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Shigella-based minimal plasmid recombinant invasion construct

Abstract

Recombinant *Shigella* minimal invasion plasmid constructs pRISM and pRISM-G and a method of inducing an immune response to *Shigella* in a subject, the method comprising administering the composition to the subject in an amount sufficient to induce an immune response to *Shigella* in the subject.

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Background/Summary

RELATED APPLICATIONS (1) This application claims priority to U.S. Provisional Application No. 63/021,463, filed May 7, 2020, which is incorporated by reference in its entirety.

SEQUENCE LISTING

(1) The instant application contains a Sequence Listing, which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on May 3, 2021, is named "WRAIR_18_28_PCT_ST25.txt" and is 113,272 bytes in size.

BACKGROUND

(2) Live, attenuated strains of bacteria have been successfully used as vaccines to protect humans and animals from disease. The successful vaccine strain is modified to reduce its ability to cause disease to an acceptable level while it retains sufficient pathogenic potential to stimulate a protective immune response in the vaccinated host. Therefore a delicate balance must be achieved as extensive attenuation generally results in reduced immunity.

(3) *Shigella* is a genus of Gram-negative bacteria belonging to the family Enterobacteriaceae and the etiologic agent of bacillary dysentery or shigellosis, the symptoms of which include abdominal pain, diarrhea, fever, vomiting, and blood or mucus in the stool. *Shigella* is transmitted by a fecal-oral route, typically through contaminated food or water. As a result, *Shigella* is more of a public health threat in developing countries where proper sanitation and hygiene are lacking.

(4) The development of an effective live, attenuated strain of *Shigella* to protect humans against bacillary dysentery (shigellosis) has been hampered by the inability to strike a balance between a need for the strain to invade the intestinal epithelium and reduction of pathogenicity to an acceptable level. Invasive strains that are protective tend to be "reactogenic," i.e., they cause diarrhea and/or fever in the host. The conventional approach to attenuate *Shigella* is to mutate or delete genes on the *Shigella* invasion plasmid in the hopes of reducing the reactogenicity of the vaccine strain while preserving immunogenicity. Levine, M. et al. 2007. Clinical trials of *Shigella* vaccines: two steps forward and one step back on a long, hard road. Nat Rev Microbial 5:540-553. This strategy is a "top-down" approach starting with a wild type, virulent strain of *Shigella*. Genes are then systematically inactivated until the strain is sufficiently attenuated to be clinically safe to administer yet still stimulate a robust immune response. For example, the attenuating mutation in *S. flexneri* 2a strain SC602 is a deletion of *virG* (*icsA*) which abolishes the ability of the bacterium to spread from cell to cell after invasion. Coster, T. S. et al. 1999. Vaccination against shigellosis with attenuated *Shigella flexneri* 2a strain SC602. Infect. Immun. 67:3437-3443. A gene on the invasion plasmid that encodes an enterotoxin and another enterotoxin gene on the chromosome were targets in construction of *S. flexneri* 2a strain CVD1207 leading to attenuation and reduced reactogenicity. Kotloff, K. L. et al. 2000. *Shigella flexneri* 2a strain CVD 1207, with specific deletions in *virG* (*icsA*), *sen*, *set*, and *guaBA*, is highly attenuated in humans. Infect Immun 68:1034-1039. A major disadvantage of this strategy is that the attenuated strains that are protective have the undesirable side effect of causing diarrhea and/or fever. Further, to achieve the appropriate balance in the approach between preserving immunogenicity and reducing reactogenicity, other genes on the invasion plasmid (also known as the virulence plasmid), and possibly the chromosome as well, that

may contribute to reactogenicity must be identified and inactivated.

(5) An alternate strategy is disclosed in U.S. Pat. No. 9,434,772. Vectors described in this application were designed starting with the minimal set of genes from the *Shigella* invasion plasmid that mediate invasion of and multiplication within host epithelial cells. These genes were cloned into a plasmid to produce *Shigella* minimal invasion plasmid (SMIP). The SMIP can be transferred into a plasmid-cured strain of *Shigella* or an enteroinvasive *Escherichia coli* (EIEC), where it can be used as a vaccine strain. Additionally, the SMIP can serve as a platform to which other genes can be added in an attempt to improve immunogenicity of the vaccine strain. This strategy is an improvement over the “top-down” strategy because it allows one to directly determine the contribution of any putative virulence gene by adding it back to the SMIP, i.e., the role of the gene can be measured in the context of the minimal gene complement required for invasion rather than in the larger context of all of the genes normally present on the invasion plasmid.

