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DESCRIPTION

FIELD OF THE INVENTION

[0001] The invention is in the field of vaccines, particularly vaccines for Noroviruses. In addition, the invention relates to methods of preparing vaccine compositions and methods of inducing an immunogenic response.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was produced with government support from the US Army Medical Research and Material Command, under contract numbers DAMD17-01-C-0400 and W81XWH-05-C-0135. The government may have certain rights to the invention.

BACKGROUND OF THE INVENTION

[0003] Noroviruses are non-cultivatable human Caliciviruses that have emerged as the single most important cause of epidemic outbreaks of nonbacterial gastroenteritis *(Glass et al.,* 2000; Hardy *et al.,* 1999). The clinical significance of Noroviruses was under-appreciated prior to the development of sensitive molecular diagnostic assays. The cloning of the prototype genogroup I Norwalk virus (NV) genome and the production of virus-like particles (VLPs) from a recombinant Baculovirus expression system led to the development of assays that revealed widespread Norovirus infections (Jiang *et al.* 1990; 1992).

[0004] Noroviruses are single-stranded, positive sense RNA viruses that contain a non-segmented RNA genome. The viral genome encodes three open reading frames, of which the latter two specify the production of the major capsid protein and a minor structural protein, respectively (Glass *et al.* 2000). When expressed at high levels in eukaryotic expression systems, the capsid protein of NV, and certain other Noroviruses, self-assembles into VLPs that structurally mimic native Norovirus virions. When viewed by transmission electron microscopy, the VLPs are morphologically indistinguishable from infectious virions isolated from human stool samples.

[0005] Immune responses to Noroviruses are complex, and the correlates of protection are just now being elucidated. Human volunteer studies performed with native virus demonstrated that mucosally-derived memory immune responses provided short-term protection from infection and suggested that vaccine-mediated protection is feasible (Lindesmith *et al.* 2003; Parrino *et al.* 1997; Wyatt *et al.,* 1974).

[0006] Although Norovirus cannot be cultivated *in vitro,* due to the availability of VLPs and their ability to be produced in large quantities, considerable progress has been made in defining the antigenic and structural topography of the Norovirus capsid. VLPs preserve the authentic confirmation of the viral capsid protein while lacking the infectious genetic material. Consequently, VLPs mimic the functional interactions of the virus with cellular receptors, thereby eliciting an appropriate host immune response while lacking the ability to reproduce or cause infection. In conjunction with the NIH, Baylor College of Medicine studied the humoral, mucosal and cellular immune responses to NV VLPs in human volunteers in an academic, investigator-sponsored Phase I clinical trial. Orally administered VLPs were safe and immunogenic in healthy adults (Ball *et al.* 1999; Tacket *et al.* 2003). At other academic centers, preclinical experiments in animal models have demonstrated enhancement of immune responses to VLPs when administered intranasally with bacterial exotoxin adjuvants (Guerrero *et al.* 2001; Nicollier-Jamot *et al.* 2004; Periwal *et al.* 2003). Collectively, these data suggest that a vaccine consisting of properly formulated VLPs represents a viable strategy to immunize against Norovirus infection.

[0007] EP 1 186 890 describes the use of Norovirus VLPs and Freud's adjuvant to formulate vaccine formulations. US 5 861 241, WO 92/16543, US 2005/155113, EP 2 360 175 and Singh et al. (Vaccine, 2006) disclose the use of aluminium salts and TLR as adjuvants.

SUMMARY

[0008] The current invention is defined, *inter alia,* by the following items:

1. 1. A composition comprising at least one Norovirus antigen and at least one adjuvant; wherein the adjuvant is selected from the group consisting of an adjuvant that is an aluminum salt and an adjuvant that is a toll-like receptor (TLR) agonist; wherein the composition is formulated as a liquid; and wherein the content of the at least one Norovirus antigen in the composition is from about 1 µg to about 200 µg per dose.
2. 2. The composition of item 1, wherein the content of the at least one Norovirus antigen in the composition is from about 1 µg to 100 µg per dose.
3. 3. The composition of item 1 or 2, wherein the content of the at least one Norovirus antigen in the composition is from about 1 µg to 50 µg per dose.
4. 4. The composition of any one of items 1 - 3, wherein said Norovirus antigen comprises peptides, proteins, Norovirus virus-like particles (VLPs), denatured VLPs, capsid monomers, capsid multimers, aggregates, or mixtures thereof.
5. 5. The composition of item 4, wherein said Norovirus is a Norovirus genogroup I or genogroup II viral strain.
6. 6. The composition of item 4 or item 5, wherein said Norovirus VLPs comprise a VP1 and/or VP2 capsid protein.
7. 7. The composition of item 4, wherein the VLPs are monovalent VLPs or multivalent VLPs.
8. 8. The composition of item 4, wherein the Norovirus VLPs are produced in a recombinant cellular expression system or a baculovirus-infected cellular expression system.
9. 9. The composition of item 1, further comprising a second Norovirus antigen.
10. 10. The composition of item 9, wherein said first and second Norovirus antigens are monovalent VLPs from different genogroups.
11. 11. The composition of item 10, wherein said first Norovirus antigen is a monovalent VLP comprising at least one capsid protein from a genogroup I Norovirus and said second Norovirus antigen is a monovalent VLP comprising at least one capsid protein from a genogroup II Norovirus.
12. 12. The composition of item 11, wherein the genogroup II Norovirus is a GII.4 Norovirus.
13. 13. The composition of item 1, wherein the composition is formulated for parenteral administration.
14. 14. The composition of item 1, further comprising mineral oil.
15. 15. The composition of item 1 for use in a method for inducing an immune response to Norovirus in a mammal.

