

METHODS AND COMPOSITIONS FOR INDUCING IMMUNE RESPONSES AGAINST CLOSTRIDIUM DIFFICILE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application Nos. 62/471,636, filed March, 15, 2017 and 62/474,434, filed March 21, 2017, the disclosures of which are each incorporated herein for all purposes.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

[0002] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: NOV_058_02WO_SeqList_ST25, date recorded: March 13, 2018, file size 187 kilobytes).

BACKGROUND

[0003] Vaccination against disease using a subunit-based vaccine is dependent on producing sufficient amounts of the protein antigen and maintaining stability of the antigen such that the protein remains effective when administered to a target population.

[0004] Complications in producing subunit vaccines arise at multiple steps during production. The target protein can be produced at low levels, or can be insoluble, resulting in economically-unfavorable production, even when the protein had particularly favorable immunogenicity profile.

[0005] Bacterial infections remain a health concern. Indeed, bacterial vaccines are increasingly sought after as bacteria evolve resistance to front-line antibiotics. Bacterial subunit vaccines rely on recombinant protein production. However, bacterial proteins can often be difficult to produce at high level due to low expression, and insolubility, and they can also suffer from reduced stability. Better approaches to producing vaccines, particularly for difficult antigen targets, would thus provide global health benefits. In particular, infection by clostridial bacteria, notably *C. difficile* remains a particular problem. *Clostridium difficile* infection (CDI) is the leading cause of nosocomial antibiotic-associated diarrhea in developed countries. Hypervirulent strains have evolved causing severe disease with increased mortality. Homologous glucosylating toxins,

TcdA and TcdB, and binary ADP-ribosylating toxin (CDT) are major virulence factors causing pathogenesis. There is an unmet need for vaccines targeting these toxins.

SUMMARY OF THE INVENTION

[0006] Disclosed herein are methods and compositions of inducing immune responses against *C.difficile*. The compositions contain polypeptides containing multiple *C. difficile* toxins, which, when administered to a subject, induce advantageous immune responses. Methods for producing the multi-toxin polypeptides are also disclosed.

BRIEF DESCRIPTION OF THE FIGURES

[0007] **Figure 1. *C. difficile* triple toxin vaccine constructs.** Figure shows illustration of *C.diff* triple toxin vaccine containing the binding domains of CDTb, Tcd B, and Tcd A with (construct 1420) and without (construct 1470) a furin cleavage site after the activation domain of CDTb.

[0008] **Figure 2. Expression and solubility of triple toxin vaccine BV1470 and BV1420.** *Spodoptera frugiperda* Sf9 insect cells were infected at a MOI of 0.1 with recombinant baculovirus BV1420 and BV1470, harvested at 48 and 72 hours postinfection, and analyzed for protein expression by SDS-PAGE and coomassie staining. An equal volume of total protein (T, cells and medium) and clarified medium (M) were mixed with 2X SDS-PAGE sample buffer and run on a 4%-12% polyacrilamide NuPage gel. Pelleted, infected cells were solubilized in 1% NP9, 25 mM Tris, 50 mM NaCL, pH 8.0 buffer. Lysed cells were centrifuged at 9000 x g for 40 min. Supernatant (S, soluble) was removed, pellet (I, insoluble) was suspended in buffer to original volume, and analyzed by SDS-PAGE as described above. Location of triple toxin protein marked with an arrow.

[0009] **Figure 3. Time course expression of triple toxin vaccine BV1470 and BV1420.** *Spodoptera frugiperda* Sf9 insect cells were infected with recombinant baculovirus BV1420 and BV1470, as described in Figure 6. Total protein, medium, soluble, and insoluble protein was analyzed by SDS-PAGE and coomassie staining at various timepoints postinfection. Location of triple toxin protein is marked with an arrow.

[0010] **Figure 4. Purification of triple toxin vaccine.** The triple toxin vaccine was purified from total cell culture of infected Sf9 cells following the addition of NP9 to a final concentration of 0.2%. NP9 extract was clarified twice and purified on consecutive Fractogel EMD TMAE, Phenyl HP, and Source 30Q columns. The triple toxin was eluted from each column and loaded onto the next column as shown. Eluted triple toxin positive fractions from the Source 30Q column were pooled and filter sterilized through a 0.2 μ M filter.

[0011] **Figure 5. Purification of triple toxin vaccine BV1470 from Sf9 cells.** Triple toxin vaccine BV1470 was purified from infected cells as described in Figure 8. Final filtered product from the Source 30Q column was analyzed for purity by SDS-PAGE and coomassie staining. Triple toxin protein was identified by western blot using anti-CDTb, anti-TcdB, and antiTcdA antibodies.

[0012] **Figure 6. Purification of triple toxin vaccine BV1420 from Sf9 cells.** Triple toxin vaccine BV1420 was purified from infected cells as described in Figure 8. Final filtered product from the Source 30Q column was analyzed for purity by SDS-PAGE and coomassie staining. Triple toxin protein was identified by western blot using anti-TcdB antibodies.

[0013] **Figure 7. Particle size distribution by volume graph for triple toxin BV1420.** Particle size of triple toxin BV1420 was determined by dynamic light scattering using a Zeta Sizer Nano. Graph of size distribution by volume is shown.

[0014] **Figure 8. Particle size distribution by intensity graph for triple toxin BV1470.** Particle size of triple toxin BV1420 was determined by dynamic light scattering using a Zeta Sizer Nano. Graph of size distribution by intensity is shown.

[0015] **Figures 9A-9D. Electronmicrographs of negative stained triple toxin BV1420.** Electron-micrograph of purified triple toxin BV1420 was diluted to approximately 10ug/ml and negatively stained with uranyl acetate.

[0016] **Figure 10. BV1420 triple toxin vaccine mouse lethal toxin challenge study 1.** Mice were immunized on day zero and day 14 with triple toxin vaccine BV1420 and challenged on day 35 with a lethal dose of Tcd A or CDT and monitored for 10 days post challenge. Mice were bleed as shown and serum analyzed for anti-toxin IgG and for toxin neutralizing antibodies. Animals were monitored for mortality and morbidity for 10 days after toxin challenge.

[0017] **Figure 11. BV1420 triple toxin vaccine mouse lethal toxin challenge study 1 - serum anti-toxin IgG responses.** Day 42 serum samples were assayed for Anti-Tcd A, anti-Tcd B, and anti-CDT IgG titers by ELISA using native toxins bound to plates.

[0018] **Figure 12. BV1420 triple toxin vaccine mouse lethal toxin challenge study 1 - toxin neutralizing antibody (TNA) titers.** Toxin neutralization titers were determined using a colorimetric Vero cell based assay. Titer indicated are the reciprocal of the highest dilution of serum that did not kill cells.

[0019] **Figure 13. BV1420 triple toxin vaccine mouse lethal toxin challenge study 1 – animal survival.** Animal survival was determined 10 days post challenge. Animals showing greater than 20% weight loss were sacrificed and recorded as dead.

[0020] **Figure 14. BV1420 triple toxin vaccine mouse lethal toxin challenge study 2 – toxin B survival.** Mice were immunized on day zero and day 14 with triple toxin vaccine BV1420 and challenged on day 35 with a lethal dose of Tcd B and monitored for 10 days post challenge. Mice were bled as shown and serum analyzed for anti-toxin IgG and for toxin neutralizing antibodies (TNA). Animals were monitored for mortality and morbidity for 10 days after toxin challenge.

[0021] **Figure 15. BV1420 triple toxin vaccine mouse lethal toxin challenge study 2 – anti-toxin IgG levels.** Day 42 serum samples were assayed for Anti-Tcd A, anti-Tcd B, and anti-CDT IgG titers by ELISA using native toxins bound to plates.

[0022] **Figure 16. BV1420 triple toxin vaccine mouse lethal toxin challenge study 2 – toxin B TNA titers.** Toxin neutralization titers were determined using a colorimetric Vero cell based assay. Titer indicated are the reciprocal of the highest dilution of serum that did not kill cells.

[0023] **Figure 17. BV1420 triple toxin vaccine mouse lethal toxin challenge study 2 – toxin B survival.** Animal survival was determined 10 days post challenge. Animals showing greater than 20% weight loss were sacrificed and recorded as dead.

[0024] **Figure 18.** Additional vaccine proteins with the TcdB gene translocation domain are shown. BV1512 is shown in the bottom diagram.

[0025] **Figure 19. Multimer Protein Expression:** Expression and western blot analysis of multimer protein BV1512.

[0026] **Figure 20. Quadrivalent Multimer Protein Expression:** Figure 25 illustrates two quadrivalent multimer proteins. In both cases, a peptide from a second TcdB strain is introduced

to broaden immunity against multiple strains. In the upper diagram, the TcdB peptide from Strain 027 is added at the C-terminus. In the lower diagram, the peptide is introduced between the TcdB protein and the TcdA(R19) protein from the first strain, strain 630.

[0027] **Figure 21. Quadrivalent Multimer Protein Expression:** Expression and western blot analysis of the quadrivalent protein shown in the upper diagram of Figure 20.

[0028] **Figure 22. Quadrivalent Multimer Protein Expression:** Expression and western blot analysis of the quadrivalent protein shown in the lower diagram of Figure 20.

