COMPOUNDS AND METHODS FOR THE TREATMENT AND PREVENTION OF BACTERIAL INFECTION

Statement as to Federally Sponsored Research

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Background of the Invention

In general, the invention features compounds and methods for the treatment of bacterial infections, such as anthrax infection.

The etiologic agent of anthrax (*Bacillus anthracis*) is a potential threat as an agent of biowarfare or bioterrorism because exposure to aerosolized *B. anthracis* spores can be lethal to mammals, such as humans. The major virulence factors produced by this organism are the poly-D-glutamic acid capsule and anthrax toxin (ATx). Both the capsule and the toxin assist in colonization and immune evasion by the bacterium. ATx alone can cause death of the host. Vaccination against the toxin protects the host against infection.

Anthrax toxin is a member of the class of bacterial toxins termed A-B toxins. A-B toxins are composed of two moieties; the A moiety is the enzymic portion of the toxin that catalyzes the toxic effect upon a cytoplasmic target within a target cell. The B moiety binds to a cellular receptor and facilitates the translocation of the A moiety across the cell membrane into the cytoplasm of the cell.

The B moieties of A-B toxins from tetanus, botulinum, diphtheria and anthrax all form channels in membranes. It has been hypothesized that these channels might act as the conduit for the membrane translocation of the A moiety. The A and B moieities of anthrax toxin are secreted from the bacterial cell as distinct polypeptides. The A and B subunits of other A-B toxins are produced as single chain polypeptides or as separate chains that are assembled into oligomeric toxins before release from the bacteria. There are two alternative A subunits of anthrax toxin called edema factor (EF) and lethal factor (LF). Noncovalent complexes of EF or LF and the B subunit, protective antigen (PA), are called edema toxin and lethal toxin, respectively. PA facilitates the translocation of both EF and LF across membranes.

PA is secreted as an 83 kDa monomeric polypeptide. Monomeric PA binds to a mammalian cell surface receptor and is proteolytically cleaved. The C-terminal 63 kDa fragment (PA63) remains bound to the cell and the N-terminal 20 kDa (PA20) dissociates from PA63. This proteolytic cleavage and subsequent dissociation of PA20 confer two new properties on PA63: (1) the ability to oligomerize into a ring-shaped heptameric SDS-dissociable structure termed prepore and (2) the ability to bind EF and LF. Oligomers containing PA63-EF, PA63-LF, or a combination of PA63-EF and PA63-LF are endocytosed and trafficked to an acidic compartment, where the PA63 prepore inserts into the membrane and forms a pore. During or after pore formation, EF and LF are translocated across the endosomal membrane into the cytoplasm. EF is a calmodulin-dependent adenylate cyclase which may protect the bacteria from destruction by phagocytes. LF is a metalloprotease that can kill macrophages or, at lower concentrations, induce macrophages to overproduce cytokines, possibly resulting in death of the host.

A crucial step in this intoxication pathway is pore formation by PA. Low pH serves as the trigger for conversion of the PA63 prepore to the pore. This conversion is accompanied by a transformation of the oligomer from an SDS-dissociable to an SDS-resistant state and formation of a transmembrane 14-strand β-barrel. These steps are believed to be necessary for translocation of EF and LF across the endosomal membrane and, thus, toxin action.

Summary of the Invention

In a first aspect, the invention provides a B moiety of a pore-forming binary A-B toxin. The B moiety has a mutation that results in inhibition of its pore-forming ability. In one desirable embodiment, this mutation results in inhibition of the pore-forming ability of the protein *in vivo*. In another desirable embodiment, the mutant B moiety lacks pore-forming ability *in vitro* and/or *in vivo*. In yet another desirable embodiment, the B moiety is anthrax protective antigen (PA). In yet another desirable embodiment, the PA mutant has an amino acid sequence that is at least 80%, 90%, 95% or 98% identical to a naturally-occurring PA protein (such as SEQ ID No.: 21; Fig. 13) and that has one of the following alterations: K397A, K397D, K397C, K397Q, D425A, D425N, D425E, D425K, F427A, K397 + D425K double mutation, K395D + K397D + D425K + D426K quadruple mutation, K397D +D425K + F427A triple mutation, F427A + ΔD2L2 double mutation, K397D + F427A +ΔD2L2 triple mutation, K397D + D425K + F427A +ΔD2L2 quadruple mutation, F427D, or F427K. In another desirable embodiment, the PA mutant has a sequence identical to any of SEQ ID Nos.: 1-11 and 13-18 (Table 1). Other desirable PA mutants include PA mutants in which residue 397 is any amino acid except lysine (SEQ ID No.: 19), PA mutants in which residue 425 is any amino acid except aspartic acid (SEQ ID No.: 20), PA mutants in which

residue 427 is any amino acid except phenylalanine (SEQ ID No.: 23), and PA mutants that have a mutation in domain 2 (residues 259-487). Still other desirable mutant B moieties include *Clostridium difficile*, *C. perfringens*, *C. spiroforme*, *C. botulinum*, *Bacillus cereus*, or *B. thuringiensis* toxins that have one or more of the alterations listed in Table 6 or that have a mutation in the region that corresponds to domain 2 of PA. Fragments of the above mutant B moieties, in which the fragment has a reduced pore-forming ability compared to a naturally-occurring B moiety of the corresponding toxin, are also included in the invention. Fusion proteins having a mutant B moiety of the invention or a fragment of such a mutant B moiety covalently bound to another polypeptide or protein are also included. Specifically excluded from the first aspect of the invention is the deletion of amino acids 302-325 (D2L2 loop) of PA.

In a second aspect, the invention features a vaccine composition having a mutant B moiety of the first aspect or ΔD2L2 PA (a PA mutant in which residues 302-325 are deleted), or a fragment thereof, in a pharmaceutically acceptable carrier. In a desirable embodiment, the vaccine can be inactivated by chemical or physical means.

In a third aspect, the invention features a method of preventing or treating bacterial infection in a mammal, such as a human. This method includes administering the vaccine of the second aspect to the mammal. In one desirable embodiment, the vaccine is administered with a pharmaceutically suitable carrier or an adjuvant. The vaccine can be administered orally, intramuscularly, intravenously, subcutaneously, by inhalation, or by any other route sufficient to provide a dose adequate to prevent or treat a bacterial infection. In another desirable embodiment, a vaccine that includes a mutant anthrax protective antigen is administered for the prevention or treatment of

anthrax infection.

In a fourth aspect, the invention provides a mutant B moiety of a pore-forming binary A-B toxin. The mutant B moiety has a mutation that results in inhibition of its pore-forming ability. The mutant B moiety also inhibits the pore-forming ability of a naturally-occurring B moiety of the corresponding toxin *in vitro* and/or *in vivo*. In one desirable embodiment, this mutation results in inhibition of the pore-forming ability of the protein *in vivo*. In another desirable embodiment, the mutant B moiety lacks pore-forming ability *in vitro* and/or *in vivo*. In yet another desirable embodiment, the B moiety is anthrax protective antigen (PA). The mutant B moiety may bind the A moiety of the corresponding toxin. For example, a PA mutant may bind the lethal factor or edema factor A moieties. The mutant B moiety may compete with a naturally-occurring B moiety for binding to a receptor on the surface of a mammalian cell. The mutant B moiety may also bind a naturally-occurring B moiety of the corresponding toxin. Such a mutant may oligomerize with a naturally-occurring B moiety to form a complex that has reduced ability to form a pore. In one desirable embodiment, the complex lacks the ability to form a pore and to translocate an A moiety (*e.g.*, EF or LF) across the membrane into the host cell cytoplasm.

In one desirable embodiment of the fourth aspect, the mutant PA has an amino acid sequence that is at least 80%, 90%, 95%, or 98% identical to a naturally-occurring PA protein (such as SEQ ID No. 21; Fig. 13) and that has one of the following alterations: K397D + D425K double mutation, ΔD2L2 (in which residues 302-325 of PA are deleted), K395D + K397D + D425K + D426K quadruple mutation, D425K, F427A, K397D +D425K + F427A triple mutation, F427A + ΔD2L2 double mutation, K397D + F427A +ΔD2L2 triple mutation, K397D + D425K + F427A +ΔD2L2 quadruple mutation, F427D, or

F427K In another desirable embodiment, the PA mutant has a sequence identical to any of SEQ ID Nos.: 8-18 (Table 1). In another embodiment, amino acid 395, 397, 425, 426, or a combination thereof, in naturally-occurring PA is mutated. In yet another embodiment, a residue in domain 2 of PA is mutated. In another desirable embodiment, the mutant has a deletion of at least 5, at least 10, or at least 20 amino acids of the residues in the D2L2 loop of PA or in the corresponding region of a B moiety of another pore-forming binary A-B toxin. The mutant can have a deletion of all or part of the D2L2 loop and a deletion of amino acids that are N– or C-terminal to the loop. Still other desirable mutant B moieties include *Clostridium difficile*, *C. perfringens*, *C. spiroforme*, *C. botulinum*, *Bacillus cereus*, or *B. thuringiensis* toxins that have one or more of the alterations listed in Table 6 or that have a mutation in the region that corresponds to domain 2 of PA. Fragments of the above mutant B moieties, in which the fragment has a reduced pore-forming ability compared to a naturally-occurring B moiety and inhibits the pore-forming ability of a naturally-occurring B moiety, are included in the invention. Fusion proteins having a mutant B moiety or a fragment of a mutant B moiety covalently bound to another polypeptide or protein are included.