(6) Inventors have discovered a different approach using PRISM and PRISM-G minimal plasmid constructs described in this application provide an improved platform for developing a live, attenuated vaccine strain of *Shigella* or enteroinvasive *Escherichia coli* (EIEC), that achieves the balance between immunogenicity and reduced reactogenicity in patients. In addition to inserting other *Shigella* or EIEC genes into the recombinant *Shigella* minimal invasion plasmid, it is also possible to insert a gene that encodes an immunogenic protein from a pathogen other than *Shigella* or EIEC or some other non-*Shigella* or non-EIEC protein of interest. In other words, a pPRISM and PRISM-G *Shigella* or EIEC strain comprising the recombinant *Shigella* minimal invasion plasmid can be used as a broad antigen delivery system for delivering any antigen of interest. *Shigella* or EIEC invasion into colonic mucosa stimulates a strong immune response in the intestinal mucosa. Furthermore, *Shigella* or EIEC invasion in the colon can also stimulate lymphoid tissues adjacent to the colon, and, thus, it could stimulate a strong mucosal immune response in the reproductive tract. Thus, the recombinant *Shigella* minimal invasion plasmid can be used as a live vaccine platform for delivery or expression of recombinant antigens in host cells. That is, the recombinant *Shigella* minimal invasion plasmid can be used as a vehicle for delivering DNA vaccines to mucosal lymphoid tissues or for expression and delivery of recombinant antigens. As such, the recombinant *Shigella* minimal invasion plasmid can be used in methods of inducing an immune response in a subject. In one embodiment, the immune response is directed to *Shigella*. In other embodiments, the immune response is directed to a bacterial, viral, fungal, parasitic, or mammalian protein of interest.

SUMMARY

(7) It has been discovered that the recombinant *Shigella* minimal invasion plasmid constructs pPRISM and pPRISM-G can be used as a vehicle for delivering DNA vaccines to mucosal lymphoid tissues or for expression and delivery of recombinant antigens. As such, the recombinant *Shigella* minimal invasion plasmid can be used in methods of inducing an immune response in a subject.

(8) In one embodiment, the immune response is directed to *Shigella*. In other embodiments, the immune response is directed to a bacterial, viral, fungal, parasitic, or mammalian protein of interest.

(9) A recombinant *Shigella* minimal invasion plasmid construct, pPRISM. In some embodiments, PRISM comprises SEQ ID NO: 1. A recombinant *Shigella* minimal invasion plasmid constructs pPRISM-G. In some embodiments, pPRISM-G comprises SEQ ID NO: 2. In one embodiment A composition comprising the *Shigella* or *E. coli* bacterium comprising a recombinant *Shigella* minimal invasion plasmid constructs PRISM and a pharmaceutically acceptable excipient. A composition comprising the *Shigella* or *E. coli* bacterium comprising a recombinant *Shigella* minimal invasion plasmid constructs pPRISM-G and a pharmaceutically acceptable excipient.

(10) A vaccine composition comprising the *Shigella* or *E. coli* bacterium comprising a recombinant *Shigella* minimal invasion plasmid constructs PRISM and at least one pharmaceutically acceptable

adjuvant.

(11) A vaccine composition comprising the *Shigella* or *E. coli* bacterium comprising a recombinant *Shigella* minimal invasion plasmid constructs pRISM-G and at least one pharmaceutically acceptable adjuvant.

(12) A method of inducing an immune response to *Shigella* in a subject, the method comprising the step of: administering a composition further comprising a recombinant *Shigella* minimal invasion plasmid construct PRISM or pRISM-G to the subject in an amount sufficient to induce an immune response to *Shigella* in the subject.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

(1) The following drawings form part of the present specification and are included to further demonstrate certain embodiments of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

(2) FIG. 1 shows a plasmid map of a recombinant *Shigella* minimal invasion plasmid construct, pRISM (SEQ ID NO: 1).

(3) FIG. 2 shows a plasmid map of a recombinant *Shigella* minimal invasion plasmid construct, pRISM-G (SEQ ID NO: 2).

(4) FIG. 3 shows PRISM construct colony D1 used for DNA preparation.

(5) FIG. 4 shows pRISM-G construct colony D1 used for DNA preparation.

(6) FIGS. 5A and 5B show growth curves of pRISM constructs compared to the virulence plasmid-cured *Shigella* strain BS103 and SMIP (FIG. 5A) and SMIP-G (FIG. 5B).

(7) FIG. 6 shows mean titers of serum IgA responses in naive guinea pigs or guinea pigs immunized with pRISM or pRISM-G assayed by ELISA from serum and eyewash samples collected on Day 0, Day 28 and Day 56. Upper and lower error bars together represent one standard deviation from the GMT. A. Serum IgA response to Invaplex. B. Serum IgA response to LPS.

(8) FIG. 7 shows mean titers of serum IgG responses in naive guinea pigs or guinea pigs immunized with PRISM or pRISM-G assayed by ELISA from serum and eyewash samples collected on Day 0, Day 28 and Day 56. Upper and lower error bars together represent one standard deviation from the GMT. A. Serum IgA response to Invaplex. B. Serum IgA response to LPS.

(9) FIG. 8 shows mean titers of mucosal IgA responses in naive guinea pigs or guinea pigs immunized with pRISM or pRISM-G assayed by ELISA from serum and eyewash samples collected on Day 0, Day 28 and Day 56. Upper and lower error bars together represent one standard deviation from the GMT. A. Serum IgA response to Invaplex. B. Serum IgA response to LPS.

(10) FIG. 9 depicts average results of two colonies/*Shigella* minimal plasmid strains for the ability to invade HeLa epithelial cells in a gentamicin protection assay. Invasiveness is plotted as percentages of the invasiveness of a wild-type 2457T Congo red-positive invasion-positive isolate. BS103 is a Congo red-negative invasion negative colony.

(11) FIG. 10 shows that pRISM and pRISM-G constructs are invasive and comparable to SMIP and SMIP-G constructs.