[0029] **Figure 23. *C. difficile* Toxins and Design of Chimeric Trivalent (T) and Quadravalent (Q) Toxin Fusion Proteins.** Figure 23A shows the illustration of the functional domains of *C. difficile* toxin A (TcdA), toxin B (TcdB), and binary toxin (CDT) used to construct the chimeric trivalent and quadravalent toxin fusion proteins. TcdA and TcdB share common functional domains including the enzymatic glucosyltransferase (GT) domain, autocatalytic cysteine protease (CP) domain, pore-forming translocation (PT) domain (orange), and receptor binding domain (RBD). The binary toxin (CDT) consists of the enzymatic ADP-ribosyltransferase component (CDTa) and receptor binding component (CDTb). CDTb contains a 42 amino acid (aa) signal sequence with two serine-type proteolytic cleavage sites (arrow) which, when cleaved, generates a 20 kDa and 75 kDa fragment. Figure 24B shows the illustration of the chimeric trivalent toxin fusion protein (T-toxin) and a chimeric quadravalent toxin fusion protein (Q-toxin). The T-toxin fusion protein consists of the full-length coding sequence for CDTb with the RBD of TcdB₍₀₀₃₎ containing 24 repeats and the truncated RBD of TcdA with 19 repeats. The expressed T-toxin fusion protein consists of 1813 aa with a molecular weight (MW) of 205 kDa. The Q toxin fusion protein consists of the full-length coding sequence for CDTb to the RBD of TcdB₍₀₀₃₎ containing 24 repeats, the RBD of TcdA truncated at 19 repeats, and the RBD of TcdB₍₀₂₇₎ containing 24 repeats. The expressed Q-toxin fusion protein consists of 2359 aa with a molecular weight of 268 kDa.

[0030] **Figures 24A-24C. Expression and Purification of T-Toxin and Q-Toxin Fusion Proteins.** SDS-PAGE of purified T-toxin (lanes 2 and 3) migrates with a molecular weight of 205 kDa and Q-toxin (lanes 4 and 5) migrates with a molecular weight of 268 kDa. Molecular weight marker (lane 1). Figure 24A shows T-toxin and Q-toxin purity was > 90% as determined by SDS-PAGE scanning densitometry. Figure 24B shows western blot analysis as probed with rabbit anti-CDTb specific antibodies. Figure 24C shows western blot analysis as probed with

chicken anti-TcdB specific antibodies. Figure 24D shows western blot analysis as probed with chicken anti-TcdA specific antibodies.

[0031] Figures 25A-25C. Immunogenicity of T-Toxin and Q-Toxin Fusion Proteins in Mice.

Groups of female C57BL/6 mice (N = 10/group) were immunized IM on Days 0 and 14 with T-toxin (100 µg) or Q-toxin (100 µg) adjuvanted with alum (50 µg), or PBS (control group). Serum was collected 18 days after the second vaccination. Figure 25A shows serum IgG titers to TcdA, TcdB₍₀₀₃₎, and CDTb determined by ELISA. Figure 25B shows toxin-neutralizing antibody titers for each toxin determined in the Vero cell assay. In Figure 25C, Mice received a lethal dose (MLD_{100%} = 2.0 µg) of TcdB₍₀₀₃₎ administered IP 21 days after the second immunization. *Significance was determined by Mantel-Cox log-rank test comparing the T-toxin or Q-toxin groups to the PBS control group.

[0032] Figures 26A-26D. Immunogenicity of T-Toxin and Q-Toxin Fusion Proteins in Hamsters.

Male hamsters (N = 8/group) were immunized IM 3 times at 21-day intervals with 30 µg Q-toxin adjuvanted with 120 µg alum, or PBS (control group). Two weeks after the third dose, samples were collected and analyzed. Figure 26A shows serum IgG titers to TcdA, TcdB₍₀₀₃₎, and CDTb determined by ELISA. Figure 26B shows toxin-neutralizing antibody titers for each toxin determined in the Vero cell assay. In Figures 26C and 26D, two weeks after the third immunization, all animals were treated with clindamycin (10 mg/kg) IP one day prior to spore challenge and were challenged by gavage with 200 cfu *C. difficile* strain 630 (C) or with 500 cfu *C. difficile* strain B1/NAP1/027 (D). Animals were observed for 8 days post challenge.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[0033] As used herein, the term “adjuvant” refers to a compound that, when used in combination with an immunogen, augments or otherwise alters or modifies the immune response induced against the immunogen. Modification of the immune response may include intensification or broadening the specificity of either or both antibody and cellular immune responses.

[0034] As used herein, the terms “immunogen,” “antigen,” and “epitope” are used interchangeably and refer to substances such as proteins, and peptides that are capable of eliciting an immune response.

[0035] As used herein, the term “fusion protein” means a protein comprised of two or more proteins or protein fragments that are joined or fused, directly or indirectly via a linking peptide, at the amino terminus of one protein and the carboxy terminus of another protein, to form a single continuous polypeptide. In some aspects, a fusion protein may be referred to as a “multivalent protein.” A multivalent protein contains proteins or protein fragments from two or more three discrete protein antigens that are fused together.

[0036] The terms “treat,” “treatment,” and “treating,” as used herein, refer to an approach for obtaining beneficial or desired results, for example, clinical results. For the purposes of this invention, beneficial or desired results may include inhibiting or suppressing the initiation or progression of an infection or a disease; ameliorating, or reducing the development of, symptoms of an infection or disease; or a combination thereof.

[0037] “Prevention,” as used herein, is used interchangeably with “prophylaxis” and can mean complete prevention of an infection or disease, or prevention of the development of symptoms of that infection or disease; a delay in the onset of an infection or disease or its symptoms; or a decrease in the severity of a subsequently developed infection or disease or its symptoms.

[0038] As used herein an “effective dose” or “effective amount” refers to an amount of an immunogen sufficient to induce an immune response that reduces at least one symptom of malaria. An effective dose or effective amount may be determined *e.g.*, by measuring amounts of neutralizing secretory and/or serum antibodies, *e.g.*, by plaque neutralization, complement fixation, enzyme-linked immunosorbent (ELISA), or microneutralization assay.

[0039] As used herein, the term “vaccine” refers to a preparation including an immunogen (*e.g.* a fusion protein described herein) derived from a pathogen, which is used to induce an immune response against the pathogen that provides protective immunity (*e.g.*, immunity that protects a subject against infection with the pathogen and/or reduces the severity of the disease or condition caused by infection with the pathogen). The protective immune response may include formation of antibodies and/or a cell-mediated response. Depending on context, the term “vaccine” may also refer to a suspension or solution of an immunogen that is administered to a vertebrate to produce protective immunity.

[0040] As used herein, the term “subject” includes humans and other animals. The subject, in one embodiment, is a human.

[0041] As used herein, the term "pharmaceutically acceptable" means being approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia, European Pharmacopeia or other generally recognized pharmacopeia for use in mammals, and more particularly in humans. These compositions can be useful as a vaccine and/or antigenic compositions for inducing a protective immune response in a vertebrate.

[0042] As used herein, the term "about" means plus or minus 10% of the indicated numerical value.

Overview

The present disclosure provides methods and compositions for achieving high expression of large proteins, particularly multivalent proteins containing multiple antigens, from insect cells. The production of high levels of proteins as disclosed herein is particularly unexpected in view of prior experiences in the field.

Multivalent Proteins

[0043] The multivalent (the multivalent protein may also be referred to herein as a multimer) proteins disclosed herein can protect against multiple pathogens and/or the effects from multiple pathogenic proteins from the same organism. For example, certain pathogens may produce multiple molecules that each negatively affects a subject. A more effective response is produced by inducing responses against multiple separate antigens.

[0044] The proteins multivalent protein contains protein portions from multiple bacterial toxins. In some aspects, the multivalent protein comprises, or consists of, portions of proteins from the same organism, such as toxins for example. In other aspects, the multivalent protein comprises, or consists of, proteins from more than one organism. In particular aspects, no two proteins of a multivalent protein are from the same organism. In some aspects, the same proteins from different strains (*i.e.*, isologs) may be used to produce the portion. Using the same protein from a different strain allows protection against multiple strains and is particularly useful in situations where virulent strains newly arise. Other examples include *C. botulinum*, which has 8 serological types, A through H. The methods and compositions disclosed herein can be used to provide a single vaccine against all 8 serotypes. Other particular examples include combination toxin vaccines to protect against cholera, diphtheria and shigella, or tetanus, pertussis and

diphtheria. Thus, in some aspects, a multimeric protein may contain portions from 2, 3, 4, 5, 6, 7, 8, 9, or 10 different proteins. The portions may be used as components to produce the multimeric immunogenic polypeptides.

[0045] Exemplary multimers and components used to produce vaccines are described in the table below. Nucleic acid sequences encoding Q-toxin and BV1512, as well as alternative nucleic acid sequences for BV1420 and BV1470, are those using standard codon conversion appropriate degenerate codons that encode the indicated amino acid.

Vaccine Construct	Components	Protein Sequence	Nucleic Acid Sequence
BV1420 (SEQ ID NO: 9; SEQ ID NO: 13)	CDTb TcdB TcdA	SEQ ID NO: 10 SEQ ID NO: 11 SEQ ID NO: 12	SEQ ID NO: 14 SEQ ID NO: 15 SEQ ID NO: 16
BV1470 (SEQ ID NO: 1; SEQ ID NO: 5)	CDTb TcdB TcdA	SEQ ID NO: 2 SEQ ID NO: 3 SEQ ID NO: 4	SEQ ID NO: 6 SEQ ID NO: 7 SEQ ID NO: 8
BV1512 (SEQ ID NO: 17)	CDTb TD TcdAR19	SEQ ID NO: 18 SEQ ID NO: 19 SEQ ID NO: 20	
Q-toxin (SEQ ID NO: 21)	CDTb TcdB003 TcdA TcdB027	SEQ ID NO: 22 SEQ ID NO: 23 SEQ ID NO: 24 SEQ ID NO: 25	

[0046] Additional vaccine constructs may use the various components above in different orientations. In addition, proteins having at least 90% identity to each of these disclosed sequences may be used as components to produce a multimer protein.