In a fifth aspect, the invention features a method of preventing or treating bacterial infection in a mammal, such as a human. This method includes administering a mutant B moiety of the fourth aspect, or a fragment thereof, that inhibits the pore-forming ability of a naturally-occurring B moiety to the mammal. In one embodiment, a PA mutant of the fourth aspect or a fragment thereof is administered to prevent or treat anthrax infection in mammals that have been exposed to *B. anthracis* spores. In another embodiment, the protein is administered prophylactically. In one desirable embodiment, the mutant B moiety is administered with a pharmaceutically

suitable carrier. The mutant may be administered orally, intramuscularly, intravenously, subcutaneously, by inhalation, or by any other route sufficient to provide a dose adequate to prevent or treat an anthrax infection. In one embodiment, the method also includes administering an anti-B moiety antibody, such as an antibody that binds a naturally-occurring B moiety but not the dominant negative mutant B moiety, to the mammal. In one particular embodiment, the antibody binds a naturally-occurring PA but not the dominant negative PA mutant.

In a sixth aspect, the invention features a nucleic acid encoding a mutant B moiety (*e.g.*, a PA mutant) of the first or fourth aspects.

In a seventh aspect, the invention features a vector having the nucleic acid of the sixth aspect.

In an eighth aspect, the invention features a purified antibody that specifically binds a naturally-occurring PA or a PA mutant protein listed in Table 1. In one embodiment, the antibody binds to the D2L2 loop, K397, D425, D426, or F427 of a PA protein. The antibody may be a monoclonal or polyclonal antibody. In a related aspect, the invention features a purified antibody that specifically binds a naturally-occurring B moiety of a pore-forming binary A-B toxin with greater affinity than it binds a B moiety of the present invention from the corresponding toxin. In another related aspect, the invention features a purified antibody that specifically binds a B moiety of the present invention with greater affinity than it binds a naturally-occurring B moiety of the corresponding toxin.

In one embodiment of the first or fourth aspects, specifically excluded are those PA molecules having as their sole alteration, a mutation to cysteine of a residue located in the hydrophilic face of a transmembrane pore. In another embodiment of these aspects, PA molecules having as their sole

alteration a mutation in an amino acid in the hydrophilic face of a transmembrane pore are specifically excluded. In a embodiment, specifically excluded from the first or fourth aspects are those PA molecules having as their sole alteration, a mutation in Glu302, His304, Asn306, Glu308, His310, Ser312, Phe313, Phe314, Asp315, Gly317, Ser319, Ser321, Gly323, or Ser325 in naturally-occurring PA. In one embodiment of these aspects, PA molecules having as their sole alteration, a mutation in the amino acid sequence of the D2L2 loop are specifically excluded. In another embodiment, PA molecules having as their sole alteration a mutation or deletion in an amino acid that forms the transmembrane pore are specifically excluded. In still another embodiment, specifically excluded is ΔD2L2 PA. In yet another embodiment, specifically excluded from one of these aspects are PA molecules having the C-terminal 63 kDA tryptic fragment (PA63) and having as their sole alteration a mutation in an amino acid that forms the transmembrane pore. In various other embodiments, specifically excluded from one of these aspects are other pore-forming binary A-B toxins that have a mutation that corresponds to a specifically excluded PA mutation. In various embodiments of these aspects, the mutant B moiety binds a naturally-occurring B moiety from the same A-B toxin with a higher equilibrium constant (*i.e.*, with greater affinity) than the naturally-occurring B-moiety binds other naturally-occurring molecules from the same A-B toxin. In desirable embodiments, the mutant PA protein binds naturally-occurring PA with at least 2, 5, 10, or 20 fold greater affinity than naturally-occurring PA binds other naturally-occurring PA molecules.

It should be understood that other pore-forming toxins, in addition to anthrax toxin, may be used in the compounds and methods of the invention. For example, pore-forming toxins, such as other A-B toxins, having mutations (*e.g.*, point mutations or deletion mutations) that inhibit the pore-forming

ability of the toxin or that inhibit the pore-forming ability of the naturally-occurring toxin are included in the invention. The pore-forming toxins with these mutants can be used in the vaccine compositions or methods of the invention to prevent or treat infection by the etiologic agent of the toxin. While not meant to limit the invention in any way, other A-B binary toxins; hetero-oligomeric toxins (AB5 toxins), such as cholera toxin; or single polypeptide A-B toxins, such as tetanus, botulinum, or diphtheria toxin can be used. Other toxins that can be used include α-hemolysin from *Staphylococcus aureus*, aerolysin from *Aeromonas hydrophila*, α-toxin from *Clostridium septicum*, and cytotoxin from *Pseudomonas aeruginosa*. The invention is also relevant to any other pore-forming toxin such as cholesterol dependent cytolysins, hexameric toxins, or heptameric toxins. Examples of hexameric and heptameric toxins include toxins that are related to the Staphylococcal α-toxin. In one embodiment, a deletion mutant of the VacA toxin from *Helicobacter pylori* is specifically excluded.

"Mutation" means an alteration in the nucleic acid sequence such that the amino acid sequence encoded by the nucleic acid sequence has at least one amino acid alteration from a naturally-occurring sequence. The mutation may, without limitation, be an insertion, deletion, frameshift mutation, or missense mutation.

"Pore-forming toxin" means a toxin which forms a transmembrane aqueous pore.

"Pore-forming A-B toxin" means a pore-forming toxin with two functional moieties; one moiety (B) which forms a pore in a host cell barrier membrane, and the other (A) traverses the membrane barrier and enzymatically modifies specific intracellular substrates of a host cell.

"Pore-forming binary A-B toxin" means a pore-forming A-B toxin in which the A and B moieties of the pore-forming toxin inhabit separate proteins, and interact during the intoxication of host cells. An example of a binary toxin is anthrax toxin.

"B moiety" means the component of a pore-forming A-B toxin which binds a specific host cell-surface receptor, interacts with the A moiety of the toxin, and aids in internalization of the A moiety into the cell. Many B moieties, such as PA, also form transmembrane pores.

"Protective antigen (PA)" means a polypeptide having at least 60%, 70%, 80%, or 90%, of at least one of the biological activities of the anthrax PA polypeptide described herein. The polypeptide may be encoded by the PA gene that was reported by Vodkin *et al.* (Cell 34:693-697, 1983). The polypeptide can be identical to wild-type PA characterized by Miller *et al.* (Biochemistry 38(32):10432-10441, 1999) (SEQ ID No.: 21) or any naturally-occurring PA polypeptide from a strain of *Bacillus anthracis*. The PA polypeptide may be cloned and expressed in a heterologous host, such as *Escherichia coli* or *Bacillus subtilis.* It is understood that homologs and analogs have the characteristics of the anthrax PA described herein and may be used in the methods of the invention.

"PA63" means the carboxy-terminal portion that results from proteolytic cleavage of a 20 kDa N-terminal segment from the PA polypeptide. PA63 forms a heptameric prepore and binds the two alternative A moieties, edema factor (EF) and lethal factor (LF). The entire complex is trafficked to the endosome, where PA63 inserts into the membrane, forms a transmembrane pore, and translocates EF and LF into the host cell cytoplasm.

"Transmembrane pore" means a transmembrane aqueous channel. For example, the transmembrane pore can be a β-barrel channel formed by alternating hydrophilic and hydrophobic residues of PA63 such that the hydrophobic residues form an exterior membrane-contiguous surface of the barrel, and the hydrophilic residues face an aqueous lumen of a pore that spans across the host cell membrane.

"Hydrophilic face of a transmembrane pore" means the amino acids of PA that face the aqueous lumen of a pore that spans across the host cell membrane.

"An amino acid that forms the transmembrane pore" means an amino acid of PA that is located in a β-barrel channel of a transmembrane pore.

"D2L2 loop" means the amphipathic loop which connects strands 2β2 and 2β3 of PA polypeptide and PA63 polypeptide as described herein.

"Inhibits the pore-forming ability" means reduces the amount of pores formed in membranes or reduces the rate or amount of an A moiety (*e.g.*, EF or LF) that is translocated into the host cell cytoplasm. This decrease in pore formation or toxin translocation is positively correlated with, and could be predicted by, a decrease in activity in the cell surface translocation, LFnDTA toxicity, or rubidium release assays described herein. This decreased activity can be correlated with a decrease in the amount of a radiolabeled ligand that is translocated into cells in the cell surface translocation assay, a decrease in the inhibition of protein synthesis due to the translocation of a ligand into cells in the LFnDTA toxicity assay, or a decrease in the release of radiolabeled ions from cells in the rubidium release assay. Additionally, this decreased activity can be correlated with a decrease in toxicity due to the translocation of a toxic ligand into cells. In one desirable embodiment, the decrease in pore formation

or translocation of an A moiety is at least 20%, more desirably at least 40%, and most desirably at least 80% relative to a naturally-occurring B moiety of the corresponding toxin. In another desirable embodiment, the decrease in pore formation or translocation of EF or LF by a PA mutant is at least 20%, more desirably at least 40%, and most desirably at least 80% relative to naturally-occurring PA63

"Lacks pore-forming ability" means does not form a significant amount of pores in membranes or does not transfer a significant amount of EF or LF into the host cell cytoplasm. This lack of significant pore-forming or toxin translocating activity is positively correlated with, and could be predicted by, a lack of significant activity in the cell surface translocation, LFnDTA toxicity, or rubidium release assays described herein. In one desirable embodiment, the amount of pores formed or the amount of toxin translocated is less than 5 times the amount detected in a control assay without PA. More desirably, the amount is less than 2 times the amount in a control assay without PA.