[0009] The present disclosure provides antigenic and vaccine formulations comprising a Norovirus antigen. In one embodiment, the formulations further comprise at least one adjuvant. The Norovirus antigen can be derived from genogroup I or genogroup II viral sequences or a consensus viral sequence. The Norovirus formulations comprise antigenic peptides, proteins or virus-like particles (VLPs). In one embodiment, the VLPs may be denatured. In another embodiment, the antigenic peptides and proteins are selected from the group consisting of capsid monomers, capsid multimers, protein aggregates, and mixtures thereof. In another embodiment, the Norovirus antigen is present in a concentration from about 0.01% to about 80% by weight. The dosage of Norovirus antigen is present in an amount from about 1 µg to about 100 mg per dose.

[0010] In another embodiment, the Norovirus VLPs are recombinant VLPs produced in an expression system using a Norovirus nucleic acid sequence, which encodes at least one capsid protein or fragment thereof. The capsid protein is selected from the group consisting of VP1 and VP2 or a combination thereof. The expression system can be a recombinant cellular expression system such as a yeast, bacterial, insect, mammalian expression system, or a baculovirus-infected cellular expression system.

[0011] In still another embodiment, the composition further comprises a delivery agent, which functions to enhance antigen uptake by providing a depot effect, increase antigen retention time at the site of delivery, or enhance the immune response through relaxation of cellular tight junctions at the delivery site. The delivery agent can be a bioadhesive, preferably a mucoadhesive selected from the group consisting of glycosaminoglycans (e.g., chondroitin sulfate, dermatan sulfate chondroitin, keratan sulfate, heparin, heparan sulfate, hyaluronan), carbohydrate polymers (e.g., pectin, alginate, glycogen, amylase, amylopectin, cellulose, chitin, stachyose, unulin, dextrin, dextran), cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides (including mucin and other mucopolysaccharides)cellulose derivatives (e.g., hydroxypropyl methylcellulose, carboxymethylcellulose), proteins (*e.g.* lectins, fimbrial proteins), and deoxyribonucleic acid. Preferably, the mucoadhesive is a polysaccharide. More preferably, the mucoadhesive is chitosan, or a mixture containing chitosan, such as a chitosan salt or chitosan base.

[0012] In yet another embodiment, the present disclosure provides a composition further comprising an adjuvant. The adjuvant may be selected from the group consisting of toll-like receptor (TLR) agonists, monophosphoryl lipid A (MPL®), synthetic lipid A, lipid A mimetics or analogs, aluminum salts, cytokines, saponins, muramyl dipeptide (MDP) derivatives, CpG oligos, lipopolysaccharide (LPS) of gram-negative bacteria, polyphosphazenes, emulsions, virosomes, cochleates, poly(lactide-co-glycolides) (PLG) microparticles, poloxamer particles, microparticles, endotoxins, for instance bacterial endotoxins and liposomes.

Preferably, the adjuvant is a toll-like receptor (TLR) agonist. More preferably, the adjuvant is MPL®.

[0013] The compositions of the present disclosure may be provided as a liquid formulation or a dry powder formulation. Dry powder formulations as described herein may contain an average particle size from about 10 to about 500 micrometers in diameter. In one embodiment, the composition is an antigenic formulation. In another embodiment, the composition is formulated for administration as a vaccine. Suitable routes of administration include mucosal, intramuscular, intravenous, subcutaneous, intradermal, subdermal, or transdermal. In particular, the route of administration may be intramuscular or mucosal, with preferred routes of mucosal administration including intranasal, oral, or vaginal routes of administration. In another embodiment, the composition is formulated as a nasal spray, nasal drops, or dry powder, wherein the formulation is administered by rapid deposition within the nasal passage from a device containing the formulation held close to or inserted into the nasal passageway. In another embodiment, the formulation is administrated to one or both nostrils.