Linkers

[0047] In some aspects, linkers may be used between one or more proteins in the multivalent proteins. In some aspects, the linker is a poly-(Gly)*n* linker, wherein *n* is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 19, or 20. In other aspects, the linker is GG, GGG, or GGGG (SEQ ID NO: 26). In yet other aspects, the linker is selected from the group consisting of: dipeptides,

tripeptides, and quadripeptides. Preferred dipeptides are Alanine-Serine (AS), Leucine-Glutamic acid (LE), Serine-Arginine (SR).

[0048] Multivalent antigens are particularly suited for protection against organisms that release multiple toxins into a subject. For example, bacteria are known to produce toxins that cause disease in humans. Thus, while the primary focus of the disclosure is *C. difficile*, the multimeric polypeptides of the disclosure may be prepared using portions of protein toxins from other species.

[0049] Toxin-producing species include *C. perfringes*, *C. botulinum*, *C. difficile*, and *C. tetani*, Bacillus (e.g., *B. anthracis*), Vibrio (e.g., *Vibrio cholerae*), Shigella, and Corynebacterium. *C. difficile* releases two enteric toxins, A and B, which are produced by toxigenic strains. Toxin A is an enterotoxin with minimal cytotoxic activity, whereas toxin B is a potent cytotoxin but has limited enterotoxic activity. A third toxin, Binary Toxin, also known as CDT, is also produced by the bacteria. Sequences encoding toxin A and B are known (Moncrief et al., Infect. Immun. 65:1105-1108 (1997); Barroso et al., Nucl. Acids Res. 18:4004 (1990); Dove et al. Infect. Immun. 58:480-488 (1990)). Sequences encoding Binary Toxin are also known (Accession Nos. ABS57477, AAB67305, AAF81761).

[0050] The usefulness of the present disclosure for protection against pathogen infection is illustrated by a trivalent protein vaccine against *C. difficile*. Figure 1 shows the structure of two exemplary multimer proteins (BV1420 and BV1470). Each multimer contains portions of three toxin proteins, Toxin A (TcdA), Toxin B (TcdB), and binary toxin (CDTb), from *C. difficile*. Triple toxin 1420 also contains a furin cleavage site. These proteins are large—over 1800 amino acids—and would not be previously have been expected to yield usable amounts of protein when expressed in insect cells. Surprisingly, however, both proteins are expressed at high levels. See Figure 3. Indeed, as Figure 5 demonstrates, the yield for BV1470 was 269 mg/L. Similarly, the yield for BV1420 was 166 mg/L.

[0051] Analysis of the purified multimer proteins confirmed they were in nanoparticle structures with peak diameters around about 16 nm for BV1420 and about 18 nm for BV1470. Notably, the distribution of diameters shown in Figures 7 and 8 illustrates that a high percentage of the multimer proteins retained nanoparticle structure after purification.

[0052] Administering the BV1420 trivalent nanoparticles to mice demonstrates that immune responses to all three proteins were obtained. Moreover, as Figure 13 illustrates the immune response obtained protected 100% of mice from lethal challenge with Toxin A and Binary toxin, as well as 67% to 83% of mice in response to lethal challenge with Toxin B. In contrast, mice in the PBS control group all died, with the exception of two mice in the binary toxin control group.

[0053] Quadrivalent toxins are also a preferred type of multimer immunogenic peptide. Figure 20 shows two illustrative examples with four portions or components arranged in sequence. Despite the substantial length of the multimer, good protein production was obtained. Fig. 22.

[0054] Figure 23 illustrates the conversion of a tri-toxin fusion protein to a quadrivalent toxin by addition of portion of a toxin from a second TcdB type. Comparing these two proteins shows that insect cell expression is able to give high level production. See Fig. 24A-D.

[0055] Thus, exemplary multimers include portions organized in various orientation. For example, starting from the N-terminus the first portion may be a TcdA portion, a TcdB portion or a CDTb portion. The second portion may be a TcdA portion, a TcdB portion or a CDTb portion. The third portion may be a TcdA portion, a TcdB portion or a CDTb portion. The fourth portion, if present, may be a TcdA portion, a TcdB portion or a CDTb portion. Thus, each portion may occupy each position. Typically, though not always, two adjacent portions are not portions from the same type of toxin. In preferred embodiments, the N-terminal portion is a CDTb portion.

Molecular Biology Techniques

[0056] The multivalent proteins disclosed herein are prepared through molecular biology approaches. General texts which describe molecular biological techniques, which are applicable to the present invention, such as cloning, mutation, cell culture and the like, include Berger and Kimmel, *Guide to Molecular Cloning Techniques*, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, Calif. (Berger); Sambrook *et al.*, *Molecular Cloning--A Laboratory Manual* (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2000 ("Sambrook") and *Current Protocols in Molecular Biology*, F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., ("Ausubel"). These texts describe mutagenesis, the use of vectors, promoters and many other relevant topics related to, *e.g.*, the cloning and mutating PfCSP, etc. Thus, the invention also encompasses using known methods of protein engineering and

recombinant DNA technology to improve or alter the characteristics of the proteins expressed on or in the fusion proteins of the invention. Various types of mutagenesis can be used to produce and/or isolate variant nucleic acids that encode for protein molecules and/or to further modify/mutate the proteins in or on the fusion proteins of the invention. They include but are not limited to site-directed, random point mutagenesis, homologous recombination (DNA shuffling), mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like. Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, *e.g.*, involving chimeric constructs, is also included in the present invention. In one embodiment, mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, *e.g.*, sequence, sequence comparisons, physical properties, crystal structure or the like.

[0057] Methods of cloning the proteins are known in the art. A gene can be cloned as a DNA insert into a vector. The term “vector” refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.

[0058] Thus, the invention comprises nucleotides that encode proteins, including chimeric molecules, cloned into an expression vector that can be expressed in a cell that induces the formation of fusion proteins of the invention. An “expression vector” is a vector, such as a plasmid, that is capable of promoting expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is “operably linked” to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter

and/or enhancer. In one embodiment, the nucleotides encode for a *Plasmodium* protein (as discussed above). In another embodiment, the expression vector is a baculovirus vector.

[0059] In some embodiments of the invention, proteins may comprise mutations containing alterations which produce silent substitutions, additions, or deletions, *e.g.*, to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by insect cells such as Sf9 cells). See, for example, U.S. Patent Publication 2005/0118191, herein incorporated by reference in its entirety for all purposes.

[0060] In addition, the nucleotides can be sequenced to ensure that the correct coding regions were cloned and do not contain any unwanted mutations. The nucleotides can be subcloned into an expression vector (*e.g.* baculovirus) for expression in any cell. The above is only one example of how the proteins can be cloned. A person with skill in the art understands that additional methods may be used.

Host Cells

[0061] The high level expression was obtained in insect cell expression systems. Non limiting examples of insect cells are, *Spodoptera frugiperda* (Sf) cells, *e.g.* Sf9, Sf21, *Trichoplusia ni* cells, *e.g.* High Five cells, and *Drosophila* S2 cells.

[0062] Vectors, *e.g.*, vectors comprising polynucleotides that encode fusion proteins, can be transfected into host cells according to methods well known in the art. For example, introducing nucleic acids into eukaryotic cells can be achieved by calcium phosphate co-precipitation, electroporation, microinjection, lipofection, and transfection employing polyamine transfection reagents. In one embodiment, the vector is a recombinant baculovirus.

Nanoparticle Production

[0063] The nanoparticles may be produced by growing host cells transformed by an expression vector under conditions whereby the recombinant proteins are expressed. In one aspect, a method of producing a multivalent protein comprises transfecting vectors encoding the protein into a suitable host cell and expressing the protein under conditions that allow nanoparticle formation. In another embodiment, the eukaryotic cell is selected from the group consisting of yeast, insect, amphibian, avian or mammalian cells. The selection of the appropriate growth conditions is within the skill or a person with skill of one of ordinary skill in the art.

[0064] Methods to grow host cells include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. Cell culture means the growth and propagation of cells in a bioreactor (a fermentation chamber) where cells propagate and express protein (e.g. recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions in a bioreactor. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, the bioreactor is a stainless steel chamber. In another embodiment, the bioreactor is a pre-sterilized plastic bag (e.g. Cellbag®, Wave Biotech, Bridgewater, N.J.). In other embodiment, the pre-sterilized plastic bags are about 50 L to 1000 L bags.

Detergent Extraction and Purification of Nanoparticles

[0065] The nanoparticles may be harvested from the host cells using detergents. Suitable detergents include non-ionic surfactants. For example, the non-ionic surfactant may be Bis(polyethylene glycol bis[imidazolylcarbonyl]), nonoxynol-9, Bis(polyethylene glycol bis[imidazolyl carbonyl]), Brij® 35, Brij®56, Brij® 72, Brij® 76, Brij® 92V, Brij® 97, Brij® 58P, Cremophor® EL, Decaethyleneglycol monododecyl ether, N-Decanoyl-N-methylglucamine, n-Decyl alpha-Dglucopyranoside, Decyl beta-D-maltopyranoside, n-Dodecanoyl-N-methylglucamine, nDodecyl alpha-D-maltoside, n-Dodecyl beta-D-maltoside, n-Dodecyl beta-D-maltoside, Heptaethylene glycol monododecyl ether, Heptaethylene glycol monododecyl ether, Heptaethylene glycol monotetradecyl ether, n-Hexadecyl beta-D-maltoside, Hexaethylene glycol monododecyl ether, Hexaethylene glycol monohexadecyl ether, Hexaethylene glycol monooctadecyl ether, Hexaethylene glycol monotetradecyl ether, Igepal CA-630, Igepal CA -630, Methyl-6-0-(N -heptylcarbonyl)-alpha-D-glucopyranoside, Nonaethylene glycol monododecyl ether, N-Nonanoyl-N-methylglucamine, N-Nonanoyl-N-methylglucamine, Octaethylene glycol monododecyl ether, Octaethylene glycol monododecyl ether, Octaethylene glycol monohexadecyl ether, Octaethylene glycol monooctadecyl ether, Octaethylene glycol monotetradecyl ether, Octyl-beta-D glucopyranoside, Pentaethylene glycol monododecyl ether, Pentaethylene glycol monododecyl ether, Pentaethylene glycol monohexadecyl ether, Pentaethylene glycol monohexyl ether, Pentaethylene glycol monooctadecyl ether, Pentaethylene glycol monooctyl ether, Polyethylene glycol diglycidyl ether, Polyethylene glycol ether W-1, Polyoxyethylene 10 tridecyl ether, Polyoxyethylene 100