"Fragment" means polypeptide having a region of consecutive amino acids that is identical to the corresponding region in a PA mutant. The fragment has either a reduced ability to form pores or translocate toxins compared to naturally-occurring PA. The fragment may also inhibit the pore-forming ability of naturally-occurring PA. This decrease in pore formation or toxin translocation is positively correlated with, and could be predicted by, a decrease in activity in the cell surface translocation, LFnDTA toxicity, or rubidium release assays described herein. This decreased activity can be correlated with a decrease in the amount of a radiolabeled ligand that is translocated into cells in the cell surface translocation assay, a decrease in the inhibition of protein synthesis due to the translocation of a ligand into cells in

the LFnDTA toxicity assay, or a decrease in the release of radiolabeled ions from cells in the rubidium release assay. In one desirable embodiment, the decrease in pore formation or translocation of EF or LF is at least 20% relative to naturally-occurring PA63. More desirably, the decrease is at least 40%, and most desirably, the decrease is at least 80%. The inhibition of the pore-forming ability of naturally-occurring PA is positively correlated with, and could be predicted by, a decrease in activity in an assay described above using an equimolar mixture of naturally-occurring PA and a PA fragment compared to using naturally-occurring PA alone. In one desirable embodiment, the decrease is at least 20, 40, 60, 80, or 99% compared to the activity using only naturally-occurring PA. Desirably, the fragment is immunogenic and induces the production of protective antibodies against naturally-occurring PA. In another desirable embodiment, the administration of the fragment to a mammal, as described in Example 9, prevents or diminishes an anthrax infection for a period of at least 1 month, more desirably 3 months, or most desirably 6 months. Examples of possible fragments include the C-terminal 63 kDA tryptic fragment of a PA mutant or a PA mutant having a deletion of amino acids that form the transmembrane pore.

By "purified antibody" is meant an antibody which is at least 60%, by weight, free from proteins and naturally-occurring organic molecules with which it is naturally associated. Desirably, the preparation is at least 75%, more desirably 90%, and most desirably at least 99%, by weight, antibody. A purified antibody may be obtained, for example, by affinity chromatography using recombinantly-produced protein or conserved motif peptides and standard techniques.

By "specifically binds" is meant an antibody that recognizes and binds to, for example, wild-type PA or a PA mutant but does not substantially recognize and bind to other non-PA molecules in a sample, *e.g.*, a biological sample, that naturally includes protein. A desirable antibody specifically binds any of the PA mutants # 1-18 in Table 1. Other desirable antibodies bind wild-type PA with at least 2, 5, 10, or 20 fold greater affinity than they bind one or more of the PA mutants in Table 1.

Sequence identity is typically measured using sequence analysis software with the default parameters specified therein (*e.g.*, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This software program matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Other features and advantages of the invention will be apparent from the following detailed description.

Brief Description of the Drawings

Fig. 1 is a schematic illustration of the intoxication pathway for ATx toxin. The PA component of ATx binds to a receptor on the surface of mammalian cells and delivers the enzymic A moieties of the toxin, edema factor (EF) and lethal factor (LF), to the cytosol, as described above.

Fig. 2A is a picture of SDS-PAGE gels showing the formation of nicked PA mutant proteins and the formation of SDS-resistant oligomers by wild-type, K397Q, and ΔD2L2 PA. Fig. 2B is a picture of a native gel showing the formation of prepores by wild-type, K397A, and D425A PA.

Fig. 3 is a bar graph showing the amount of ^{86}Rb released from ^{86}RB loaded cells after incubation with wild-type, K397A, or D425A PA compared to the no PA control.

Fig. 4A is a bar graph showing the similar level of ^{35}S-LFn (N-terminal 1-255 amino acid PA binding domain of LF) binding by cells that have been incubated with wild-type, K397A, or D425A PA. Fig. 4B is a graph showing the reduction in translocation of ^{35}S-LFn into cells that is mediated by K397A or D425A PA compared to wild-type PA.

Fig. 5 is a graph showing the percent of ^{3}H-Leu in the TCA insoluble fraction (protein fraction) after incubation of cells with wild-type, K397A, or D425A PA in the LFnDTA toxicity assay. Translocation of LFnDTA, which contains LFn fused to the A-chain of diptheria toxin, into the cell leads to ribosylation of EF-2, resulting in the inhibition of protein synthesis and a decrease in the amount of ^{3}H-Leu in the protein fraction.

Fig. 6A is a bar graph showing the similar binding of ^{35}S-LFn to cells incubated with wild-type, ΔD2L2, the double mutant K397D + D425K, or a mixture of wild-type and ΔD2L2 or K397D + D425K PA. Fig. 6B is a bar graph showing the reduction of wild-type PA-mediated translocation of ^{35}S-LFn by ΔD2L2 or K397D + D425K PA.

Fig. 7 is a graph showing the higher percent of ^{3}H-Leu in the TCA insoluble fraction after incubation of ΔD2L2 or K397D + D425K PA and wild-type PA compared to wild-type PA alone. This result corresponds to a decrease in wild-type PA-mediated inhibition of protein synthesis in the

LFnDTA toxicity assay.

Fig. 8A is a graph showing the decrease in wild-type PA-mediated inhibition of protein synthesis in the LFnDTA toxicity assay. Increasing concentrations of mutant PA proteins relieve the wild-type PA-mediated inhibition of ^{3}H-Leu uptake into the TCA insoluble fraction. Fig. 8B is a graph showing that much higher amounts of PA-SSR relative to wild-type PA are required to relieve the wild-type PA-mediated inhibition of ^{3}H-Leu uptake compared to the amounts required for the mutants listed in Fig. 8A.

Fig. 9 is a graph showing the decrease in wild-type PA-mediated inhibition of protein synthesis in the LFnDTA toxicity assay due to the presence of increasing concentrations of a dominant negative PA mutant. The effect of the dominant negative mutants K397D + D425K (□), ΔD2L2 (■), F427A (○), D425K (Δ), and K397D (◊) and the control mutant SSSR (♦) are shown in this figure.

Fig. 10 is a graph showing the decrease in wild-type PA-mediated inhibition of protein synthesis in the LFnDTA toxicity assay due to the presence of increasing concentrations of one of the following dominant negative PA mutants: K397D + D425K (■), F427A + ΔD2L2 (□), K397D + D425K + F427A (○), and K397D + F427A + ΔD2L2 (▲).

Fig. 11 is a bar graph showing the inhibition of protein synthesis by a hetero-heptamer formed by mixing wild-type PA with a mutant PA (K397D + D425K, ΔD2L2, F427A, or D425K) and then cleaving the PA molecules with trypsin. Inhibition of protein synthesis by an equivalent amount of a 1:1 mixture of the corresponding mutant and wild-type homo-heptamers was also measured.

Fig. 12 is a bar graph showing the effect of the dominant negative mutants K397D + D425K, ΔD2L2, F427A, and D425K on the low-pH triggered translocation of ^{35}S LFN across the plasma membrane. The results presented are the mean of three experiments ± SEM.

Fig. 13 is the amino acid sequence of wild-type PA protein used for the assays described herein (SEQ ID No.: 21). The PA mutant proteins described herein are based on this wild-type sequence.

Fig. 14 is the polynucleotide sequence encoding the wild-type PA protein used for the assays described herein (SEQ ID No.: 22).

Fig. 15 is an alignment of the amino acid sequence of PA with other binary A-B toxins that have ADP ribosyltransferase activity. The amino acid sequences of toxins from *Clostridium difficile* ("cdADPRT"), *C. perfringens* ("cpiota"), *C. spiroforme* ("csiota'), and *C. botulinum* ("cbc2") are listed. The *C. perfringens* and *C. spiroforme* toxins are frequently referred to as iota toxins while the botulinum toxin is referred to as C2. Additionally, the alignment includes the sequence of the toxin produced by *Bacillus cereus* ("VIP1"), which is frequently referred to as VIP for vegetative insecticidal protein.

Fig. 16 is an alignment of the amino acid sequence of PA with the amino acid sequences of toxins from *Clostridium difficile* ("cdADPRT"), *C. perfringens* ("cpiota"), *C. spiroforme* ("csiota'), and *C. botulinum* ("cbc2"). This alignment shows the complete sequences of the toxins.

Detailed Description

We have found a means by which infection by A-B toxin producing bacteria can be halted. Thus, the invention provides a composition for use as an antidote to particular bacterial infections, including anthrax and gangrene. Because the composition is safe and immunogenic, it may also be used as a

vaccine.

The multiple mutants of anthrax PA were constructed, expressed, purified, and assayed to determine whether they have reduced activity compared to wild-type PA. In particular, these mutants were assayed for the ability to bind PA ligands and receptors; to form prepores, SDS-resistant oligomers, and pores; and to translocate ligands across membranes. Based on the x-ray structure of PA, the mutated residues are predicted to project into the lumen of the PA prepore. PA mutants, or fragments thereof, with reduced or no detectable ability to form pores in membranes can be used as vaccines for the induction of protective antibodies to prevent anthrax infection. In addition, these mutants might be more effective than wild-type PA in treating anthrax infection because of their reduced ability to translocate EF and LF secreted by *Bacillus anthracis* in the infected mammal.

These point mutants and the previously reported deletion mutant lacking residues 302-325 of putative membrane spanning loop 2 of domain 2 (ΔD2L2) (Miller *et al.*, Biochemistry 38:10432-10441, 1999) were further characterized to determine whether they could act as dominant negative inhibitors by reducing the pore formation of wild-type PA. This inhibition could result from the binding of ligands or receptors by the mutants so that fewer molecules were available for wild-type PA to bind. The mutants could also form oligomers with wild-type PA that have reduced or no detectable ability to form pores and translocate ligands. Dominant negative PA mutants, and fragments thereof, could be used as vaccines to elicit protective antibodies for the prevention or treatment of anthrax infection, as described above. Additionally, mutants or fragments with dominant negative activity could be used as therapeutics to treat anthrax infection by inhibiting the activity of PA secreted by *Bacillus anthracis* in the infected mammal. Because dominant negative mutants can

induce the production of protective antibodies and inhibit the activity of PA produced by the infecting bacteria, they can be used as a combination vaccine/therapeutic that is particularly effective in treating individuals suffering from, or at risk of developing, anthrax infection. Besides the need to abrogate toxin action as quickly as possible, it is also important to vaccinate individuals who have been exposed to aerosolized *B. anthracis* spores. This vaccination is essential to guard against delayed contraction of anthrax by germination of spores that can remain in the body for prolonged periods (at least a month).