DETAILED DESCRIPTION

(12) Reference will now be made in detail to various exemplary embodiments. It is to be understood that the following detailed description is provided to give the reader a fuller understanding of certain embodiments, features, and details of aspects of the invention, and should not be interpreted as a limitation of the scope of the invention. In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

(13) *Shigella* is a genus of Gram-negative bacteria belonging to the family Enterobacteriaceae and the etiologic agent of bacillary dysentery or shigellosis, the symptoms of which include abdominal pain, diarrhea, fever, vomiting, and blood or mucus in the stool. There are four species of *Shigella*: *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei* (also referred to as serogroups A, B, C, and D, respectively). *Shigella* is transmitted by a fecal-oral route, typically through contaminated food or water. *Shigella* is very closely related to enteroinvasive *Escherichia coli* (EIEC), which also cause dysentery, with recent evidence suggesting that *Shigella* and EIEC are derived from multiple origins of *E. coli* and form a single pathovar. Yang et al., (2005) Nucleic Acids Res., 33 (19): 6445-58. Certain O-antigens associated with EIEC are identical to those found in *Shigella* spp. and many plasmid-associated virulence determinants are common to both EIEC and *Shigella*. Id. *Shigella* and EIEC invade colonic epithelial multiply intracellularly, and spread to neighboring cells. The genetic elements responsible for these invasive properties are located on the bacterial chromosome and on a large (about 220 kb) plasmid called the invasion or virulence plasmid that is unique to the virulent strains of *Shigella* and EIEC. *Shigella* variants that have lost the invasion plasmid also lose their invasive properties. In addition to the invasion plasmid, many chromosomal genes also contribute to virulence, including set1A, set1B (enterotoxin), iucA-D (aerobactin production and transport), iutA (iron uptake), pic (serine protease/mucinase), and sigA (protease).

(14) Virulence of *Shigella* is dependent on temperature. At 37° C. *Shigella* are fully virulent, whereas at 30° C. *Shigella* lose the ability to both invade epithelial cells and provoke keratoconjunctivitis in guinea pigs. Maurelli et al., (1984) Infect. Immun. 43 (1): 195-201. When *Shigella* grows at 37° C., the VirF protein induces the expression of the VirB protein, which in turn activates the ipa, mxi, and spa promoters, causing the activation of the spa and mxi operons. This results in the synthesis of a protein complex called the Mxi-Spa translocon. Contact between the bacterium and the host epithelial cell activates the translocon, leading to the secretion of invasion plasmid antigens (Ipa). IpaA, IpaB, and IpaC form a complex that interacts with the cellular membrane and induces a signal cascade that causes the bacterium to be internalized into the cytoplasm via an endosome. The Ipa proteins also mediate the release of the bacterium from the endosome.

(15) A cell entry region of about 30 kb is generally conserved in invasion plasmids and includes the mxi-spa gene locus, the virB gene, the ipa gene locus, and the ipg gene locus. Yang et al., (2005) Nucleic Acids Res., 33 (19) 6445-58. See FIG. 5. The cell entry region is bracketed by insertion sequence 100 and insertion sequence 600 in all invasion plasmids, suggesting the transmission of a common invasion plasmid in *Shigella* or the transmission of a cell entry region to all invasion plasmids from a common source. Yang et al., (2005) Nucleic Acids Res., 33 (19): 6445-58. The cell entry region has the markings of a typical pathogenicity island (PAI), such as G+C content distinctly different from the surrounding genes, indicating genetic instability. The remaining .about. 190 kb of virulence plasmid DNA sequence has a mosaic character. It contains genes related to IncFIIA-type plasmids. In addition, the plasmid is littered with copies of IS elements and remnants of IS elements that account for 53% of the open reading frames on the plasmid as well as a class of virulence genes known as the osp genes. These virulence genes encode secreted effector molecules that are transported by the type III secretion system (T3SS). Given the unusual mosaic makeup of the virulence plasmid, it is likely that it was formed by sequential gene acquisition and possibly gene duplication and rearrangement. The presence of three separate and distinct ospD genes on the *S. flexneri* 2a plasmid is evidence that argues for gene duplication after gene acquisition. In any event, it is apparent that the virulence plasmid present in contemporary strains of *Shigella* spp. arose in a step-wise fashion.

(16) In addition to invasion, a fully virulent strain of *Shigella* has several other hallmark phenotypes, including intracellular replication, intercellular spread, the ability to induce apoptosis in macrophages, the ability to protect a host mammalian cell from apoptosis, the ability to signal

polymorphonuclear leukocyte migration across a model monolayer of polarized epithelial cells, and the ability to generate a strong inflammatory reaction in the guinea pig model for keratoconjunctivitis (Sereny test). Post-invasion phenotypes are important for full virulence and the state of the art for identifying genes responsible for these phenotypes has been to inactivate a gene and test for loss or reduction of virulence phenotypes.