stearate, Polyoxyethylene 20 isohexadecyl ether, Polyoxyethylene 20 oleyl ether, Polyoxyethylene 40 stearate, Polyoxyethylene 50 stearate, Polyoxyethylene 8 stearate, Polyoxyethylene bis(imidazolyl carbonyl), Polyoxyethylene 25 propylene glycol stearate, Saponin from Quillaja bark, Span® 20, Span® 40, Span® 60, Span® 65, Span® 80, Span® 85, Tergitol Type 15-S-12, Tergitol Type 15-S-30, Tergitol Type 15-S-5, Tergitol Type 15-S-7, Tergitol Type 15-S-9, Tergitol Type NP-10, Tergitol Type NP-4, Tergitol Type NP-40, Tergitol, Type NP-7 Tergitol Type NP-9, Tergitol Type TMN-10, Tergitol Type TMN-6, Tetradecyl-beta-D-maltoside, Tetraethylene glycol monodecyl ether, Tetraethylene glycol monododecyl ether, Tetraethylene glycol monotetradecyl ether, Triethylene glycol monodecyl ether, Triethylene glycol monododecyl ether, Triethylene glycol monohexadecyl ether, Triethylene glycol mono-octyl ether, Triethylene glycol monotetradecyl ether, Triton CF-21, Triton CF-32, Triton DF-12, Triton DF-16, Triton GR-5M, Triton QS-15, Triton QS-44, Triton X-100, Triton X-102, Triton X-15, Triton X- 151, Triton X-200, Triton X-207, Triton® X-100, Triton® X-114, Triton® X-165, Triton® X-305, Triton® X-405, Triton® X-45, Triton® X-705-70, TWEEN® 20, TWEEN® 21, TWEEN® 40, TWEEN® 60, TWEEN® 61, TWEEN® 65, TWEEN® 80, TWEEN® 81, TWEEN® 85, Tyloxapol, n-Undecyl beta-D-glucopyranoside, semi-synthetic derivatives thereof, or combinations thereof. Tergitol NP-9 is a preferred detergent.

[0066] Once the host cells have grown for 48 to 72 hours, the cells are isolated from the media and a detergent-containing solution is added to solubilize the cell membrane, releasing the nanoparticles in a detergent extract. The detergent may be added to a final concentration of about 0.1% to about 1.0%. For example, the concentration may be about 0.1%, about 0.2%, about 0.3%, about 0.5%, about 0.7%, about 0.8%, or about 1.0 %. In certain aspects, the range may be about 0.1% to about 0.3%. Preferably, the concentration is about 0.2%.

[0067] The nanoparticles may then be isolated using methods that preserve the integrity thereof, such as centrifugation. In some aspects, gradient centrifugation, such as using cesium chloride, sucrose and iodixanol, may be used. Other techniques may be used as alternatives or in addition, such as standard purification techniques including, e.g., ion exchange and gel filtration chromatography.

[0068] In one aspect, the detergent extract is added to multiple columns sequentially. For example, the first column may be an ion chromatography column, such as TMAE, the second column may be a hydrophobic interaction column, such as Phenyl HP, and the third column may

be a strong anion exchange column such as a Source 30Q column. Increased purity may be obtained by repeating the three-step procedure.

[0069] The following provides a general procedure for making isolating and purifying proteins. A person of skill in the art would understand that there variations that can be utilized

[0070] Production is initiated by seeding Sf9 cells (non-infected) into shaker flasks, allowing the cells to expand and scaling up as the cells grow and multiply (for example from a 125-ml flask to a 50 L Wave bag). The medium used to grow the cell is formulated for the appropriate cell line (preferably serum free media, e.g. insect medium ExCell-420, JRH). Next, the cells are infected with recombinant baculovirus at the most efficient multiplicity of infection (e.g. from about 1 to about 3 plaque forming units per cell). Once infection has occurred, the fusion proteins (and, optionally, other immunogens) are expressed from the virus genome. Usually, infection is most efficient when the cells are in mid-log phase of growth ($4-8 \times 10^6$ cells/ml) and are at least about 90% viable.

[0071] Proteins of the disclosure can be harvested approximately 48 to 96 hours post infection. In some aspects, harvesting takes place at about 48 hours, about 72 hours, or between about 48 and about 72 hours. Typically, harvesting takes place when the levels of VLPs in the cell culture medium are near the maximum but before extensive cell lysis. The Sf9 cell density and viability at the time of harvest can be about 0.5×10^6 cells/ml to about 1.5×10^6 cells/ml with at least 20% viability, as shown by dye exclusion assay.

[0072] To solubilize the particles, directly add Tergitol NP9 to cell culture to final concentration of 0.2% NP9/25 mM Tris/50 mM NaCl/pH8.0. Incubate at room temperature for 1 hour then centrifuge the lysate at 9000 g for 30 min twice. Collected the supernatant containing the nanoparticles. The supernatant is then added to in Buffer A and eluted in Buffer B (Buffer A: 25mM Tris pH 8.0 /50 mM NaCl Buffer B: 25 mM Tris pH 8.0/1M NaCl). The eluate is applied to Phenyl HP columns (Buffer A: 350 mM Na-Citrate/25 mM Tris pH7.5 and Buffer B: 5 mM Tris pH8.0) and then to a Source 30Q column (Buffer A: 25 mM Tris pH8.0/250 mM NaCl Buffer B: 25 mM Tris pH8.0/1M NaCl). The pooled fractions containing the product are passed through a 2 micron filter. See Figures 8-10.

[0073] The procedures described above enable a purity of at least about 90%, at least about 95% or about 98% at a yield of 150 mg/L to about 300 mg/L. Purity may be measured by gel-based approaches that indicate total protein.

[0074] The intact baculovirus can be inactivated, if desired. Inactivation can be accomplished by chemical methods, for example, formalin or β -propiolactone (BPL). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods known in the art, as exemplified above. Methods of inactivation comprise incubating the sample containing the VLPs in 0.2% of BPL for 3 hours at about 25 °C to about 27 °C. The baculovirus can also be inactivated by incubating the sample containing the VLPs at 0.05% BPL at 4 °C for 3 days, then at 37 °C for one hour.

[0075] The above techniques can be practiced across a variety of scales. For example, T-flasks, shake-flasks, spinner bottles, up to industrial sized bioreactors. The bioreactors can comprise either a stainless steel tank or a pre-sterilized plastic bag (for example, the system sold by Wave Biotech, Bridgewater, N.J.). A person with skill in the art will know what is most desirable for the particular circumstance.

Protein Size and Yield

[0076] The yield for the multimer proteins using the methods disclosed herein is remarkable. In some cases, the yield is about 150 mg/L to about 300 mg/L. In some embodiments, the yield is about 40 mg/L, about 60 mg/L, about 80 mg/L, about 100 mg/L, about 150 mg/L, about 200 mg/L, about 250 mg/L, or about 300 mg/L. In particular aspects, the yield ranges from about 40 mg/L to about 300 mg/L, from about 80 mg/L to about 250 mg/L, or about 100 mg/mL to about 300 mg/L.

[0077] Large multimer proteins disclosed herein typically range from about 1500-2500 amino acids. In some aspects, they range from about 1500 to about 2000 amino acids. In other aspects, they range from about 1800 to about 2000 amino acids.

[0078] The multimer proteins form nanoparticles having a typical diameter of about 11 nm to about 35 nm. The diameter range may be about 15 nm to about 25 nm. Illustrative examples of multimer protein nanoparticles in these ranges are shown in Figure 9.

[0079] Importantly, even though the proteins are large, they remain soluble. For example, the purified multimer protein may be about 80% soluble, about 85% soluble, about 90% soluble, about 95% soluble, about 97% soluble, or about 99% soluble. In some aspects, solubility is about 90% to about 99% or about 90% to about 95%.

Modified Antigens and Polypeptides

[0100] The antigens disclosed herein encompass variations and mutants of those antigens. In certain aspects, the antigen may share identity to a disclosed antigen. Generally, and unless specifically defined in context of a specifically identified antigens, the percentage identity may be at least 80%, at least 90%, at least 95%, at least 97%, or at least 98%. Percentage identity can be calculated using the alignment program Clustal Omega, available at www.ebi.ac.uk/Tools/msa/clustalo using default parameters.

[0101] In particular aspects, the protein contained in the nanoparticles consists of that protein. In other aspects, the protein contained in the nanoparticles comprise that protein. Extensions to the protein itself may be for various purposes.

[0102] In some aspects, the antigen may be extended at the N-terminus, the C-terminus, or both. In some aspects, the extension is a tag useful for a function, such as purification or detection. In some aspects the tag contains an epitope. For example, the tag may be a polyglutamate tag, a FLAG-tag, a HA-tag, a polyHis-tag (having about 5-10 histidines), a Myc-tag, a Glutathione-S-transferase-tag, a Green fluorescent protein-tag, Maltose binding protein-tag, a Thioredoxin-tag, or an Fc-tag. In other aspects, the extension may be an N-terminal signal peptide fused to the protein to enhance expression. While such signal peptides are often cleaved during expression in the cell, some nanoparticles may contain the antigen with an intact signal peptide. Thus, when a nanoparticle comprises an antigen, the antigen may contain an extension and thus may be a fusion protein when incorporated into nanoparticles. For the purposes of calculating identity to the sequence, extensions are not included. In some aspects, the antigen may be truncated. For example, the N-terminus may be truncated by about 10 amino acids, about 30 amino acids, about 50 amino acids, about 75 amino acids, about 100 amino acids, or about 200 amino acids. For example, the C-terminus may be truncated by about 10 amino acids, about 30 amino acids, about 50 amino acids, about 75 amino acids, about 100 amino acids, or about 200 amino acids.