In this study, several mutants of PA were identified that lack the ability to form pores in membranes and translocate ligands and, thus, are potential vaccines for the prevention or treatment of anthrax infection (Table 1). Mutants # 1-12 were able to be proteolytically activated, to form the SDS-dissociable PA63 prepore state, and to bind a cellular receptor, EF, and LF. Some of the mutations prevented the conversion of the prepore to an SDS-resistant state (Table 1). These mutants (K397A, K397C, K397D, D425A, D425N, D425K D425E, D425K, K397D + D425K, and K395D + K397D + D425K + D426K) are also defective in pore formation and membrane translocation. The other class of mutants (ΔD2L2 PA, K397Q, and F427A) forms SDS-resistant oligomers but does not undergo membrane insertion and pore formation. These results were unexpected.

Table 1. Activity of Protective Antigen Mutants

Mutant #	SEQ ID No.	Mutation	Forms SDS-resistant oligomer?	Forms channels?	Dominant negative?
1	1	K397A	No	No	No
2	2	K397D	No	No	No
3	3	K397C	No	No	No
4	4	K397Q	Yes	No	No
5	5	D425A	No	No	No
6	6	D425N	No	No	Not determined
7	7	D425E	No	No	No
8	8	D425K	No	No	Yes
9	9	F427A	Yes	No	Yes
10	10	K397D + D425K	No	No	Yes
11	11	K395D + K397D + D425K + D426K	No	No	Yes
12	12	ΔD2L2	Yes	No	Yes
13	13	K397D + D425K + F427A	Not determined	Not determined	Yes
14	14	F427A + ΔD2L2	Not determined	Not determined	Yes
15	15	K397D + F427A + ΔD2L2	Not determined	Not determined	Yes
16	16	K397D + D425K + F427A + ΔD2L2	Not determined	Not determined	Yes
17	17	F427D	Not determined	Not determined	Yes
18	18	F427K	Not determined	Not determined	Yes

These PA mutants were constructed as described in Example 1.

Several of the mutants (ΔD2L2, K397D + D425K double mutant, K395D+ K397D + D425K + D426K quadruple mutant, D425K, F427A, K397D +D425K + F427A triple mutant, F427A + ΔD2L2 double mutant, K397D + F427A +ΔD2L2 triple mutant, K397D + D425K + F427A +ΔD2L2 quadruple mutant, F427D, and F427K) inhibit the wild-type PA-mediated translocation of ligands across membranes. The ΔD2L2 and K397D + D425K PA mutants were shown to form oligomers with wild-type PA that are unable to translocate ligands. These results were unexpected. The presence of a single molecule of these mutants within a heptameric prepore may be sufficient to block conversion to the pore. This ability to block the pore formation by wild-type PA, coupled with the ability to compete with wild-type PA for the binding of cellular receptors and to remove EF and LF from circulation, makes these mutants particularly attractive for use in the treatment and prevention of anthrax infection.

Mutation of other residues in PA could also inhibit pore formation or produce dominant negative activity. For example, residues that electrostatically interact with the charged side-chains of Lys397 or Asp425 may also be required for pore formation by PA, and the mutation of one or a combination of these residues may inhibit pore formation and result in dominant negative activity. Additionally, the deletion of smaller portions of the 302-325 D2L2 loop or the deletion of amino acids flanking the loop and part or all of the 302-325 region could produce these results.

The ability to obtain mutants of PA with no detectable ability to form pores or translocate ligands and mutants that serve as dominant negative inhibitors of wild-type PA suggests that similar mutants could be obtained in other toxins, such as α-hemolysin from *Staphylococcus aureus*, aerolysin from *Aeromonas hydrophila*, α-toxin from *Clostridium septicum*, cytotoxin from

Pseudomonas aeruginosa, hetero-oligomeric toxins (AB5 toxins), or in the B moieties of tetanus, botulinum, or diphtheria toxins. Additionally, these results underscore the possibility of identifying dominant negative forms of a number of other oligomeric virulence factors, ranging from toxins to adhesins.

In anthrax toxin and other oligomeric systems in which the assembly process occurs in contact with the extracellular milieu, exogenously added mutant subunits can in principle be incorporated into the final structure, raising the possibility that such subunits could be used therapeutically. Systemic anthrax, although rare as a natural disease, is feared as an agent of biological warfare and terrorism, and dominant negative PA would seem to be a worthy candidate for a therapeutic. Assuming that administered dominant negative PA intermixes freely with wild-type PA produced in the body by *B. anthracis*, the proteins should co-assemble on cells to form inactive, dead-end complexes, thereby blocking the actions of both LF and EF. Besides preventing overt symptoms, dominant negative mutants may also protect professional phagocytes from destruction, thereby aiding the host in eradicating the infection. No significant side effects have been observed following injection of wild-type PA into humans, and thus a mutant inactive form of the protein should pose no hazard.

Dominant negative PA may also be useful as a basis for a new vaccine against anthrax. As its name connotes, PA induces protective antibodies against anthrax, and indeed is the major immunogen of the vaccine currently licensed in the United States. The ΔD2L2, K397D + D425K, and F427A mutants described herein exhibit little or no diminution in immunogenicity relative to wild-type PA in Fisher rats. We have also found mutants that are unexpectedly dominant negative, such that administration of a 0.25:1 ratio of mutant to wild-type PA did not result in any detectable symptoms of anthrax

infection in a rat model. Purified wild-type PA is under consideration as a replacement for the currently licensed vaccine, and if a dominant negative form of PA proves efficacious therapeutically, it might fulfill this role as well, eliminating the need to develop two almost identical pharmaceuticals.

The following examples are to illustrate the invention. They are not meant to limit the invention in any way. Unless otherwise noted, the data for the K397A and D425A PA mutants is representative of the data obtained for PA mutants number 1-12 listed in Table 1.

Example 1: General methods

Cell culture, media and chemicals

Chinese hamster ovary-K1 (CHO-K1) cells were obtained from American type culture collection. The cells were grown in HAM's F-12 supplemented with 10% calf serum, 500 units/mL penicillin G, 2 mM L-glutamine and 500 units/mL streptomycin sulfate and maintained at 5% CO_2 in a humidified atmosphere. Cells were seeded into 24- or 96-well microtiter plates (Costar, Cambridge, MA) 16 - 18 hours prior to the experiment. All media for cell culture was obtained from Gibco BRL unless noted otherwise. All chemicals were obtained from Sigma Chemical Co. unless specified.

Construction and purification of PA proteins

The ΔD2L2 PA mutant, which does not contain amino acids 302-325 of PA, was expressed and purified as described previously (Miller *et al.*, Biochemistry 38:10432-10441, 1999). The point mutations # 1-11 from Table 1 were constructed using the QuickChange method of site directed mutagenesis, following the manufacturer's protocol (Stratagene, La Jolla, CA). The plasmid of Miller *et al.* (*supra*) encoding wild-type PA was used as the

template. The point mutants were cloned into a pET22-b(+) (Novagen) expression vector and transformed into BL21(DE3) (Novagen) for expression. The point mutants were expressed and purified as previously described (Miller, 1999). Briefly, cultures were grown in LB at 37 °C to an A_{600} of 1.0. Expression of the recombinant protein was induced by the addition of β-D-isopropylthiogalactopyranoside to 1 mM. Following induction, the cells were grown for an additional 3 hours at 30 °C and harvested by centrifugation for 10 minutes at 8000 x *g*.

The proteins were released from the periplasm by osmotic shock. The cells were resuspended in 20 mM Tris-HCl, pH 8.0, 30% glucose and 1 mM EDTA and incubated at room temperature for 10 minutes with continuous stirring. The cells were harvested again by centrifugation, resuspended in 5 mM $MgSO_4$ containing 20 mM Benzamidine, and incubated at 4 °C for 10 minutes with constant stirring. After the cells were again pelleted by centrifugation at 8000 x *g*, the perplasmic extract was decanted. Tris-HCL pH 8.0 was added to a final concentration of 20 mM, and the entire sample was loaded onto a Q-sepharose HP column. The unbound protein was washed off the column with buffer A (20 mM Tris, pH 8.0). The bound protein was eluted with a 0% - 25% buffer B linear gradient (20 mM Tris, pH 8.0, 1 M NaCl). The PA containing fractions were concentrated, and the buffer was exchanged using a pd-10 column (Amersham-Pharmacia) containing buffer A. The PA-containing eluate was loaded onto a Mono-Q column and eluted with a 0 - 25% buffer B gradient. PA containing fractions were analyzed by SDS-PAGE and stored at -80 °C. Proteins concentrations were determined using the Bio-Rad protein assay kit based on the manufacturer's protocol. All liquid chromatography was performed using an AKTA-purifier liquid chromatography system (Amersham-Pharmacia).

The other PA mutants (# 13-18 from Table 1) were constructed, expressed, and purified similarly.

Proteolytic activation of PA

Trypsin was used to proteolytically cleave PA83 to nicked PA (nPA). PA was diluted to a concentration of 0.5 mg/ml for the prepore-forming assay or 0.2 mg/ml for the other assays. Trypsin was added to a final trypsin to PA ratio of 1:1,000 (w:w), and the mixture was incubated at room temperature for 20 minutes, followed by inhibition of the trypsin with a 10 molar excess of soybean trypsin inhibitor.