(17) The present disclosure provides a recombinant vector comprising the cell entry region of a *Shigella* or EIEC invasion plasmid and a virF gene from a *Shigella* or EIEC invasion plasmid and optionally a selection gene. In one embodiment, the cell entry region is from a *Shigella flexneri* invasion plasmid. In yet another embodiment, the recombinant vector comprises an ipa gene locus from a *Shigella* or EIEC invasion plasmid, an ipg gene locus from a *Shigella* or EIEC invasion plasmid, a virB gene from a *Shigella* or EIEC invasion plasmid, a mxi-spa gene locus from a *Shigella* or EIEC invasion plasmid, and a virF gene from a *Shigella* or EIEC invasion plasmid. The recombinant vector is capable of directing the expression of genes that have been inserted into the vector. Thus, in certain aspects, the recombinant vector further comprises a nucleic acid encoding one or more foreign proteins, including, for example, one or more bacterial viral, fungal, parasitic, or mammalian proteins, or an immunogenic portion thereof. The recombinant vector can be used to express any protein of interest. In one embodiment, the protein is from an enterohemorrhagic or enterotoxigenic *E. coli*, including, but not limited to the intimin protein, the colonization factor antigen I fimbrial adhesion, or the heat labile toxin (LT) subunit B. In another embodiment, the protein is from *Salmonella*, including, for example, a type III secretion system-1 secreted protein.

(18) The nucleic acid encoding the one or more bacterial, viral, fungal, parasitic, or mammalian proteins, or an immunogenic portion thereof or the one or more *Shigella* or EIEC proteins, or an immunogenic portion thereof, may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures known in the art. For example, DNA may be inserted into an appropriate restriction endonuclease site(s) in the vector using techniques known in the art. As compared to other mutated *Shigella* invasion plasmids (Coster, T. S. et al., Infect. Immun. 67:3437-3443; Kotloff, K. L. et al. 2000. Infect Immun 68:1034-1039. Levine, M. M. et al., Nat Rev Microbiol 5:540-553), the recombinant vectors provided in this application contain a substantially smaller portion of the invasion plasmid. In one embodiment, the recombinant vector comprises no more than about 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 kilobase pairs of a *Shigella* or EIEC invasion plasmid. Preferably the recombinant vector comprises no more than about 30-50 kilobase pairs of the invasion plasmid. The recombinant vectors disclosed herein can also be much smaller in overall size than the mutated invasion plasmids generated using a conventional approach. Thus, in certain embodiments, the recombinant vector has no more than about 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200 kilobase pairs. Preferably the recombinant vector has no more than about 40-100 kilobase pairs. More preferably the recombinant vector has no more than about 40-60 kilobase pairs.

(19) Suitable vectors may be chosen or constructed to contain appropriate regulatory sequences, including promoter sequences, terminator sequences, enhancer sequences, a selection gene, and other sequences. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, kanamycin, chloramphenicol, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase. Such selection genes are well known in the art. Construction of suitable vectors containing one or more of these regulatory sequences employs standard ligation techniques which are known to the skilled artisan. See e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press (2001). Many established techniques used with vectors, including the manipulation, preparation, mutagenesis, sequencing, and transfection of DNA, are described in Current Protocols in Molecular

Biology, Ausubel et al. eds., John Wiley & Sons (2010).

(20) The invention further provides a host cell that comprises at least one recombinant vector described herein. Systems for cloning and expressing polypeptides in a variety of host cells are known in the art. Suitable host cells include mammalian cells, insect cells, plant cells, yeast cells, or prokaryotic cells, e.g., *E. coli*. Preferably the host cell is a bacterium. In some embodiments, the host cell is a *Shigella* (e.g., *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*) or EIEC bacterium comprising the recombinant vector described in this application. In one embodiment, the *Shigella* or EIEC bacterium is a plasmid-cured bacterium that does not contain an invasion plasmid. A further aspect of the disclosure provides a method of introducing the recombinant vector into a host cell. For eukaryotic cells, suitable transfection techniques may include calcium phosphate, DEAE-Dextran, electroporation, or liposome-mediated transfection. For bacterial cells, suitable techniques may include, but are not limited to, calcium chloride transformation, electroporation, and transfection using bacteriophage. These techniques are well known in the art. See e.g., Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons (2010). DNA introduction may be followed by a selection method (e.g., antibiotic resistance) to select cells that contain the vector.

(21) The vectors and bacterial strains disclosed in this invention provide an improved platform for developing an attenuated vaccine strain of *Shigella* or EIEC that achieves the balance between immunogenicity and reduced reactogenicity in a subject. In one embodiment, the attenuated strain is a plasmid-cured *Shigella* or EIEC comprising a recombinant vector as described in this application (hereinafter referred to as “vaccine strain”). As noted above, in one embodiment, the recombinant vector of the vaccine strain further comprises a nucleic acid encoding one or more foreign proteins, including, for example, one or more bacterial, viral, fungal, parasitic, or mammalian proteins, or an immunogenic portion thereof. As noted above, in another embodiment, the recombinant vector of the vaccine strain further comprises a nucleic acid encoding one or more *Shigella* or EIEC proteins, or an immunogenic portion thereof. Also disclosed is a composition comprising the vaccine strain, at least one pharmaceutically acceptable excipient, and optionally an adjuvant (hereinafter referred to as “vaccine composition”).