Pharmaceutical Compositions

[0103] The pharmaceutical compositions disclosed herein comprise a multimer protein and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include any pharmaceutical agent that can be administered to a subject without undue toxicity, irritation, or allergic reaction. Pharmaceutically acceptable carriers may also include one or more

pharmaceutically acceptable excipient. A pharmaceutically acceptable excipient is any excipient that is useful in preparing a pharmaceutical composition that is generally safe and non-toxic, and is acceptable for veterinary as well as human pharmaceutical use.

[0080] The pharmaceutical compositions useful herein contain a pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to the vertebrate receiving the composition, and which may be administered without undue toxicity and an immunogen; for example a multimer fusion protein.

[0104] In some aspects, formulations may include a pharmaceutically acceptable carrier or excipient. Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in Remington's Pharmaceutical Sciences (Mack Pub. Co. N.J. current edition). The formulation may be adapted to suit the mode of administration. In an exemplary embodiment, the formulation is suitable for administration to humans, is sterile, non-particulate and/or non-pyrogenic.

[0105] The composition may also contain wetting agents, or emulsifying agents, or pH buffering agents, or mixtures thereof. The composition can be a solid form, such as a lyophilized powder suitable for reconstitution (e.g., with water or saline), a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulations may include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

Adjuvants

[0106] The immunogenicity of a particular composition may be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Adjuvants have been used experimentally to promote a generalized increase in immunity against antigens (e.g., U.S. Pat. No. 4,877,611). Immunization protocols have used adjuvants to stimulate responses for many years, and as such, adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the

duration of antigen presentation. The inclusion of any adjuvant described in Vogel et al., "A Compendium of Vaccine Adjuvants and Excipients (2nd Edition)," herein incorporated by reference in its entirety for all purposes, is envisioned within the scope of this disclosure. [0107] Exemplary adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant. Other adjuvants comprise GMCSP, BCG, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL), MF-59, RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween® 80 emulsion. In other preferred aspects, Alum such as 2% Alhydrogel ($\text{Al}(\text{OH})_3$) is used. In some aspects, the adjuvant may be a paucilamellar lipid vesicle; for example, Novasomes®. Novasomes® are paucilamellar nonphospholipid vesicles ranging from about 100 nm to about 500 nm. They comprise Brij 72, cholesterol, oleic acid and squalene. Novasomes have been shown to be an effective adjuvant (see, U.S. Pat. Nos. 5,629,021, 6,387,373, and 4,911,928).

Saponin Adjuvants

[0108] Adjuvants containing saponin may also be combined with the immunogens disclosed herein. Saponins are glycosides derived from the bark of the *Quillaja saponaria* Molina tree. Typically, saponin is prepared using a multi-step purification process resulting in multiple fractions. As used, herein, the term "a saponin fraction from *Quillaja saponaria* Molina" is used generically to describe a semi-purified or defined saponin fraction of *Quillaja saponaria* or a substantially pure fraction thereof.

Saponin fractions

[0109] Several approaches for producing saponin fractions are suitable. Fractions A, B, and C are described in U.S. Pat. No. 6,352,697 and may be prepared as follows. A lipophilic fraction from Quil A, a crude aqueous *Quillaja saponaria* Molina extract, is separated by chromatography and eluted with 70% acetonitrile in water to recover the lipophilic fraction. This lipophilic fraction is then separated by semi-preparative HPLC with elution using a gradient of from 25% to 60% acetonitrile in acidic water. The fraction referred to herein as "Fraction A" or "QH-A" is, or corresponds to, the fraction, which is eluted at approximately 39% acetonitrile. The fraction referred to herein as "Fraction B" or "QH-B" is, or corresponds to, the fraction,

which is eluted at approximately 47% acetonitrile. The fraction referred to herein as "Fraction C" or "QH-C" is, or corresponds to, the fraction, which is eluted at approximately 49% acetonitrile. Additional information regarding purification of Fractions is found in U.S. Pat. No. 5,057,540. When prepared as described herein, Fractions A, B and C of *Quillaja saponaria* Molina each represent groups or families of chemically closely related molecules with definable properties. The chromatographic conditions under which they are obtained are such that the batch-to-batch reproducibility in terms of elution profile and biological activity is highly consistent.

[0110] Other saponin fractions have been described. Fractions B3, B4 and B4b are described in EP 0436620. Fractions QA1-QA22 are described EP03632279 B2, Q-VAC (Nor-Feed, AS Denmark), *Quillaja saponaria* Molina Spikoside (Isconova AB, Ultunaallén 2B, 756 51 Uppsala, Sweden). Fractions QA-1, QA-2, QA-3, QA-4, QA-5, QA-6, QA-7, QA-8, QA-9, QA-10, QA-11, QA-12, QA-13, QA-14, QA-15, QA-16, QA-17, QA-18, QA-19, QA-20, QA-21, and QA-22 of EP 0 3632 279 B2, especially QA-7, QA-17, QA-18, and QA-21 may be used. They are obtained as described in EP 0 3632 279 B2, especially at page 6 and in Example 1 on page 8 and 9.

[0111] The saponin fractions described herein and used for forming adjuvants are often substantially pure fractions; that is, the fractions are substantially free of the presence of contamination from other materials. In particular aspects, a substantially pure saponin fraction may contain up to 40% by weight, up to 30% by weight, up to 25% by weight, up to 20% by weight, up to 15% by weight, up to 10% by weight, up to 7% by weight, up to 5% by weight, up to 2% by weight, up to 1% by weight, up to 0.5% by weight, or up to 0.1% by weight of other compounds such as other saponins or other adjuvant materials.

ISCOM Structures

[0112] Saponin fractions may be administered in the form of a cage-like particle referred to as an ISCOM (Immune Stimulating COMplex). ISCOMs may be prepared as described in EP0109942B1, EP0242380B1 and EP0180546 B1. In particular embodiments a transport and/or a passenger antigen may be used, as described in EP 9600647-3 (PCT/SE97/00289).

Matrix Adjuvants

[0113] In some aspects, the ISCOM is an ISCOM matrix complex. An ISCOM matrix complex comprises at least one saponin fraction and a lipid. The lipid is at least a sterol, such as cholesterol. In particular aspects, the ISCOM matrix complex also contains a phospholipid. The

ISCOM matrix complexes may also contain one or more other immunomodulatory (adjuvant-active) substances, not necessarily a glycoside, and may be produced as described in EP0436620B1.

[0114] In other aspects, the ISCOM is an ISCOM complex. An ISCOM complex contains at least one saponin, at least one lipid, and at least one kind of antigen or epitope. The ISCOM complex contains antigen associated by detergent treatment such that that a portion of the antigen integrates into the particle. In contrast, ISCOM matrix is formulated as an admixture with antigen and the association between ISCOM matrix particles and antigen is mediated by electrostatic and/or hydrophobic interactions.

[0115] According to one embodiment, the saponin fraction integrated into an ISCOM matrix complex or an ISCOM complex, or at least one additional adjuvant, which also is integrated into the ISCOM or ISCOM matrix complex or mixed therewith, is selected from fraction A, fraction B, or fraction C of Quillaja saponaria, a semipurified preparation of Quillaja saponaria, a purified preparation of Quillaja saponaria, or any purified sub-fraction e.g., QA 1-21.

[0116] In particular aspects, each ISCOM particle may contain at least two saponin fractions. Any combinations of weight % of different saponin fractions may be used. Any combination of weight % of any two fractions may be used. For example, the particle may contain any weight % of fraction A and any weight % of another saponin fraction, such as a crude saponin fraction or fraction C, respectively. Accordingly, in particular aspects, each ISCOM matrix particle or each ISCOM complex particle may contain from 0.1 to 99.9 by weight, 5 to 95% by weight, 10 to 90% by weight 15 to 85% by weight, 20 to 80% by weight, 25 to 75% by weight, 30 to 70% by weight, 35 to 65% by weight, 40 to 60% by weight, 45 to 55% by weight, 40 to 60% by weight, or 50% by weight of one saponin fraction, e.g. fraction A and the rest up to 100% in each case of another saponin e.g. any crude fraction or any other faction e.g. fraction C. The weight is calculated as the total weight of the saponin fractions. Examples of ISCOM matrix complex and ISCOM complex adjuvants are disclosed in U.S Published Application No. 2013/0129770.

[0117] In particular embodiments, the ISCOM matrix or ISCOM complex comprises from 5-99% by weight of one fraction, e.g. fraction A and the rest up to 100% of weight of another fraction e.g. a crude saponin fraction or fraction C. The weight is calculated as the total weight of the saponin fractions.

[0118] In another embodiment, the ISCOM matrix or ISCOM complex comprises from 40% to 99% by weight of one fraction, e.g. fraction A and from 1% to 60% by weight of another fraction, e.g. a crude saponin fraction or fraction C. The weight is calculated as the total weight of the saponin fractions.

[0119] In yet another embodiment, the ISCOM matrix or ISCOM complex comprises from 70% to 95% by weight of one fraction e.g., fraction A, and from 30% to 5% by weight of another fraction, e.g., a crude saponin fraction, or fraction C. The weight is calculated as the total weight of the saponin fractions. In other embodiments, the saponin fraction from *Quillaja saponaria* Molina is selected from any one of QA 1-21.