Cell surface translocation assay

A cell surface translocation assay to measure the PA-mediated translocation of radiolabeled LFn (N-terminal 1-255 amino acid PA binding domain of LF) was performed as previously described (Wesche *et al.*, Biochemistry 37:15737, 1998). Briefly, nPA ($2x10^{-8}$ M) was first bound to CHO cells, followed by ^{35}S LFn which binds to the PA63 on the cell surface. Excess LFn was removed, and the cells were washed and subjected to a pH 5.0 pulse at 37°C. The low pH pulse mimics the acidification of the endosome and results in the PA-mediated translocation of LFn across the plasma membrane and into the cell. The samples were treated with pronase which proteolytically degrades extracellular ^{35}S-LFn, but not ^{35}S-LFn that has been translocated into the cell. The cells were then washed and lysed. To determine the total amount of ^{35}S-LFn that bound to the cells, some of the cells were not treated with pronase. Following lysis, the amount of ^{35}S-LFn in the supernatant was determined using a scintillation counter. The percent translocation was calculated as follows:

(DPM protected from pronase)/(DPM bound to cells) x 100 = % translocated.

To determine if mutant PA proteins inhibit the translocation of LFn by wild-type PA, this assay was also performed using equimolar amounts of mutant and wild-type PA that were combined prior to trypsinization and diluted to 2 x 10^{-8} M PA (1x10^{-8} M of each protein) before being added to cells. When PA at a concentration of 1x10^{-8} M was used as a control, the translocation efficiency was only slightly affected by the drop in PA compared to the assay above with 2x10^{-8} M wild-type PA, suggesting that any decrease in translocation and binding was not the result of the drop in the concentration of wild-type PA.

Inhibition of protein synthesis

LFnDTA inhibition of protein synthesis was used as another method to measure PA-mediated translocation of ligands into cells (Milne *et al.*, Mol. Microbiol. 15:66, 1995). For assaying PA mutants # 1-12 in Table 1, CHO-K1 cells were plated at 2.5 x 10^4 cells/well in a 96 well plate 16 hours prior to the addition of PA protein. PA83 (1x10^{-12} M to 1x10^{-7} M) was incubated with cells in the presence of 1 x 10^{-8} M LFnDTA for 4 hours. The media was then removed and replaced with leucine free HAM's F-12 media supplemented with ^{3}H-Leu at 1 mCi/ml. After a one hour incubation, the cells were washed with ice cold PBS followed by ice-cold trichloro acetic acid (10%) to precipitate proteins. The quantity of ^{3}H-leu incorporated into the TCA insoluble material was determined using a scintillation counter and was used as a measure of the amount of newly synthesized protein.

Mutant PA proteins were also tested in this assay to see if they relieved the wild-type PA-mediated inhibition of ^{3}H-Leu uptake. Wild-type PA was added to CHO cells at a concentration of 1x10^{-9} M with 1x10^{-8} M LFnDTA.

Increasing amounts of one of the mutants were also added. The cells were incubated with the toxin for 4 hours and the samples were processed as described above.

The PA mutants listed in Fig. 9 were tested similarly. CHO-K1 cells (2.5 x 10^4 cells/well) in a 96-well plate were incubated for 18 hours at 37°C with wild-type PA (100 pM) in the presence of LFN-DTA (100 pM) and various amounts of individual PA mutants (K397D + D425K, ΔD2L2, F427A, D425K, K397D, or SSSR). The medium was then removed and replaced with leucine-free HAM F-12 supplemented ^{3}H-Leu at 1 μCi/ml. After incubation for one hour at 37°C, the cells were washed with ice-cold PBS followed by ice-cold 10% trichloroacetic acid (TCA). The quantity of ^{3}H-Leu incorporated into the TCA-precipitable material was measured and is expressed as percent of that incorporated in the absence of PA. At the concentrations of wild-type PA and LFnDTA chosen, protein synthesis was inhibited by about 90% in the absence of mutant PA (dotted line). The mean of three experiments ± SEM is reported. Similar results were seen when the initial incubation was four hours, instead of 18 hours. The
K397D + D425K + F427A, F427A + ΔD2L2, and K397D + F427A + ΔD2L2 PA mutants listed in Fig. 10 were tested similarly.

The PA-mediated inhibition of protein synthesis by hetero-heptamers of wild-type and mutant PA was compared to that of mixtures of the corresponding homo-heptamers. Homo-heptamers of wild-type PA63 and K397D + D425K, ΔD2L2, F427A, K397D, and D425K mutants, were prepared as described above. Putative hetero-heptamers were prepared by mixing each mutant PA with wild-type PA in a 1:1 ratio before trypsinization and column chromatography (Fig. 11). Wild-type PA (1 nM), hetero-heptamer (H) (final concentration 2 nM), or an equimolar mixture (M)

(1 nM each) of the corresponding mutant homo-heptamer and wild-type-heptamer, was incubated with CHO-K1 cells in the presence of LFnDTA (100 pM) for 18 hours, and inhibition of protein synthesis was measured as described above for Fig. 9. Heptamer concentrations are expressed in terms of monomeric PA63 subunits. Protein synthesis is expressed as the percent of a control without PA. The mean of three experiments ± SEM is reported. Similar results were seen after a four hour incubation.

Prepore and SDS-resistant oligomer formation

The formation of prepores and SDS-resistant oligomers was measured by incubating nPA with an equimolar amount of LFn for 30 minutes at room temperature. To determine whether prepores had formed, the samples were subjected to electrophoresis in a 4-12% native gradient gel (FMC) using 50 mM CHES, pH 9.0, 2 mg/ml CHAPS as the running buffer. To determine whether low pH induced the formation of SDS-resistant heptamers, 100 mM sodium acetate, pH 4.5 was added until the pH of the solution reached 5.0, and then the sample was incubated at room temperature for 30 minutes. The sample was then dissolved in SDS-PAGE sample buffer and run on a 4-12% SDS-PAGE gradient gel. Proteins in the gels were visualized with coomassie brilliant blue.

Rubidium release

CHO-K1 cells were plated at a density of 2 x 10^5 cells/well and incubated at 37 °C for 24 hours. The media was then aspirated and replaced with media containing 1 μCi/ml ^{86}RbCl and incubated for 16 hours. The cells were chilled on ice for 20 minutes, and the media was removed. The cells were washed, and nPA (2 x 10^{-8}M) in HEPES buffered media was added. The

cells were incubated with nPA for 2 hours on ice, followed by the addition of ice cold pH 5.0 buffer. After 30 minutes, samples from the supernatant were collected and counted in a scintillation counter to determine the amount of released ^{86}Rb.

This standard assay may also be used to determine the effect of other pore-forming toxins on the amount of released ^{86}Rb. Thus, other mutant toxins of the present invention may be tested in this assay to determine whether they have a reduced ability to form transmembrane pores.

Example 2: Failure of most mutants to form SDS-resistant oligomers

All PA mutants # 1-12 in Table 1 and wild type PA proteins were proteolytically nicked with trypsin as described above, forming nicked PA (nPA) proteins that migrated as lower molecular species when analyzed by SDS-PAGE (Figure 2A). Formation of SDS-dissociable prepores by PA mutants # 1-12 in Table 1 was detected by the decreased mobility in native gels of heptameric PA63 complexed with LFn compared to monomeric nPA (Figure 2B). The formation of prepores by the K397A and D425A PA mutants was further supported by the elution of the prepores from a MonoQ column at a higher salt concentration than that which elutes monomeric PA. The nPA mutants were also analyzed for the formation of SDS-resistant oligomers. As a positive control, wild-type PA was treated with LFn. The low pH pulse converted wild-type PA into SDS-resistant oligomers, which migrated as high molecular weight complexes when analyzed by SDS-PAGE. ΔD2L2 (PA lacking residues 302-325) and K397Q (Figure 2B). Wild-type, K397Q, F427A, and ΔD2L2 PA formed SDS-resistant oligomers when treated with low pH (Figure 2A and Table 1).

Example 3: Failure of PA mutants to form pores in membranes

The failure of most of the PA mutants to form SDS-resistant oligomers suggested that pore formation in cell membranes would also be inhibited. Pore formation was assayed by binding nPA proteins to cells loaded with the radioactive potassium analogue, ^{86}Rb, pulsing with low pH, and measuring the release of ^{86}Rb into the surrounding media, as described in Example 1. Wild-type nPA induced the release of ^{86}Rb due to the insertion of nPA into the membrane forming ion permeable pores. In contrast, none of the mutants # 1-12 in Table 1 induced ^{86}Rb release (Figure 3 and Table 1). Thus, the inability of most PA mutants to form SDS-resistant oligomers (Example 2) correlates with an inability of these mutants to form pores in cell membranes.

Example 4: Failure of PA mutants to translocate LFn across membranes

Pore formation is a requisite step in the PA dependent translocation of ligands (*i.e.*, LF, EF or LFn) across membranes. A cell surface translocation assay was used to directly measure the translocation of PA ligands into the cytoplasm of the cell (Example 1). None of the PA mutants # 1-12 in Table 1 had a significantly decreased ability to bind LFn (Fig. 4A); however, all of the assayed mutants had a significantly reduced ability to translocate LFn in this assay (Figs. 4B and 12). The SSSR control mutant caused little inhibition under these conditions. These data suggest that the mutants retain structural integrity and the ability to bind to the cellular receptor and LFn but are not able to form pores or translocate ligands across membranes.

Example 5: Failure of PA mutants to translocate LFnDTA across membranes

Another method used to measure translocation of PA ligands across

membranes is the LFnDTA toxicity assay (Example 1). In this assay, CHO cells are treated with PA and a ligand containing LFn fused to the A-chain of diphtheria toxin DTA (LFnDTA). The translocated A-chain of diphtheria toxin ADP ribosylates the cytoplasmic protein EF-2, resulting in the inhibition of protein synthesis and the induction of cell death. This assay is a measure of translocation of a ligand from an endosomal compartment as opposed to a cell surface, as measured in Example 4. After incubation with LFnDTA and wild-type or mutant PA, cells were washed and incubated in leucine-free media supplemented with ^{3}H-leucine. If protein synthesis is not inhibited, ^{3}H-leucine will be incorporated into newly synthesized proteins. If protein synthesis is inhibited by LFnDTA, little ^{3}H will be incorporated. All of the mutants tested did not significantly inhibit protein synthesis in this assay (Figure 5). This result further supports the hypothesis that the lack of significant pore formation by PA mutants results in decreased membrane translocation of PA ligands by these mutants.