(22) The pharmaceutically acceptable excipient can be chosen from, for example, diluents such as starch, microcrystalline cellulose, dicalcium phosphate, lactose, sorbitol, mannitol, sucrose, methyl dextrans; binders such as povidone, hydroxypropyl methylcellulose, dihydroxy propylcellulose, and sodium carboxymethylcellulose; and disintegrants such as crospovidone, sodium starch glycolate, croscarmellose sodium, and mixtures of any of the foregoing. The pharmaceutically acceptable excipient can further be chosen from lubricants such as magnesium stearate, calcium stearate, stearic acid, glyceryl behenate, hydrogenated vegetable oil, glycerine fumerate and glidants such as colloidal silicon dioxide, and mixtures thereof. In some embodiments of the present invention, the pharmaceutically acceptable excipient is chosen from microcrystalline cellulose, starch, talc, povidone, crospovidone, magnesium stearate, colloidal silicon dioxide, sodium dodecyl sulfate, and mixtures of any of the foregoing. The excipients of the present invention can be intragranular, intergranular, or mixtures thereof.

(23) The vaccine composition can be formulated as freeze-dried or liquid preparations according to any means suitable in the art. Non-limiting examples of liquid form preparations include solutions, suspensions, syrups, slurries, and emulsions. Suitable liquid carriers include any suitable organic or inorganic solvent, for example, water, alcohol, saline solution, buffered saline solution, physiological saline solution, dextrose solution, water propylene glycol solutions, and the like, preferably in sterile form. After formulation, the vaccine composition can be incorporated into a sterile container which is then sealed and stored at a low temperature (e.g., 4° C.), or it can be freeze dried. The vaccine composition can be formulated in either neutral or salt forms.

Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example,

hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

(24) The vaccine composition can comprise agents that enhance the protective efficacy of the vaccine, such as adjuvants. Adjuvants include any compound or compounds that act to increase an immune response to *Shigella* or to any antigen delivered by the recombinant vector, thereby reducing the quantity of antigen necessary in the vaccine, and/or the frequency of administration necessary to generate a protective immune response. Adjuvants can include for example, emulsifiers, muramyl dipeptides, pyridine, aqueous adjuvants such as aluminum hydroxide, chitosan-based adjuvants, and any of the various saponins, oils, and other substances known in the art, such as Amphigen, LPS, bacterial cell wall extracts, bacterial DNA, CpG sequences, synthetic oligonucleotides and combinations thereof (Schijns et al. (2000) Curr. Opin. Immunol. 12:456), Mycobacterialphlei (*M. phlei*) cell wall extract (MCWE) (U.S. Pat. No. 4,744,984), *M. phlei* DNA (M-DNA), and *M. phlei* cell wall complex (MCC). Compounds which can serve as emulsifiers include natural and synthetic emulsifying agents, as well as anionic, cationic and nonionic compounds. Among the synthetic compounds, anionic emulsifying agents include, for example, the potassium, sodium and ammonium salts of lauric and oleic acid, the calcium, magnesium and aluminum salts of fatty acids, and organic sulfonates such as sodium lauryl sulfate. Synthetic cationic agents include, for example, cetyltrihethylammonium bromide, while synthetic nonionic agents are exemplified by glycerylestere (e.g., glyceryl monostearate), polyoxyethylene glycol esters and ethers, and the sorbitan fatty acid esters (e.g., sorbitan monopalmitate) and their polyoxyethylene derivatives (e.g., polyoxyethylene sorbitan monopalmitate). Natural emulsifying agents include acacia, gelatin, lecithin and cholesterol.

(25) Other suitable adjuvants can be formed with an oil component, such as a single oil, a mixture of oils, a water-in-oil emulsion, or an oil-in-water emulsion. The oil can be a mineral oil, a vegetable oil, or an animal oil. Mineral oils are liquid hydrocarbons obtained from petrolatum via a distillation technique, and are also referred to in the art as liquid paraffin, liquid petrolatum, or white mineral oil. Suitable animal oils include, for example, cod liver oil, halibut oil, menhaden oil, orange roughy oil and shark liver oil, all of which are available commercially. Suitable vegetable oils, include, for example, canola oil, almond oil, cottonseed oil, corn oil, olive oil, peanut oil, safflower oil, sesame oil, soybean oil, and the like. Freund's Complete Adjuvant (PCA) and Freund's Incomplete Adjuvant (FIA) are two common adjuvants that are commonly used in vaccine preparations, and are also suitable for use in the present invention. Both FCA and FIA are water-in-mineral oil emulsions; however, FCA also contains a killed *Mycobacterium* sp. Immunomodulatory cytokines can also be used in the vaccine compositions to enhance vaccine efficacy, for example, as an adjuvant. Non-limiting examples of such cytokines include interferon alpha (IFN- α), interleukin-2 (IL-2), and granulocyte macrophage-colony stimulating factor (GM-CSF), or combinations thereof. The vaccine composition can be prepared using techniques well known to those skilled in the art including, but not limited to, mixing, sonication and microfluidation. The adjuvant can comprise from about 10% to about 80% (v/v) of the vaccine composition, more preferably about 20% to about 50% (v/v), and more preferably about 20% to about 30% (v/v), or any integer within these ranges.