[0120] In addition to particles containing mixtures of saponin fractions, ISCOM matrix particles and ISCOM complex particles may each be formed using only one saponin fraction. Compositions disclosed herein may contain multiple particles wherein each particle contains only one saponin fraction. That is, certain compositions may contain one or more different types of ISCOM-matrix complexes particles and/or one or more different types of ISCOM complexes particles, where each individual particle contains one saponin fraction from *Quillaja saponaria* Molina, wherein the saponin fraction in one complex is different from the saponin fraction in the other complex particles.

[0121] In particular aspects, one type of saponin fraction or a crude saponin fraction may be integrated into one ISCOM matrix complex or particle and another type of substantially pure saponin fraction, or a crude saponin fraction, may be integrated into another ISCOM matrix complex or particle. A composition or vaccine may comprise at least two types of complexes or particles each type having one type of saponins integrated into physically different particles.

[0122] In the compositions, mixtures of ISCOM matrix complex particles and/or ISCOM complex particles may be used in which one saponin fraction *Quillaja saponaria* Molina and another saponin fraction *Quillaja saponaria* Molina are separately incorporated into different ISCOM matrix complex particles and/or ISCOM complex particles.

[0123] The ISCOM matrix or ISCOM complex particles, which each have one saponin fraction, may be present in composition at any combination of weight %. In particular aspects, a composition may contain 0.1% to 99.9% by weight, 5% to 95% by weight, 10% to 90% by weight, 15% to 85% by weight, 20% to 80% by weight, 25% to 75% by weight, 30% to 70% by weight, 35% to 65% by weight, 40% to 60% by weight, 45% to 55% by weight, 40 to 60% by

weight, or 50% by weight, of an ISCOM matrix or complex containing a first saponin fraction with the remaining portion made up by an ISCOM matrix or complex containing a different saponin fraction. In some aspects, the remaining portion is one or more ISCOM matrix or complexes where each matrix or complex particle contains only one saponin fraction. In other aspects, the ISCOM matrix or complex particles may contain more than one saponin fraction.

[0124] In preferred compositions, the saponin fraction in a first ISCOM matrix is Fraction A (a "Fraction A Matrix") and the saponin fraction in a second ISCOM matrix or ISCOM complex particle is Fraction C (a "Fraction C Matrix"). Thus, preferred compositions comprise, as an adjuvant, a Fraction A Matrix adjuvant and a Fraction C Matrix adjuvant. The amounts of each Matrix in the composition may vary. For example, the amount of Fraction A Matrix may be about 80% (w/w), about 85% (w/w), about 90% (w/w), about 92% (w/w), or about 95% (w/w) with the remainder Fraction C Matrix. A suitable example of a suitable 85:15 Fraction A Matrix and Fraction C Matrix combination is Matrix-M™ (Novavax AB, Uppsala, Sweden), a mixture of Fraction A Matrix and Fraction C Matrix at a ratio of about 85 to about 15.

[0125] Other saponin fractions, such as QS-7 and QS-21 fractions, their production and their use is described in U.S. Pat. Nos. 5,057,540; 6,231,859; 6,352,697; 6,524,584; 6,846,489; 7,776,343, and 8,173,141. These fractions may be used in the methods and compositions disclosed herein.

Immune Stimulators

[0126] Compositions of the disclosure may also be formulated with "immune stimulators." These are the body's own chemical messengers (cytokines) to increase the immune system's response. Immune stimulators include, but are not limited to, various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (e.g., granulocyte-macrophage (GM)-colony stimulating factor (CM)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immunostimulatory molecules may be administered in the same formulation as the compositions of the disclosure, or may be administered separately. Either the protein or an expression vector encoding the protein may be administered to produce an immunostimulatory effect. Thus, in one embodiment, the disclosure comprises antigenic and vaccine formulations comprising an adjuvant and/or an immune stimulator.

Methods of Inducing Immune Responses

[0081] Also provided in the present disclosure are methods of eliciting an immune response against pathogens. The method involves administering an immunologically effective amount of a composition comprising a multimer protein to a subject. Administration of an immunologically effective amount of the composition of the disclosure elicits an immune response specific for epitopes present on the fusion protein. Such an immune response can include B cell responses and/or T cell responses. When administered to a subject, the multimer proteins preferably induce neutralizing antibodies. Preferably, the immune response includes elements that are specific for at least one conformational epitope present each protein contained in the multimer protein.

Administration

[0127] Administration may be by any suitable route. Suitable routes include parenteral administration (e.g., intradermal, intramuscular, intravenous and subcutaneous), epidural, and mucosal (e.g., intranasal and oral or pulmonary routes or by suppositories), transdermally or intradermally. Administration may be by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucous, colon, conjunctiva, nasopharynx, oropharynx, vagina, urethra, urinary bladder and intestinal mucosa, etc.) and may be administered together with other biologically active agents. In some aspects, intranasal or other mucosal routes of administration may result in an antibody or other immune response that is substantially higher than other routes of administration. Administration can be systemic or local.

[0128] In some aspects, administration may be by injection using a needle and syringe, by a needle-less injection device. In other aspects, administration is by drops, large particle aerosol (greater than about 10 microns), or by spray into the upper respiratory tract.

[0129] In some aspects, a pharmaceutical pack or kit comprising one or more containers filled with one or more of the components of the formulations is provided. In a particular aspect, the kit may include two containers, a first container containing a multimer protein, and a second container containing an adjuvant. Associated with such container(s) may be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale

for human administration. Formulations may also be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of composition.

[0130] In some aspects, administration may be targeted. For example, the compositions may be administered in such a manner as to target mucosal tissues in order to elicit an immune response at the site of immunization. Mucosal tissues such as gut associated lymphoid tissue (GALT) can be targeted for immunization by using oral administration of compositions which contain adjuvants with particular mucosal targeting properties. Additional mucosal tissues can also be targeted, such as nasopharyngeal lymphoid tissue (NALT) and bronchial-associated lymphoid tissue (BALT).

[0131] In some aspects, multiple compositions may be administered each having different collections of antigens. Where more than one multimer protein is administered, the proteins may be co-administered simultaneously to the same position of the subject; for example, by injection of material from one or more containers containing multimer proteins. In other aspects, they may be co-administered sequentially at different sites within a short space of time; for example, one administration may be in the thigh, and a second administration may be in the arm, with both administrations occurring within a short period (e.g. up to 30 minutes).

[0132] Human clinical studies can be performed to determine the preferred effective dose for humans by a skilled artisan. Such clinical studies are routine and well known in the art. The precise dose to be employed will also depend on the route of administration. Effective doses may be extrapolated from dose-response curves derived from in vitro or in vivo test systems. Dose may be adjusted based on, e.g., age, physical condition, body weight, sex, diet, time of administration, and other clinical factors.

[0133] While stimulation of immunity with a single dose is possible, additional dosages may be administered, by the same or different route, to achieve the desired effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood, as necessary to maintain sufficient levels of protection against infections. Similarly, adults who are particularly susceptible to repeated or serious infections, such as, for example, health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function may require multiple immunizations to establish and/or maintain

protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of protection.

[0134] The vaccine compositions may also be used for preparing antibodies against the toxins useful for passive administration therapies. *See* Casadevall. "Passive Antibody Administration (Immediate Immunity) as a Specific Defense Against Biological Weapons," *Emerging Infectious Diseases*. 2002;8(8):833-841.

EXAMPLES

EXAMPLE 1

C. difficile Triple Toxin Vaccine Constructs

[0082] Two triple toxin vaccines were constructed. A diagram of the protein structures is shown in Figure 1. Triple toxin 1420 (also referred to as BV1420) contains, from N-terminus to C-terminus, an Activation domain peptide, a mature CDTb peptide, a TcdB RBD peptide, and a TcdA RBD peptide containing 19 repeats (R19). A furin cleavage site (RARRRKKR; SEQ ID NO:27) was located between the activation domain and mature CDTb peptides. Figures 2 and 3 show the protein and genetic sequence of BV1470, respectively. Linker sites at either end of the TcdB peptide.

EXAMPLE 2

Expression of Triple Toxin Vaccine

[0083] Sf9 cells were transformed with a baculovirus vector expressing the triple vaccine as a single transcript. Expression data from the Sf9 cells is shown in Figure 2. Figure 2 shows expression of each proteins harvested at 48 hours and at 72 hours. Remarkably, even though each protein is over 200kDa, high level production is achieved. Figure 7 shows a time course of expression from 48 hours to 96 hours. The data shows that, for both proteins, the protein is highly soluble.

EXAMPLE 3

Purification of Triple Toxin Vaccine

[0084] To solubilize and purify the particles, Tergitol (NP9) was directly added to cell culture to final concentration of 0.2% NP9/25 mM Tris/50 mM NaCl/pH8.0. Incubate at room temperature for 1 hour then centrifuge the lysate at 9000 g for 30 min twice. Collected the supernatant containing the nanoparticles. The supernatant is then added to in Buffer A and eluted in Buffer B (Buffer A: 25mM Tris pH 8.0 /50 mM NaCl Buffer B: 25 mM Tris pH 8.0/1M NaCl). The eluate is applied to Phenyl HP columns (Buffer A: 350 mM Na-Citrate/25 mM Tris pH7.5 and Buffer B: 5 mM Tris pH8.0) and then to a Source 30Q column (Buffer A: 25 mM Tris pH8.0/250 mM NaCl Buffer B: 25 mM Tris pH8.0/1M NaCl). The pooled fractions containing the product are passed through a 2 micron filter. See Figures 4-6. Purification of 1470 from Sf9 yielded 269 mg/liter of protein. Purification of 1420 from Sf9 cells yielded 166 mg/liter.