Example 6: Inhibition of wild-type PA pore formation by PA mutants

Since all of the PA mutants # 1-12 in Table 1 were defective in pore formation, they were tested to determine whether they could form inactive hetero-oligomers with wild-type PA thus inhibiting PA-mediated translocation of ligands across membranes. ΔD2L2, K397D + D425K, and K395D + K397D + D425K + D426K PA inhibited wild-type PA in this manner. When mixed with an equimolar amount of wild-type PA, each of these three mutants markedly inhibited translocation of ^{35}S-LFn into the cells in the cell surface translocation assay (Figure 6). ^{35}S-LFn binding to cells was not inhibited (Figure. 6).

Example 7: Inhibition of wild-type PA pore formation by PA mutants

The effect of these mutant proteins on PA mediated LFnDTA toxicity was also measured. When the ΔD2L2, K397D + D425K double mutant, or K395D + K397D + D425K + D426K quadruple mutant PA was mixed with an equimolar amount of wild-type PA in the LFnDTA assay, there was an approximately 2-log decrease in the wild-type PA-mediated inhibition of ^{3}H-Leu (Fig. 7). Thus, the mutants inhibited PA-mediated translocation by 99%. The activity retained in the presence of the mutant proteins is probably the result of heptamers containing 7 wild-type PA molecules and 0 mutant PA molecules (WT_7Mut_0). Using Pascal's triangle, 1% of the heptamers formed from the equimolar mixture of wild-type and mutant PA are expected to be 100% wild-type (WT_7Mut_0) (Table 2). This calculated result agrees with the 1% experimentally measured residual activity present in the mixture. Inhibition studies in which various ratios of wild-type to ΔD2L2 or K397D + D425K mutant PA were tested in the LFnDTA assay indicate that the only active species in the mix is probably WT_7Mut_0. Thus, the majority of heptamers containing one molecule of ΔD2L2 or K397D + D425K PA are inactive (Table 2), further supporting the dominant negative nature of these inhibitors.

Table 2. Predicted and Measured Compositions of PA Oligomers Formed from Various Ratios of Mutant to Wild-type PA

	Predicted % of the total heptamer population			**Activity Retained**	
Mutant:WT (mole:mole)	WT_7Mut_0	WT_6Mut_1	WT_5Mut_2	ΔD2L2 Mix	K397D + D425A Mi
1:1	0.78%	6%	22%	0.7% ± .2	0.9% ± .0(
0.75:1	2	10.4	23.5%	3.8% ± 2	1.2% ± .2
0.5:1	5.8	25.8	56.8	13.5% ± .5	5.8% ± 3.(
0.25:1	21	57	85	14.3% ± 2	10% ± 2

The predicted values represent the percent of the total heptamers that are expected to have at least the indicated number of wild-type molecules in the mixtures containing varying ratios of mutant and wild-type PA. The WT_7Mut_0 column represents the percent of the total heptamers that are expected to contain seven wild-type PA molecules. The WT_6Mut_1 column represents the percent of the total heptamers that are expected to contain at least six wild-type PA molecules (*i.e.*, the heptamers that either contain six wild-type PA molecules and one mutant PA molecule or contain seven wild-type PA molecules and zero mutant PA molecules. Similarly, the WT_5Mut_2 column represents the percent of the total heptamers that are expected to contain at least five wild-type PA molecules These values were calculated using Pascal's triangle. The values listed under" Activity Retained" are the actual experimental values seen in these mixtures.

A titration of mutant with wild-type PA in the LFnDTA assay was performed to further characterize the inhibition of wild-type PA. Increasing amounts of one of the mutants was added to incubations of cells with wild-type PA and LFnDTA (Fig. 8A). The mutant PA-SSSR, which has the furin recognition site mutated from ^{164}RKKR^{167} to ^{164}SSSR^{167}, was included as a control. Since this mutant cannot be nicked by furin or other furin-like proteases and thus can not form pores, the mutant can only inhibit PA by competing for the receptor. Both ΔD2L2 and K397D + D425K greatly inhibited PA mediated translocation. Most importantly these mutants do not inhibit solely by competing for the receptor since far less protein is required by these mutants to see 50% inhibition than is required by PA-SSSR (Fig. 7B). The single mutant constituents of K397D + D425K do not inhibit as well as the double mutant but inhibit better than PA-SSSR. Taken together these data suggest that ΔD2L2, K397D + D425K, and K395D + K397D + D425K + D426K PA are dominant negative inhibitors of wild-type PA.

The dominant negative inhibitory activity of the F427A, D425K, K397D + D425K + F427A, F427A + ΔD2L2, K397D + F427A + ΔD2L2 PA mutants was also measured. For this assay, increasing amounts of the mutant forms of PA were mixed with a constant amount of wild-type PA as described above. The most potent member of this group, the K397D + D425K + F427A triple mutant, almost completely blocked toxin action at a 1:1 ratio of mutant:wild-type PA. The D2L2, K397D + D425K, F427A, F427A + ΔD2L2, and K397D + F427A + ΔD2L2 PA mutants also had inhibitory activity. The K397D + D425K + F427A + ΔD2L2, F427D, and F427K PA mutants also exhibited dominant negative activity in the LFnDTA toxicity assay. In contrast, another translocation-deficient mutant, K397D, caused virtually no inhibition at a 1:1 ratio, showing that not all mutants of this type are strongly

inhibitory (Fig. 9). The SSSR control mutant caused no detectable inhibition of toxin action, even in 10-fold excess over wild-type PA, implying that competition for receptors did not contribute significantly to the inhibitory activities of the other mutants.

The hypothesis that inhibition by the dominant negative mutants depends upon the ability of their PA63 moieties to form hybrid complexes with wild-type PA63 was tested using purified homo- and hetero-heptamers. PA in solution can be cleaved at the furin site by mild trypsinization, and the resulting fragments can be separated by chromatography of the trypsin-nicked molecule on an anion-exchange column (Miller *et al.*, Biochemistry 38, 10432, 1999). Purified PA63 isolated by this method is heptameric, indicating that the oligomerization equilibrium is greatly in favor of this form, and may be structurally similar or identical to the prepore. Purified homo-heptamers were prepared from wild-type PA and each of the K397D + D425K, ΔD2L2, F427A, D425K, and K397D translocation-deficient PA mutants. Putative hetero-heptamers were prepared by mixing each mutant PA 1:1 with wild-type PA, followed by trypsinization of the mixture and chromatography of the products on an anion-exchange column.

The LFnDTA-dependent inhibition of protein synthesis by each hetero-heptamer and by an equivalent amount of a 1:1 mixture of the corresponding mutant and wild-type homo-heptamers was measured. Hetero-heptamers containing the K397D + D425K, ΔD2L2, F427A and D425K mutants did not mediate the action of LFnDTA, whereas the corresponding mixtures of homo-heptamers were highly active (Fig. 11). In contrast, the putative hetero-heptamer formed by mixing K397D with wild-type PA was as active as the mixture of homo-K397D PA and homo-wild-type PA. These results are consistent with the properties of these mutants in the

experiment of Fig. 9 and support the notion that PA63 from the dominant negative mutants inactivates the wild-type protein by co-oligomerizing with it. The absence of inhibitory activity of K397D in the hetero-heptamer preparation may reflect a defect either in ability to co-oligomerize with the wild-type protein or in ability to inhibit its activity within a heptamer. The finding that mutant homo-heptamers did not inhibit the activity of the wild-type indicates that little competition for receptors and little or no subunit exchange among heptamers occurred under the conditions of the experiment.

As described above, the fact that the K397D + D425K double mutant almost completely blocked activity in these LFnDTA toxicity assays suggests both that a single molecule of the mutant inactivates a heptamer and that oligomerization is stochastic. The ΔD2L2, D425K, and F427A mutants appear to be slightly less inhibitory, implying that more than one molecule of these mutants per heptamer may be required for inactivation and/or that their co-oligomerization with wild-type PA may not be purely stochastic. Other factors, such as the order of addition of B moieties to a growing heptamer complex (*e.g.*, the B moiety that is added first or last) may also effect inactivation. It is not intended that the invention be limited by any proposed mechanism for inhibition set forth in the specification.

Example 8: Formation of SDS-resistant oligomers containing mutant and wild-type PA

To examine the interaction of ΔD2L2 and K397D + D425K mutants with wild-type PA, an equimolar ratio of mutant to wild-type PA was mixed, nicked with trypsin, and analyzed by SDS-PAGE for SDS-resistant oligomer formation. When either mutant was mixed with wild-type PA, a new species of SDS-resistant PA was formed. In contrast to wild-type PA alone which

produces a diffuse high molecular weight smear in the gel, the mixture of mutant and wild-type PA results in the formation of a sharp high molecular weight band. This sharp band also differs from what is seen for either of the mutants alone: K397D + D425K alone does not form an SDS-resistant oligomer, and ΔD2L2 PA alone forms an oligomer which migrates farther in the gel than the band formed when wild-type PA is also present. Although the exact composition or nature of this band has not been determined, this band further suggests that the mutants interact with wild-type PA in SDS-resistant oligomers resulting in a change in the mobility of the oligomer in the gel.