(26) The vaccine composition can be administered to any animal, and preferably is a mammal such as a human, mouse, rat, hamster, guinea pig, rabbit, cat, dog, monkey, cow, horse, pig, and the like. Humans are a preferred embodiment. Administration of the vaccine composition can be by infusion or injection (e.g., intravenously, intramuscularly, intracutaneously, subcutaneously, intrathecal, intraduodenally, intraperitoneally, and the like). The vaccine composition can also be administered intranasally, vaginally, rectally, orally, intratonsillar, or transdermally. Additionally, the vaccine composition can be administered by "needle-free" delivery systems. The effective amount of the

vaccine composition may be dependent on any number of variables, including without limitation, the species, breed, size, height, weight, age, overall health of the patient, the type of formulation, or the mode or manner of administration. The appropriate effective amount can be routinely determined by those of skill in the art using routine optimization techniques and the skilled and informed judgment of the practitioner and other factors evident to those skilled in the art. Preferably, a therapeutically effective dose of the vaccine composition described herein will provide the therapeutic preventive benefit without causing substantial toxicity to the subject. The vaccine composition can be administered to a patient on any schedule appropriate to induce and/or sustain an immune response against *Shigella* or any other protein of interest. For example, patients can be administered a vaccine composition as a primary immunization as described and exemplified herein, followed by administration of a secondary immunization, or booster, to bolster and/or maintain protective immunity. The vaccine administration schedule, including primary immunization and booster administration, can continue as long as needed for the patient, for example, over the course of several years, to over the lifetime of the patient. The frequency of primary vaccine and booster administration and dose administered can be tailored and/or adjusted to meet the particular needs of individual patients, as determined by the administering physician according to any means suitable in the art. The vaccine composition may be administered prophylactically (before exposure to *Shigella*, EIEC, or other foreign pathogen) or therapeutically (after exposure to *Shigella*, EIEC, or other foreign pathogen).

(27) In another aspect, the vaccine strain or vaccine composition can be used in a method of inducing an immune response. The immune response can be induced in a naive subject who has not previously been exposed to *Shigella* or EIEC (or some other foreign pathogen). Alternatively, the immune response can be induced in a subject who has been previously exposed to *Shigella* or EIEC (or some other foreign pathogen) and used to enhance an existing immune response. In one embodiment, the method comprises administering to a subject a vaccine strain or a vaccine composition, wherein the administration of the vaccine strain or the vaccine composition induces an immune response against a protein, or immunogenic portion thereof, encoded by the recombinant vector in the vaccine strain or the vaccine composition.

(28) In certain embodiments, the recombinant vector comprises a nucleic acid encoding a foreign protein, or an immunogenic portion thereof, as discussed previously. In other embodiments, the recombinant vector comprises a nucleic acid encoding a *Shigella* or EIEC protein, or an immunogenic portion thereof, as discussed previously. Alternatively, the recombinant vector may comprise a first nucleic acid encoding a foreign protein, or immunogenic portion thereof, and a second nucleic acid encoding a *Shigella* or EIEC protein, or immunogenic portion thereof. One embodiment is directed to a method of inducing an immune response against *Shigella* or EIEC in a subject, the method comprising administering to the subject a vaccine strain or a vaccine composition in an amount sufficient to induce an immune response to *Shigella* or EIEC in the subject. In certain aspects, the vaccine strain or the vaccine composition induces significantly less transepithelial migration of polymorphonuclear neutrophils (PMNs), as compared to the 2457T *Shigella flexneri* strain or, in certain embodiments, does not induce transepithelial migration of PMNs. Transepithelial migration of PMNs can be measured using routine methods, such as those described in this application.

(29) Another embodiment is directed to a method of inducing an immune response to a bacterial, viral, fungal, parasitic, or mammalian protein in a subject, the method comprising administering to the subject a vaccine strain or a vaccine composition in an amount sufficient to induce an immune response to the bacterial, viral, fungal, parasitic, or mammalian protein in the subject. In these methods of inducing an immune response, the immune response can be measured using routine methods in the art, such as those disclosed in this application. These routine methods include, but are not limited to, measuring an antibody response, such as an antibody response directed against the protein encoded by the recombinant vector, and measuring cellular proliferation, including, for

example, by measuring tritiated thymidine incorporation or cytokine (e.g., IFN-gamma.) production.

(30) Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

Example 1 pRISM Construct

(31) Construction of a recombinant *Shigella* minimal invasion plasmid construct, PRISM (SEQ ID NO: 1.)

(32) A core plasmid called SMIP for *Shigella* minimum invasion plasmid was constructed comprising the following features, as summarized in FIG. 1 (SEQ ID NO: 1).

Example 2 pRISM-G Construct

(33) Construction of a recombinant *Shigella* minimal invasion plasmid construct, pRISM-G A core plasmid called SMIP for *Shigella* minimum invasion plasmid was constructed comprising the following features, as summarized in FIG. 2 (SEQ ID NO: 2).

(34) PRISM construct colony D1 used for DNA preparation (FIG. 3). PRISM-G construct colony D1 used for DNA preparation (FIG. 4).

(35) HeLa cell invasion assay: Live attenuated strains of *Shigella* are expected to retain the ability to invade nonphagocytic cells in order to generate protective immune responses in the host. The *Shigella* minimal plasmid constructs that invade and become intracellular will be protected from the effects of gentamicin whereas extracellular bacteria will be killed. PRISM and pRISM-G minimal plasmid constructs in a non-invasive *Shigella* background were evaluated for their invasiveness by the gentamycin protection assay with Hela cells. Alongside, wild type invasive *S. flexneri* 2a strain 2457T, non-invasive strain BS103 (positive and negative controls respectively) and SMIP and SMIP-G were also tested.