EXAMPLE 4

Analysis of Triple Toxin Vaccine Particles

[0085] Particle size distribution by volume graph for triple toxin BV1420 was analyzed by dynamic light scattering using a Zeta Sizer Nano. Graph of size distribution by volume is shown in Figure 7. The average diameter was ~30 nm. Figure 8 shows particle size distribution by intensity graph for triple toxin BV1470. The average diameter was ~18 nm.

[0086] Figure 9 shows various electronmicrographs of negative stained triple toxin BV1420. Electron-micrograph of purified triple toxin BV1420 was diluted to approximately 10ug/ml and negatively stained with uranyl acetate.

EXAMPLE 5

C. difficile Triple Toxin Vaccine: Lethal Toxin challenge and Animal Survival

[0087] Figure 10 provides the results of a mouse trial of the Triple Toxin Vaccine against Toxin A and Binary Toxin. Groups 1-6 were administered BV1420 antigen (30 µg) or PBS as shown. Groups 1 and 4 contain 50 µg Alum OH; Groups 2 and 5 contained 50 µg Alum OH and 50 µg ISCOM Matrix M adjuvant. Mice were immunized at Day 0 and Day 14, with bleeds at Day 0, 14, and 32. Mice were challenged with Toxin A or Binary Toxin at Day 35.

[0088] Figure 11 shows serum IgG responses. PBS did not induce antibodies, as expected. The Triple Toxin Vaccines, either with Alum OH or with both Alum OH and Matrix M induced Titers

ranging from about 10^4 to about 10^6 against Toxin A, Toxin B, and CDTb. Figure 12 establishes that the antibodies neutralized both Toxin A and CTDb. Figure 13 shows animal survival for the 6 groups. Groups 1, 2, 4, and 5 showed 100% survival. Except for two mice in the binary toxin challenge, all the animals in the control PBS groups died. These data establish that the triple toxin vaccine protects against the effect of the toxins.

[0089] In a second challenge study, for Toxin B, several constructs were produced and tested alone or in combination. Group 1 mice were administered BV1420 (30 μ g) with Alum OH. Group 2 mice were administered BV1470 (30 μ g) with Alum OH, Group 3 was administered a tandem protein containing rotavirus VP6 and the TcdB RBD (10 μ g) with Alum OH. Group 4 mice were administered BV1470 and VP6/TcdB RBD. Group 5 was administered Toxoid B (10 μ g). Group 6 was the control and was administered PBS. Anti-IgG response is shown in Figure 15. High titers antibodies were obtained in each case. Each of the groups containing the Toxin A peptide induced high titer anti-Toxin A responses ranging between 10^4 and about 10^5 . All groups were administered the Toxin B peptide and each demonstrated high titer ranging between 10^4 and about 10^6 . Each of the groups containing the Binary Toxin peptide induced high titer responses ranging between 10^5 and about 10^6 . Figure 16 establishes that the antibodies were produced that neutralized both Toxin B, with the Toxoid B showing higher levels.

[0090] Survival of the Groups 1-6 mice is shown in Figure 17. All mice in the PBS control Group died by Day 3, with 5 of 6 dead within one day. Toxin B survival was 100%. For groups 1 to 4, survival rates ranged from 67% to 83%.

EXAMPLE 6

Additional Triple Toxin Vaccines

[0091] Additional vaccines can be produced while obtaining high expression levels. Figures 18 and 19 shows additional trivalent vaccine proteins with the TcdB gene translocations gene. BV1512 is shown in the bottom diagram. Figures 18 shows additional vaccines structures: Multimer Protein Sequence: Sequence of BV1512 multimer vaccine protein showing CDTb protein separated from the Translocation Domain (TD) by an A-S linker and the TD separated from the TcdAR19 portion by an S-R linker. Figure 19 shows expression of the multimer protein BV1512 from Sf9 cells.

EXAMPLE 7**Quadrivalent Vaccines**

[0092] Multimer proteins containing four peptides were produced. Fig. 20. In this example, a peptide from a second TcdB strain was introduced to broaden immunity against an additional *C. difficile* strain. The first quadrivalent multimer protein (CBAB, or pCDTb/TcdB₆₃₀/TcdAR19/TcdB₀₂₇) included a TcdB peptide from Strain 027 added at the C-terminus (See Fig. 20, upper diagram). In a second quadrivalent multimer peptide, a TcdB peptide from Strain 027 peptide was introduced between the TcdB protein and the TcdA(R19) protein from the first strain, strain 630 (See Fig. 20, lower diagram). Figure 21 shows expression of the CBBA quadrivalent multimer from Sf9 cells as described above. The data shows that the yield obtained was 42 mg/L. A second protein (CBBA, or pCDTb/TcdB₆₃₀/TcdAR19/TcdB₀₂₇ as shown in Figure 26) was also produced in the Sf9 system and achieved 40 mg/L yield. See Fig. 22.

EXAMPLE 8**Design, Expression, and Purification of T-toxin and Q-toxin fusion proteins**

[0093] Chimeric fusion proteins were constructed to encode RBD of *C. difficile* TcdA, TcdB₍₀₀₃₎, TcdB₍₀₂₇₎, and CDTb. The RBD amino acid sequence for TcdA was derived from *C. difficile* strain VPI 10463 (ATCC 43255), NCBI P16154 (toxintype 0, ribotype 003); TcdB₍₀₀₃₎ from strain VPI 10463 (ATCC 43255), NCBI P18177 (toxintype 0, ribotype 003); TcdB₍₀₂₇₎ from strain CD196, NCBI WP_009888442.1 (toxintype III, ribotype 027); and CDTb from strain CD196, GenBank ABS57477.1 (toxintype III, ribotype 027).

[0094] The coding sequences for TcdA RBD (truncated with 19 of 38 repeats), TcdB₍₀₀₃₎ and TcdB₍₀₂₇₎ RBDs (24 repeats each), and CDTb were codon optimized for expression in insect cells (GenScript).

[0095] The nucleotide sequences encoding the CDTb gene fragment (amino acids 1-835), TcdA RBD (1314 base pairs [bp], 6816-8130 bp), and TcdB₍₀₀₃₎ RBD (1608 bp, 5493-7098 bp) were obtained by PCR amplification from the synthesized gene. PCR-amplified gene fragments were digested with restriction enzyme: CDTb with BamHI/NheI; TcdB₍₀₀₃₎ RBD with NheI/XbaI; and

TcdA RBD with XbaI/HindIII. After digestion, the three genes were ligated into the BamHI and HindIII sites of pFastBac1 (Invitrogen). The plasmid encoding the three RBDs was used to construct a recombinant *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcMNPV) baculovirus using the Bac-to-Bac baculovirus expression system (Invitrogen) in *Spodoptera frugiperda* (Sf9) insect cells to express the trivalent fusion protein, hereafter referred to as T-toxin (Figure 23B).

[0096] TcdB₍₀₂₇₎ RBD (1608 bp, 5493-7098) digested with SpeI/HinIII was fused to the C-terminus of the trivalent fusion gene to form the plasmid and baculovirus construct encoding the RBD of all four toxins, which was similarly expressed in Sf9 cells to produce the quadravalent fusion protein, hereafter referred to as Q-toxin (Figure 23B; SEQ ID NO: 21).

[0097] The construct thus contains **pCDTb**: 835 amino acid from 1-835; Strain: CD196; toxinotype: III, ribotype: 027; GenBank: ABS57477.1; **TcdB₀₀₃**: 536 amino acid from 838-1373; NCBI: P18177, STRAIN=ATCC 4325 / VPI 10463, Toxinotype 0, Ribotype: 087; **TcdA**: 438 amino acid from 1376-1813; NCBI: P16154, STRAIN=ATCC 4325 / VPI 10463, Toxinotype 0; Ribotype: 087, and **TcdB₀₂₇**: 536 amino acid from 1815-2351; NCBI: 013315, strain CD196; toxinotype: III, ibotype: 027. Each of the portion is separated by a two amino acid linker: AS between the pCDTb portion and the TcdB003 portion, SR between the TcdB003 portion and the TcdA portion, TS between the TcdA portion and the TcdB027 portion.

[0098] Fusion proteins were extracted by detergent lysis in a buffer comprising 0.2% Tergitol NP-9 in 25 mM Tris buffer (pH 8.0), 250 mM NaCl and 2 µg/mL leupeptin. Lysates were purified by centrifugation, and the fusion proteins were purified with Fractogel EMD TMAE, phenyl HP and 30Q column chromatography. Purified T-toxin and Q-toxin were formulated in 25 mM Tris and 250 mM NaCl (pH 8.0) at approximately 4.0 mg/mL and stored at < -60°C. Recovery of purified T-toxin and Q-toxin was 267 and 154 mg/L, respectively. T-toxin and Q-toxin migrate in SDS-PAGE gels with molecular weights of 205 kDa and 268 kDa, respectively, and purity of > 90% (Figure 23A). Western blot analysis with toxin-specific antibodies confirmed expression of CDTb, TcdB, and TcdA in each fusion protein (Figure 23B-D).

EXAMPLE 9

Immunogenicity of T-toxin and Q-toxin Fusion Proteins in Mice

[0099] To evaluate immunogenicity of T-toxin and Q-toxin fusion proteins, Mouse studies were conducted in accordance with Noble Life Sciences' Institutional Animal Care and Use Committee (IACUC) approved protocols. Female C57BL/6 mice (6-8 weeks old) were immunized IM on Days 0 and 14 with T-toxin (30 or 100 µg) or Q-toxin (100 µg) formulated with 50 µg aluminum hydroxide (alum), or PBS (control). Serum was collected 18 days after the second dose. Mice were challenged intraperitoneally (IP) 3 weeks after the second immunization with a 100% minimal lethal dose (MLD_{100%}) of TcdA, TcdB₍₀₀₃₎, or CDTa and CDTb.