Example 9: Toxin inhibition *in vivo*

The properties displayed by the dominant negative mutants *in vitro* imply that they should inhibit toxin action *in vivo*. To test this hypothesis, activities of three of these mutants (K397D + D425K, ΔD2L2, and F427A) were measured in a classical *in vivo* model for anthrax toxin action, the Fisher 344 rat (Ivins *et al.*, Appl. Environ. Microbiol. 55:2098, 1989). Male rats (250-300 g) injected intravenously with a mixture of 8 μg LF and 40 μg PA (approximately 10 times the minimal lethal dose) become moribund after about 90 minutes (Table 3). When wild-type PA was replaced with any of the dominant negatives mutants, the animals showed no symptoms of intoxication during the two week time period before the animals were sacrificed. When a dominant negative PA was added to the wild-type PA/LF mixture before injection, either at a 1:1 ratio relative to wild-type PA (40 μg dominant negative PA) or at a 0.25:1 ratio (10 μg dominant negative PA), the injected animals also survived without symptoms. The SSSR mutant had little effect on the activity of the toxin. These results are consistent with our *in vitro* results and demonstrate that the dominant negative mutants can ablate anthrax toxin

action *in vivo*, even at a sub-stoichiometric (0.25:1) ratio to wild-type PA.

Table 3: Inhibition of wild-type PA by PA mutants *in vivo*

Quantity of protein (μg)					
WT	ΔD2L2	K397D + D425K	F427A	SSR	TTM
40	-	-	-	-	90 ± 11 min
-	40	-	-	-	Survived
-	-	40	-	-	Survived
-	-	-	40	-	Survived
40	40	-	-	-	Survived
40	-	40	-	-	Survived
40	-	-	40	-	Survived
40	-	-	-	40	100 ± 3 min
40	10	-	-	-	Survived
40		10	-	-	Survived
40	-	-	10	-	Survived

The ability of the K397D + D425K + F427A triple mutant ("Triple") to inhibit the activity of wild-type PA *in vivo* was compared to that of the K397D + D425K double mutant ("Double") (Table 4). This experiment was performed as described above using rats injected with a mixture of 40 μg wild-type PA, 10 μg LF, and either PBS or a dominant negative PA mutant.

Table 4: Inhibition of wild-type PA by PA mutants *in vivo*

	Animals	amount of mutant PA	TTM
PBS	2	-	~100 minutes
Double	2	40 μg	Survived
Triple	2	40 μg	Survived
Double	4	4 μg	Survived
Triple	4	4 μg	Survived

The anti-PA and the neutralizing antibody titer generated by vaccination of rats with K397D + D425K, ΔD2L2, or F427A PA was also measured. For this determination, groups of six animals were vaccinated three times each at 0, 3, and 6 weeks with 50 μg of protein in 200 μl of Ribi Tri-Mix adjuvant (Sigma) by intramuscular injection into the hind-quarters. Two days prior to the first injection and 14 days following each injection, blood was drawn from each animal and the serum was collected. Sixteen days following the final injection the rats were challenged with a lethal dose of LF (30 μg PA + 6 μg LF) by IV injection as described in Table 5. The mean anti-PA antibody titers in the serum were determined in a standard ELISA assay against PA. The titers are reported as the reciprocal of the geometric mean of the dilution at which the reactivity of the serum ends. Neutralizing antibodies were titered in an LFnDTA assay at 1 x 10^{-10} M PA and 1x10^{-10} M LFnDTA. Antibody dilutions were incubated with PA at 37 °C for one hour prior to starting the assay. Protein synthesis inhibition was measured using the LFnDTA toxicity assay as described above. The neutralizing titers are represented as the reciprocal of the geometric mean dilution required to inhibit PA activity by 50%. As illustrated

in Table 5, the K397D + D425K, ΔD2L2, and F427A PA mutants exhibited little or no diminution in immunogenicity relative to wild-type PA in Fisher rats. The neutralizing and anti-PA antibody titers after three injections were similar, regardless of immunogen employed, and all vaccinated animals survived challenge with a lethal dose of wild-type PA plus LF administered 16 days after the last injection.

Table 5: Anti-PA and the neutralizing antibody titer generated by vaccination of rats with PA mutants

	Animals	Anti-PA Titer	Neutralizing Titer	TTM
PBS	6	〈 10	〈 10	74.2 ± 1.5
WT	5	43,300	2,490	Survived
ΔD2L2	6	47,500	3,350	Survived
K397D + D425K	6	65,500	2,260	Survived
F427A	6	132,000	6,090	Survived

Example 10: Antibodies to PA

Antibodies to a PA protein may be used as therapeutics and/or diagnostics. Antibodies may be produced using standard methods by immunologically challenging a B-cell-containing biological system, *e.g.*, an animal such as a mouse or rabbit, with a PA protein or a fragment thereof to stimulate production of an anti-PA antibody by the B-cells, followed by isolation of the antibody from the biological system. For the generation of

monoclonal antibodies, the spleen may be harvested from the animal with the highest ELISA-determined immune response to the PA protein, and the B-cells fused to NS-1 myeloma cells to generate hybridomas. Hybridomas that secrete antibodies which bind PA may be selected using a standard ELISA assay or by western blotting. Monoclonal cell lines producing a high antibody titer and specifically recognizing a PA protein are saved.

The cell lines may also be screened to identify lines that produce antibodies which bind naturally-occurring PA with greater affinity than a mutant PA protein. These antibodies may be generated by administering to animals fragments of naturally-occurring PA that contain residues such as K397, D425, D426, or F427. The resulting antibodies may then be screened to determine which antibodies bind naturally-occurring PA but do not bind a mutant PA protein in which one or more of residues K397, D425, D426, or F427 is mutated or deleted. For example, the antibodies may be applied to a column containing an immobilized mutant PA protein, and the antibodies that do not bind the mutant PA protein may be selected. Antibodies may also be generated that are reactive with residues in the D2L2 loop; these antibodies may be produced by administering a fragment of PA containing the D2L2 loop to an animal, as described above. Antibodies that are reactive with residues in the D2L2 loop of naturally-occurring PA may also be screened to select the antibodies that do not bind a mutant PA protein in which one or more residues in the D2L2 loop are deleted. Alternatively, antibodies may be generated that bind a mutant PA with greater affinity than a naturally-occurring PA molecule by administering a fragment of a mutant PA to an animal as described above and selecting the antibodies with greater affinity for the mutant PA form. These antibodies may bind a residue in a mutant PA that is not present in a naturally-occurring PA.

Anti-PA antibodies may be used to measure PA protein in a biological sample such as serum, by contacting the sample with the antibody and then measuring immune complexes as a measure of the PA protein in the sample. Thus, these antibodies may be used in kits to determine whether a subject has been exposed to anthrax toxin.

Antibodies to PA can also be used as therapeutics for the treatment or prevention of anthrax infection. If a anti-PA antibody that binds wild-type PA but does not bind a dominant negative PA mutant is administered to a subject for passive immunization against anthrax infection, a dominant negative PA mutant may also be administered to the same subject as a therapeutic to inhibit the activity of wild-type PA. Because the administered anti-PA antibody does not react with the therapeutic dominant negative PA mutant, the anti-PA antibody should not reduce the ability of the dominant negative PA mutant to inhibit wild-type PA. Additionally, an anti-PA antibody that does not react with a therapeutic dominant negative PA mutant may be used to determine the amount of wild-type PA present in a sample from a subject who has been treated with the dominant negative PA mutant.

Similar antibodies may be generated for other mutant B moieties of the present invention.

Example 11: Administration of PA proteins and fragments

It is not intended that the administration of the PA proteins or fragments of the invention be limited to a particular mode of administration, dosage, or frequency of dosing; the present mode contemplates all modes of administration, including oral, intramuscular, intravenous, subcutaneous, by inhalation, or any other route sufficient to provide a dose adequate to prevent or treat an anthrax infection. One or more of the mutant PA proteins or

fragments may be administered to a mammal in a single dose or multiple doses. When multiple doses are administered, the doses can be separated from one another by, for example, one week to one month. It is to be understood that for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgement of the person administering or supervising the administration of the compositions.

The pharmaceutical compositions containing one or more PA proteins or fragments of the invention can be prepared as described previously in Remingtion's Pharmaceutical Sciences by E. W. Martin. Pharmaceutical stabilizing compounds, delivery vehicles, carrier vehicles, or adjuvants may be used. For example, human serum albumin or other human or animal proteins can be used. Phospholipid vesicles or liposomal suspensions are possible pharmaceutically acceptable carriers or delivery vehicles. Adjuvants that can be used in the invention include aluminum compounds, such as aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate. These compositions can be prepared according to methods known to those skilled in the art.

Other mutant B moieties or fragments of the invention may be administered similarly.

Example 12: Other pore-forming mutants

The crystal structure of PA identified four domains of PA (Petosa *et al.*, Nature 385(6619): 833-838, 1997). Domain 2 (residues 259-487) contains a large flexible loop that may undergo a major conformational change during conversion from the prepore to the pore. Mutation, deletion, or insertion of one or more amino acids in this region may result in inhibition of the pore-forming ability of the protein *in vivo* and/or result in the ability of the PA

mutant to inhibit the pore-forming ability of naturally-occurring PA. For example, residues in domain 2 of PA that are identical to the corresponding residues in one or more other pore-forming toxins (such as toxins from *Clostridium difficile*, *C. perfringens*, *C. spiroforme*, *C. botulinum*, *Bacillus cereus*, or *B. thuringiensis;* Figs. 15 and 16) may be mutated. These residues may be mutated or deleted in PA to generate dominant negative PA mutants. The following residues of domain 2 in PA are invariant among the binary A-B toxins listed in Figs. 15 and 16: A259, P260, V262, V264, M266, E267, S272 E275 T298, N353, N361, N363 R365, Y366, N368, G370, T371, Y375, V377, P389, T380, T381, V384, T393, I394, P407, Y411, P412, A420, D425, F427, I432, N435, Q438, L450, T452, Q454, G457, G474, W477, and I484. These residues may be mutated to any other amino acid. For example, the residues may be changed to an amino acid with a smaller side chain such as glycine or alanine, or the residues may be changed to an amino acid with a larger or branched side chain such as tryptophan, leucine, or methionine. Additionally, charged residues may be changed to residues with a neutral side chain or residues with a side chain of the opposite charge. Other examples of residues that may be used to replace a naturally-occurring residue are listed in Table 1.

