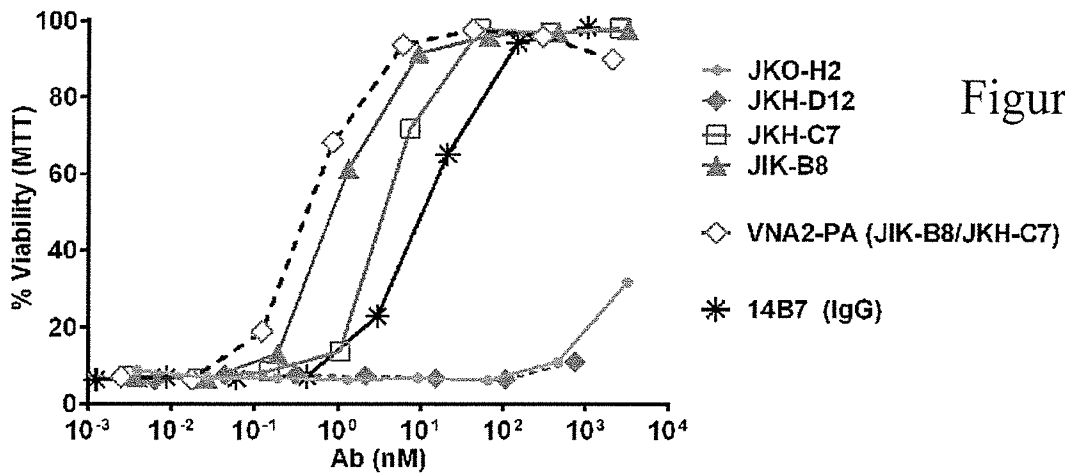


Figure 7C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/64872

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/395, A61K 39/40 (2016.01)
CPC - A61K 39/39591

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 39/395, A61K 39/40 (2016.01)
CPC - A61K 39/39591

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/178.1; 530/358, 530/387.3, 530/391.3, 530/391.5

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
pubWEST; Google Scholar; PatBase
search terms - Aflatoxin, AFB1, B1, antibod*, treat, treat$, therap$, Botulin, Anthrax

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2013/0058962 A1 (SHOEMAKER et al.) 02 October 2014 (02.10.2014) abstract; para [0013].	1-12
A	CN 103866401 A (OIL CROPS RES INST CAAS) 18 June 2014 (18.06.2014) abstract; Claim 8; SEQ ID NO 7.	1-12
A	US 2006/0149041 A1 (SILENCE) 06 July 2006 (06.07.2006) para [0009]; [0019]; SEQ ID NO: 34.	1-12

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 2016	15 APR 2016

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/64872

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1 In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-12, directed to a pharmaceutical composition. The pharmaceutical composition will be searched to the extent that the amino acid sequence encompasses SEQ ID NO: 1 (corresponding to nucleotide sequence SEQ ID NO: 2). It is believed that claims 1-12 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1. Additional amino acid sequences will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected amino acid sequences. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the ?+? group(s) will result in only the first claimed invention to be searched. An exemplary election would be SEQ ID NO: 3 (claims 1-12).

--continued in next supplemental page attached hereto--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-12 limited to SEQ ID NO: 1 and 2

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

continuation of Box No III:

Group II+: Claims 13, 14 and 25, directed to a method for treating a subject. Group II+ will be searched upon payment of additional fees. The method for treating a subject may be searched, for example, to the extent that the amino acid sequence encompasses SEQ ID NO: 1 for an additional fee and election as such. Additional the amino acid sequences will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected the amino acid sequences. Failure to clearly identify how any paid additional invention fees are to be applied to the ?+? group(s) will result in only the first claimed invention to be searched. Another exemplary election would be SEQ ID NO: 3 (claims 13, 14 and 25).

Group III: Claims 15-24, directed to a method of identifying a therapeutic binding protein for treating a subject at risk for exposure to or exposed to at least one disease agent.

The inventions listed as Groups I+, II+ and III do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features
Group I+ includes the special technical feature of a pharmaceutical composition, which is not required by Groups II+ or III.
Group II+ includes the special technical feature of a method for treating a subject at risk for exposure to or exposed to at least one disease agent, which is not required by Groups I+ or III.
Group III includes the special technical feature of a method of identifying a therapeutic binding protein for treating a subject at risk for exposure to or exposed to at least one disease agent, which is not required by Groups I+-II+.

The inventions of Group I+ and II+ each include the special technical feature of a unique amino acid sequence. Each amino acid sequence is considered a distinct technical feature.

Shared technical features
Groups I+, II+ and III include the common technical feature of a binding protein for treating a subject at risk for exposure to or exposed to at least one disease agent. Groups I+ and II+ further share the common technical feature of wherein the binding protein comprises at least one amino acid sequence. However, these shared technical features do not represent a contribution over prior art, because the shared technical features are anticipated by reference US 2013/0058962 A1 to Shoemaker et al., (herinafter Shoemaker).

Shoemaker teaches a binding protein for treating a subject at risk for exposure to or exposed to at least one disease agent (abstract), wherein the binding protein comprises at least one amino acid sequence (para [0013]).

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+, II+ and III inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.