(36) Gentamicin protection assay with Hela cells was performed as follows. HeLa monolayers were grown to semiconfluence in a 75 cm.^{sup.2} flask in complete medium (cMEM) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 2 mM pyruvate. One flask was trypsinized with 0.25% trypsin and concentration of cells was adjusted to 2×10.^{sup.5} cells/ml in cMEM. Twenty four well-plates were seeded with 2 ml of Hela cells and grown overnight at 37° C. in 5% CO.₂. Log phase cultures of bacteria (grown in LB medium with appropriate antibiotics for strain maintenance if needed) were added at an estimated multiplicity of infection of 10. After addition of bacteria, the plate was centrifuged in a Sorvall swinging bucket rotor at 500×g for 5 minutes at 25° C. and the plate was incubated at 37° C. in 5% CO.₂ for 1.5 h. The plates were washed three times with Hank's balanced salt solution and then incubated with cMEM containing 50 µg/ml gentamycin for 2 h at 37° C. in 5% CO.₂. Hela cells were then lysed in a 0.1% Triton X-100 solution in PBS for 10 minutes. The bacteria in the lysate were plated on Tryptic Soy Agar plates and bacterial colonies counted after growth at 37° C. for 18 h. The figure below shows that pRISM and pRISM-G constructs are invasive and comparable to SMIP and SMIP-G constructs.

(37) *Shigella* minimal plasmid strains were tested for the ability to invade HeLa epithelial cells in a gentamicin protection assay. Invasiveness is plotted as percentages of the invasiveness of a wild-type 2457T Congo red-positive invasion-positive isolate. BS103 is a Congo red-negative invasion negative colony. Average for two colonies/strain are shown in FIG. 9.

(38) HeLa cell invasion assay: Live attenuated strains of *Shigella* are expected to retain the ability to invade nonphagocytic cells in order to generate protective immune responses in the host. The *Shigella* minimal plasmid constructs that invade and become intracellular will be protected from

the effects of gentamicin whereas extracellular bacteria will be killed. PRISM and pRISM-G minimal plasmid constructs in a non-invasive *Shigella* background were evaluated for their invasiveness by the gentamicin protection assay with Hela cells. Alongside, wild type invasive *S. flexneri* 2a strain 2457T, non-invasive strain BS103 (positive and negative controls respectively) and SMIP and SMIP-G were also tested.

(39) Gentamicin protection assay with Hela cells was performed as follows. HeLa monolayers were grown to semiconfluence in a 75 cm^{sup.2} flask in complete medium (cMEM) containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 2 mM pyruvate. One flask was trypsinized with 0.25% trypsin and concentration of cells was adjusted to 2×10^{sup.5} cells/ml in cMEM. Twenty four well-plates were seeded with 2 ml of Hela cells and grown overnight at 37° C. in 5% CO₂. Log phase cultures of bacteria (grown in LB medium with appropriate antibiotics for strain maintenance if needed) were added at an estimated multiplicity of infection of 10. After addition of bacteria, the plate was centrifuged in a Sorvall swinging bucket rotor at 500×g for 5 minutes at 25° C. and the plate was incubated at 37° C. in 5% CO₂ for 1.5 h. The plates were washed three times with Hank's balanced salt solution and then incubated with cMEM containing 50 µg/ml gentamicin for 2 h at 37° C. in 5% CO₂. Hela cells were then lysed in a 0.1% Triton X-100 solution in PBS for 10 minutes. The bacteria in the lysate were plated on Tryptic Soy Agar plates and bacterial colonies counted after growth at 37° C. for 18 h. FIG. 10 shows that pRISM and pRISM-G constructs are invasive and comparable to SMIP and SMIP-G constructs.

(40) *Shigella* minimal plasmid strains were tested for the ability to invade HeLa epithelial cells in a gentamicin protection assay. Invasiveness is plotted as percentages of the invasiveness of a wild-type 2457T Congo red-positive invasion-positive isolate. BS103 is a Congo red-negative invasion negative colony. Average for two colonies/strain are shown.

(41) Growth curves of pRISM constructs compared to the virulence plasmid-cured *Shigella* strain BS103 and SMIP and SMIP-G. PRISM and SMIP constructs were transformed into BS103 (FIGS. 5A and 5B).

(42) Immunogenicity and Efficacy of pRISM and pRISM-G Constructs:

(43) Guinea pigs were immunized ocularly on Day 0 and day 14 with ~1×10^{sup.8} CFUs of either PRISM or PRISM-G while the control group received only saline. Eyes were observed and scored for disease 4 days post-treatment. Serum and eye wash samples were collected from all the guinea pigs on Day 0 (pre-immune sample) and Day 28 (2 weeks after the second immunization) for immune responses. One month after the second immunization, the three groups were ocularly challenged with ~1×10^{sup.5} CFUs of virulent *Shigella flexneri* strain 2457T to determine vaccine efficacy. The eyes were scored for disease (intensity of keratoconjunctivitis or Sereny reaction) for 4 days post-challenge. The protective efficacy of the pRISM constructs were assessed using a scoring criteria established in the lab. The guinea pig eyes which were immunized with PRISM or PRISM-G showed an overall protective efficacy of 92% (p<0.0557) and 100% (p<0.0096) respectively compared to the control group. The guinea pigs were euthanized at the end of the scoring period on Day 56 when serum samples were collected by heart bleed.