In addition to anthrax toxin, the present invention is relevant to other pore-forming toxins. These toxins may also be mutated to generate toxins with reduced or negligible ability to oligomerize, to form transmembrane channels, or to translocate a ligand. Additionally, dominant negative mutants of other pore-forming mutants may be generated. For example, mutations that correspond to the PA mutations described herein may be made in other toxins that are homologous to PA (such as toxins from *Clostridium difficile*, *C. perfringens*, *C. spiroforme*, *C. botulinum*, *Bacillus cereus*, or *B. thuringiensis*)

(Figs. 15 and 16 and Table 6). Residues in other toxins that correspond to residues in domain 2 of PA may be mutated as described above. Additionally, at least 1, 3, 5, 8, 10, 15, 20, or 24 of the amino acids in the region that corresponds to the D2L2 loop of PA may be deleted in other pore-forming toxins. Also, one or more point mutations may be made at residues that correspond to the mutated PA residues described herein.

Any of these mutant forms of pore-forming toxins may be administered to a mammal for the treatment or prevention of infection by the pathogens (*e.g.*, bacteria) that produce the corresponding toxin.

Table 6. Mutations in other pore-forming toxins that correspond to the mutations in anthrax PA which are described herein. The residues in the other pore-forming toxins that correspond to the residues that were mutated in PA may also be mutated to any other amino acid.

anthrax PA	*C. difficile* toxin	*C. perfringens* toxin	*C. spiroforme* toxin	*C. botulinum* toxin	*B. cereus* toxin
K397A	Q425A	Q424A	Q428A	Q398A	K879A
K397D	Q425D	Q424D	Q428D	Q398D	K879D
K397C	Q425C	Q424C	Q428C	Q398C	K879C
K397Q	Q425Q	Q424Q	Q428Q	Q398Q	K879Q
D425A	D453A	D452A	D456A	D426A	D907A
D425N	D453N	D452N	D456N	D426N	D907N
D425E	D453E	D452E	D456E	D426E	D907E
D425K	D453K	D452K	D456K	D426K	D907K
F427A	F455A	F454A	F458A	F428A	F909A
K397D + D425K	Q425D + D453K	Q424D + D452K	Q428D + D456K	Q398D + D426K	K879D + D907K
K395D + K397D + D425K + D426K	K423D + Q425D + D453K + Q454K	K422D + Q424D + D452K + Q453K	K426D + Q428D + D456K + Q457K	K396D + Q398D + D426K + Q427K	T877D K879D + D907K + D908K
ΔD2L2	Δ340–358	Δ339–357	Δ343–361	Δ307–331	Δ797–816
K397D + D425K + F427A	Q425D + D453K + F455A	Q424D + D452K + F454A	Q428D + D456K + F458A	Q398D + D426K + F428A	K879D + D907K + F909A
F427A + ΔD2L2	F455A + Δ340–358	F454A + Δ339–357	F458A + Δ343–361	F428A + Δ307–331	F909A + Δ797–816
K397D + F427A + ΔD2L2	Q425D + F455A + Δ340–358	Q424D + F454A + Δ339–357	Q428D + F458A + Δ343–361	Q398D + F428A + Δ307–331	K879D + F909A + Δ797–816
K397D + D425K + F427A + ΔD2L2	Q425D + D453K + F455A + Δ340–358	Q424D + D452K + F454A + Δ339–357	Q428D + D456K + F458A + Δ343–361	Q398D + D426K + F428A + Δ307–331	K879D + D907K + F909A + Δ797–816
F427D	F455D	F454D	F458D	F428D	F909D
F427K	F455K	F454K	F458K	F428K	F909K

Alternatively, random mutagenesis may be performed on nucleic acids encoding pore-forming mutants (such as cholesterol dependent cytolysins or hexameric or heptameric toxins related to the Staphylococcal α-toxin) using standard molecular biology methods. The encoded mutant toxins may be expressed and optionally purified using standard methods. The rubidium release assay described herein may be used to identify mutant toxins with a reduced ability to form a transmembrane channel. Additionally, animal models may be used to identify dominant negative toxin mutants that reduce the toxicity of the corresponding wild-type toxin when both the mutant and wild-type toxins are administered to the animal.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the appended claims.

Other embodiments are within the claims.

Claims

1. A B moiety of a pore-forming binary A-B toxin, wherein said B moiety comprises a mutation that inhibits its pore-forming ability, wherein said mutation is not the deletion of amino acids 302-325 of anthrax protective antigen (SEQ ID NO. 12).

2. The B moiety of claim 1, wherein said B moiety is anthrax protective antigen.

3 The B moiety of claim 1, wherein said B moiety lacks pore-forming ability.

4. The B moiety of claim 1, having an amino acid sequence that is at least 80% identical to SEQ ID No.: 21 and that has an alteration selected from the group consisting of:

a) K397A;

b) K397D;

c) K397C;

d) K397Q;

e) D425A;

f) D425N;

g) D425E;

h) D425K;

i) F427A;

j) K397 + D425K double mutation;

k) K395D + K397D + D425K + D426K quadruple mutation;

l) K397D +D425K + F427A triple mutation;

m) F427A + ΔD2L2 double mutation;

n) K397D + F427A +ΔD2L2 triple mutation;

o) K397D + D425K + F427A +ΔD2L2 quadruple mutation;

p) F427D; and

q) F427K.

5. A vaccine composition comprising a B moiety of a pore-forming binary A-B toxin or a fragment thereof in a pharmaceutically acceptable carrier, wherein said B moiety comprises a mutation that inhibits its pore-forming ability.

6. The vaccine composition of claim 5, wherein said B moiety is anthrax protective antigen.

7. The vaccine composition of claim 5, wherein said B moiety is inactivated by chemical or physical means.

8. A method of preventing bacterial infection in a mammal, said method comprising administering to said mammal a vaccine comprising a B moiety of a pore-forming binary A-B toxin or a fragment thereof in a pharmaceutically acceptable carrier, wherein said B moiety comprises a mutation that inhibits its pore-forming ability.

9. A method of treating bacterial infection in a mammal, said method comprising administering to said mammal a vaccine comprising a B moiety of a pore-forming binary A-B toxin or a fragment thereof in a pharmaceutically acceptable carrier, wherein said B moiety comprises a mutation that inhibits its pore-forming ability.

10. The method of claim 8 or 9, wherein said vaccine is administered with an adjuvant.

11. A mutant B moiety of a pore-forming binary A-B toxin, wherein said mutant B moiety comprises a mutation that inhibits its pore-forming ability, and wherein said mutant B moiety inhibits the pore-forming ability of a naturally-occurring B moiety of said toxin.

12. The mutant B moiety of claim 11, wherein said mutant B moiety is anthrax protective antigen.

13. The mutant B moiety of claim 12, having the ability to bind lethal factor or edema factor.

14. The mutant B moiety of claim 11, having the ability to compete with said naturally-occurring B moiety for binding to a receptor on the surface of a mammalian cell.

15. The mutant B moiety of claim 11, having the ability to bind said naturally-occurring B moiety.

16. The mutant B moiety of claim 11, having the ability to oligomerize with said naturally-occurring B moiety to form a complex that has reduced ability to form a pore.

17. The mutant B moiety of claim 16, wherein said complex lacks the ability to form a pore.

18. The mutant B moiety of claim 11, having an amino acid sequence that is at least 80% identical to SEQ ID No.: 21 and that has an alteration selected from the group consisting of:

a) K397D + D425K double mutation;

b) ΔD2L2;

c) K395D + K397D + D425K + D426K quadruple mutation;

d) D425K;

e) F427A;

f) K397D +D425K + F427A triple mutation;

g) F427A + ΔD2L2 double mutation;

h) K397D + F427A +ΔD2L2 triple mutation;

i) K397D + D425K + F427A +ΔD2L2 quadruple mutation;

h) F427D; and

i) F427K.

19. The mutant B moiety of claim 11, comprising a deletion of at least 5 amino acids of the D2L2 loop.

20. A method of preventing bacterial infection in a mammal, said method comprising administering to said mammal a mutant B moiety of a pore-forming binary A-B toxin or a fragment thereof, wherein said mutant B moiety comprises a mutation that inhibits its pore-forming ability, and wherein said mutant B moiety inhibits the pore-forming ability of a naturally-occurring B moiety of said toxin.

21. A method of treating bacterial infection in a mammal, said method comprising administering to said mammal a mutant B moiety of a pore-forming binary A-B toxin or a fragment thereof, wherein said mutant B moiety comprises a mutation that inhibits its pore-forming ability, and wherein said mutant B moiety inhibits the pore-forming ability of a naturally-occurring B moiety of said toxin.

22. The method of claim 8, 9, 20, or 21, wherein said B moiety or said mutant B moiety is anthrax protective antigen and said bacterial infection is an anthrax infection.

23. The method of claim 8, 9, 20, or 21, further comprising administering to said mammal an antibody that binds said naturally-occurring B moiety.

24. The method of claim 8, 9, 20, or 21, wherein said mammal is a human.

25. The method of claim 24, wherein said mammal has been exposed to *B. anthracis* spores.

26. An purified antibody that specifically binds a naturally-occurring B moiety of a pore-forming binary A-B toxin with greater affinity than a mutant B moiety of said toxin, wherein said mutant B moiety comprises a mutation that inhibits its pore-forming ability.

27. The antibody of claim 26, wherein said mutant B moiety inhibits the pore-forming ability of a naturally-occurring B moiety of said toxin.