[00100] Mouse sera was evaluated for antibodies to the toxins by ELISA. A 96-well MaxiSorp microtiter plates (Thermo Scientific) were coated with each toxin (2 µg/mL) overnight at 2-8°C. Five-fold serial dilutions of sera were added to plates in duplicate. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotech). 3,3',5, 5'-tetramethylbenzidine (TMB) substrate (Sigma) was added and the reaction stopped with TMB Stop Buffer (Scytek Laboratories). Plates were read at 450 nm with a SpectraMax Plus plate reader (Molecular Devices); results were analyzed using SoftMax Pro software. Titers were reported as the reciprocal dilution that resulted in a reading of 50% the maximum OD_{450nm}. Titer values recorded as below the lower limit of detection (LLOD) were assigned a titer 50 for calculating GMT. Mouse serum IgG titers following immunization were high for TcdA, TcdB, and CDT and comparable between T-toxin and Q-toxin (Figure 25A).

[00101] Vero cells (CCL-81, ATCC) were maintained in DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS) and antibiotics (Gibco). Two-fold serial dilutions of mouse sera were prepared in 96-well, flat-bottom tissue culture plates (Thermo Scientific). An equal volume (50 µL) of assay medium (1x DMEM with 5% heat-inactivated FBS, 1x NEAA, 0.3% dextrose, 1x penicillin/streptomycin/glutamine, 0.006% Phenol Red) containing 2x minimum cytotoxic dose of TcdA, TcdB, or CDT was added to diluted serum and incubated for 1 hour at 37°C. Vero cells (7.5×10^4 cells/mL) suspended in 50 µL medium and 150 µL sterile mineral oil (Sigma) were added and plates were incubated for 6-7 days at 37°C. After incubation, plates were observed for well color. Media and toxin-treated control wells were red/reddish-pink; cell control wells were yellow/yellow-orange. For each sample dilution, the last well that was yellow/yellow-orange was recorded as the endpoint neutralizing-antibody titer. Titer values recorded as <LLOD were assigned a value of 5 for calculating GMT. Toxin-neutralizing

antibody (TNA) titers to each of the three toxins were comparable between the T-toxin and Q-toxin fusion proteins (Figure 25B).

[00102] Three weeks after the second immunization, mice were challenged TcdB₍₀₀₃₎. The group vaccinated with Q-toxin had 80% survival ($p = 0.0043$), while 65% ($p = 0.018$) of the T-toxin group survived challenge. In contrast, only 20% survived toxin challenge in the control group (Figure 25C).

EXAMPLE 10

Immunogenicity of T-toxin and Q-toxin Fusion Proteins in Hamsters

[00103] Golden Syrian hamsters (HsdHan:Aura; Harlan Laboratories), males aged 5-7 weeks and 70 to 100 grams, received 3 immunizations at 3-week intervals with 30 μ g Q-toxin and 120 μ g alum, or PBS (control), administered IM in alternating thighs. Two weeks after the third immunization serum was collected and animals were treated with 10 mg/kg clindamycin IP. One day later, animals were challenged by gavage with strain 630 or NAP1 and were observed for 8 days.

[00104] Hamster sera was evaluated for antibodies to the toxins by ELISA. A 96-well MaxiSorp microtiter plates (Thermo Scientific) were coated with each toxin (2 μ g/mL) overnight at 2-8°C. Five-fold serial dilutions of sera were added to plates in duplicate. Bound antibodies were detected with horseradish peroxidase-conjugated rabbit anti-hamster IgG (Southern Biotech). 3,3',5, 5'-tetramethylbenzidine (TMB) substrate (Sigma) was added and the reaction stopped with TMB Stop Buffer (Scytek Laboratories). Plates were read at 450 nm with a SpectraMax Plus plate reader (Molecular Devices); results were analyzed using SoftMax Pro software. Titers were reported as the reciprocal dilution that resulted in a reading of 50% the maximum OD_{450nm}. Titer values recorded as below the lower limit of detection (LLOD) were assigned a titer 50 for calculating GMT. Hamsters immunized thrice at 3-week intervals with Q-toxin produced high IgG titers to the TcdA, TcdB, and CDTb toxins (Figure 26A).

[00105] Vero cells (CCL-81, ATCC) were maintained in DMEM supplemented with 20% heat-inactivated fetal bovine serum (FBS) and antibiotics (Gibco). Two-fold serial dilutions of hamster sera were prepared in 96-well, flat-bottom tissue culture plates (Thermo Scientific). An equal volume (50 μ L) of assay medium (1x DMEM with 5% heat-inactivated FBS, 1x NEAA, 0.3% dextrose, 1x penicillin/streptomycin/glutamine, 0.006% Phenol Red) containing 2x

minimum cytotoxic dose of TcdA, TcdB, or CDT was added to diluted serum and incubated for 1 hour at 37°C. Vero cells (7.5×10^4 cells/mL) suspended in 50 µL medium and 150 µL sterile mineral oil (Sigma) were added and plates were incubated for 6-7 days at 37°C. After incubation, plates were observed for well color. Media and toxin-treated control wells were red/reddish-pink; cell control wells were yellow/yellow-orange. For each sample dilution, the last well that was yellow/yellow-orange was recorded as the endpoint neutralizing-antibody titer. Titer values recorded as < LLOD were assigned a value of 5 for calculating GMT. TNA titers to each of the three toxins were comparable between the T-toxin and Q-toxin fusion proteins (Figure 31B).

[00106] After clindamycin treatment, animals infected with *C. difficile* strain 630 had 90% survival (Figure 26C), while animals infected with NAP1 had 75% survival (Figure 31D). All animals in the placebo group died within 48-72 hours following infection with either strain.

INCORPORATION BY REFERENCE

[00107] Each of the patents and published applications identified herein are incorporated herein for all purposes.

CLAIMS:

1. A multivalent immunogenic polypeptide comprising portions of at least four *C. difficile* toxin proteins.
2. The multivalent immunogenic polypeptide of claim 1, wherein the portions are selected from the group of toxins consisting of Binary toxin (CDT), Toxin A (TcdA) protein and Toxin B (TcdB).
3. The multivalent immunogenic polypeptide of claim 2, wherein the portions are from a CDT protein, a Toxin A protein and two Toxin B proteins, wherein the Toxin B proteins are from distinct *C. difficile* strains.
4. The multivalent immunogenic polypeptide of claim 3, wherein at least one Toxin B portion is from an epidemic strain.
5. The multivalent immunogenic polypeptide from claim 4, wherein the epidemic strain is the NAP1 strain (TcdB₍₀₂₇₎).
6. The multivalent immunogenic polypeptide of claim 3, wherein one of the Toxin B portion is from 630 strain (TcdB₍₀₆₃₎).
7. The multivalent immunogenic polypeptide of claim 3, wherein the portions are oriented with the Toxin A portion between the two Toxin B portions.
8. The multivalent immunogenic polypeptide of claim 3, wherein the CDT portion is N-terminal to one or both of the Toxin B portions.
9. The multivalent immunogenic polypeptide of claim 3, wherein the CDT portion has an amino acid sequence that comprises or consists of SEQ ID NO:22 or an amino acid sequence having at least 90% homology to the sequence..
10. The multivalent immunogenic polypeptide of claim 3, wherein one of the Toxin B portions has an amino acid sequence that comprises or consists of SEQ ID NO:23 or an amino acid sequence having at least 90% homology to the sequence.
11. The multivalent immunogenic polypeptide of claim 3, wherein the second Toxin B portion has an amino acid sequence that comprises or consists of SEQ ID NO:25 or an amino acid sequence having at least 90% homology to the sequence..

12. The multivalent immunogenic polypeptide of claim 3, wherein the Toxin A portion has an amino acid sequence that comprises or consists of SEQ ID NO:24 or an amino acid sequence having at least 90% homology to the sequence.
13. The multivalent immunogenic peptide of claim 1 wherein the portions are separated by a two amino acid linker, a three amino acid linker, or a four amino acid linker.
14. The multivalent immunogenic peptide of claim 13 wherein the portions are separated by a two amino acid linker and the linker is selected from the group consisting of Alanine-Serine (AS), Leucine-Glutamic acid (LE), and Serine-Arginine (SR).
15. The multivalent immunogenic polypeptide of claim 1 wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:21.
16. A nucleic acid molecule comprising a polynucleotide encoding the polypeptide of any of claims 1 to 15.
17. A method of preparing the polypeptide of claim 1 comprising
 - (a) expressing the polypeptide in an insect host cell,
 - (b) purifying the polypeptide in the presence of a non-ionic detergent in the form of a nanoparticle, and
 - (c) suspending the nanoparticle in a pharmaceutically acceptable carrier, excipient, or diluent.
18. The method of claim 17, wherein the an insect host is an Sf9 cell.
19. The method of claim 17 or claim 18, wherein the insect host cell is transfected with a recombinant baculovirus construct under suitable conditions for expression of the polypeptide.
20. A vaccine composition comprising the immunogenic polypeptide of any of claims 1 to 15, and a pharmaceutically acceptable carrier, excipient, or diluent.
21. The vaccine composition of claim 20 wherein the composition comprises an adjuvant.
22. The vaccine composition of claim 20 or 21 wherein the adjuvant is a saponin-based adjuvant.
23. The vaccine composition of any of claims 20 to 22 wherein the saponin-based adjuvant contains Fraction A Matrix and Fraction C Matrix.

24. The vaccine composition of any of claims 20 to 23 wherein the amount of Fraction A Matrix is about 85% to about 92% and the remainder is Fraction C Matrix.
25. Use of a polypeptide according to any of claims 1 to 15 or the nucleic acid according to claim 16, for the manufacture of a medicament for the prevention or treatment of Clostridium Difficile infection.
26. A nanoparticle comprising the multivalent immunogenic polypeptide of any of claims 1 to 15 and a non-ionic detergent.