(44) SBA is a measure of antibodies capable of killing *Shigella* in a serotype-specific manner in the presence of exogenous complement. SBA titers are used as a measure of immune response and vaccine efficacy. Serum bactericidal activity (SBA) assay was conducted on serum samples from Day 0, day 28 and Day 56 and eye wash samples of guinea pigs on Day 0 and Day 28. Results of the assays are shown below in Table 1.

(45) TABLE-US-00001

Construct	Day	0	20	20	Day	28	869	43	2991	150	Day	56	4868	234
PRISM	Day 0	20	20	Day 28	458	23	2430	122	Day 56	8956	448	Control	Day 0	20
PRISM-G	Day 0	20	20	Day 28	23	2430	122	Day 56	8956	448	Control	Day 0	20	20
	Day 28	23	0	20	Day 56	399	20							

*Killing Index when 50% of bacteria are killed by the serotype specific antibody

(46) SBA titers clearly show a serotype-specific killing response after receiving two doses of the

vaccine on day 28 in serum and mucosal samples of guinea pigs immunized with pPRISM and pRISM-G constructs.

(47) IgA and IgG to LPS and Invaplex (IVP, an aqueous extract of virulent *Shigella* containing a macromolecular complex of LPS, IpaB and IpaC) were also assayed by ELISA from serum and eyewash samples collected on Day 0, Day 28 and Day 56. Results are shown below (FIGS. 6-8).

(48) Serum IgG to LPS: On day 28 PRISM and pRISM-G immunized guinea pig sera had a 5.6 fold and 3 fold increase in mean titer respectively compared to control sera. On day 56 there was an increase in the IgG responses. PRISM sera showed 15 fold increase and pRISM-G sera showed 7.5 fold increase in mean titer compared to control.

(49) Serum IgG to IVP: pRISM sera showed 4.75 fold increase and pRISM-G sera showed 4 fold increase over control on day 56. On day 28 no significant increase was seen.

(50) Serum IgA to IVP: On day 28 PRISM sera had about 2 fold increase in mean titer over control whereas pRISM-G sera IgA titers were comparable to control. On day 56 PRISM and pRISM-G sera did not show any significant difference compared to control sera.

(51) Mucosal IgA to LPS: On day 28 PRISM and pRISM-G samples had ~25 fold increase in mean titer compared to control samples.

(52) Mucosal IgA to IVP: pRISM and pRISM-G samples had a ~2fold increase in mean titer compared to control samples.

(53) While specific aspects of the subject disclosure have been discussed, the above specification is illustrative and not restrictive. Many variations of the disclosure will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the disclosure should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

Claims

1. A recombinant *Shigella* minimal invasion plasmid construct, PRISM, comprising SEQ ID NO: 1, wherein the PRISM optionally comprises a selection gene or recombinant *Shigella* minimal invasion plasmid construct, pRISM-G, comprising SEQ ID NO: 2, wherein the pRISM-G optionally comprises a selection gene.
2. A recombinant *Shigella* minimal invasion plasmid construct of claim 1, wherein the PRISM or pRISM-G comprises a *Shigella* cell-entry region from a *Shigella flexneri* bacterium.
3. The recombinant *Shigella* minimal invasion plasmid construct of claim 1, wherein the selection gene is an antibiotic resistance gene.
4. A *Shigella* or *E. coli* bacterium comprising a recombinant *Shigella* minimal invasion plasmid construct, PRISM, comprising SEQ ID NO: 1, or a recombinant *Shigella* minimal invasion plasmid construct, pRISM-G, comprising SEQ ID NO: 2.
5. A composition comprising the *Shigella* or *E. coli* bacterium of claim 4 and a pharmaceutically acceptable excipient.
6. The composition of claim 5, further comprising an adjuvant.
7. A method of inducing an immune response in a subject, the method comprising administering a composition comprising a recombinant *Shigella* minimal invasion plasmid construct, pRISM, comprising SEQ ID NO: 1, or a recombinant *Shigella* minimal invasion plasmid construct, pRISM-G, comprising SEQ ID NO: 2, or a *Shigella* or *E. coli* bacterium comprising either pRISM or pRISM-G, to the subject in an amount sufficient to induce the immune response.
8. The method of claim 7, wherein the immune response is against a pathogen.
9. The method of claim 8, wherein the pathogen is a virus, bacterium, fungus or parasite.
10. The method of claim 9, wherein the pathogen is *Shigella* or *E. coli*.
11. The method of claim 7, wherein the pRISM or pRISM-G comprise a nucleic acid encoding a bacterial, viral, fungal, parasitic, or mammalian protein of interest, or an immunogenic portion

thereof.

12. The *Shigella* or *E. coli* bacterium of claim 4, wherein the pRISM or pRISM-G comprise a nucleic acid encoding a bacterial, viral, fungal, parasitic, or mammalian protein of interest, or an immunogenic portion thereof.