[0014] The present disclosure also provides methods for generating an immune response to Norovirus in a subject, comprising administering to the subject an antigenic formulation or a vaccine comprising the Norovirus composition. In one embodiment, the antigenic formulations and vaccines comprising the Norovirus composition find use in generating antibodies to one or more Norovirus antigens. In another embodiment, the Norovirus vaccine formulations may be used to treat Norovirus infections.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Figure 1 illustrates an *in vitro* antigen specific proliferation assay of murine cervical lymph node cells following *in vivo* intranasal immunization with 10 µg VLP.

Figure 2 illustrates *in vitro* antigen-specific proliferation assay of splenocytes following *in vivo* intranasal immunization with 10 µg VLP.

Figure 3 illustrates *in vitro* antigen-specific proliferation assay of splenocytes following *in vivo* intraperitoneal immunization with 25 µg VLP.

Figure 4 illustrates VLP-specific IgG or IgA from antibody secreting cells (ASCs) measured by ELISPOT assay.

Figure 5 illustrates VLP-specific IgG measured by ELISA.

Figure 6 illustrates the result of a potency assay for serum IgG response against Norwalk VLPs.

Figure 7 depicts the results of a potency assay comparing serum IgG responses against Norwalk VLPs in mice immunized with either a liquid formulation of the antigen or a formulation reconstituted from dry powder. The graph shows potency versus concentration of Norwalk VLPs in the different formulations.

Figure 8 shows the serum IgG response in rabbits on day 21 (left panel) and day 42 (right panel) following administration of different formulations of Norovirus VLP vaccine.

Figure 9 illustrates the serum IgG response in rabbits immunized intranasally with either a liquid formulation or a dry powder formulation of Norwalk VLPs.

Figure 10 depicts the stability of dry powder formulation as measured by quantitative SDS-PAGE analysis and size exclusion chromatography (SEC). Regression analysis indicates no statistical trends in either the total or intact µg VLP per 10 mg dry powder over 1 year. The percent aggregate is a calculation assuming that VLP protein not detected by SEC, compared to the total VLP protein by quantitative SDS-PAGE, is aggregated.

Figure 11 illustrates the results of an ELISA assay of anti-Norovirus antibody response in mice immunized i.p. with multiple Norovirus antigens. The thin arrows indicate booster injections with formulations containing only Norwalk VLPs. The thick arrows denote booster injections with formulations containing both Norwalk and Houston VLPs.

Figure 12 illustrates an ELISA assay of anti-Norovirus antibody response in mice immunized i.p. with either Norwalk VLPs, Houston VLPs, or a combination of Norwalk and Houston VLPs.

Figure 13 shows the presence of Norwalk VLP-specific long-lived plasma cells in splenocytes **(A),** cervical lymph nodes **(B),** and bone marrow (C) in mice 114 days after intranasal immunization with Norwalk VLPs in mice.

Figure 14 depicts the Norwalk-specific memory B cell response in splenocytes of mice immunized intranasally with Norwalk VLPs. **Panel A** shows IgA antibody secreting cells on day 0 (left graph) and day 4 in culture with Norwalk VLPs (right graph). **Panel B** shows the IgG antibody secreting cells on day 0 (left graph) and day 4 in culture with Norwalk VLPs (right graph). The difference in the number of cells between day 0 and day 4 indicates the level of memory B cell expansion and differentiation.

Figure 15 shows the ELISPOT assay results of peripheral blood mononuclear cells isolated from rabbits immunized intranasally with a Norwalk VLP vaccine formulation. The left panel shows the number of Norwalk VLP-specific antigen secreting cells (ASCs) at day 0 (day of tissue harvest), while the right panel illustrates the number of Norwalk VLP-specific ASCs after 4 days in culture with Norwalk VLPs. The difference in the number of cells between day 0 and day 4 indicates the memory B cell response.

Figure 16 shows the ELISPOT assay results of splenocytes harvested from rabbits immunized intranasally with a Norwalk VLP vaccine formulation. The left panel shows the number of Norwalk VLP-specific antigen secreting cells (ASCs) at day 0 (day of tissue harvest), while the right panel illustrates the number of Norwalk VLP-specific ASCs after 4 days in culture with Norwalk VLPs. The difference in the number of cells between day 0 and day 4 indicates the memory B cell response.

Figure 17 shows the ELISPOT assay results of bone marrow cells harvested from the tibias of rabbits immunized intranasally with a Norwalk VLP vaccine formulation. The left panel shows the number of Norwalk VLP-specific antigen secreting cells (ASCs) at day 0 (day of tissue harvest), while the right panel illustrates the number of Norwalk VLP-specific ASCs after 4 days in culture with Norwalk VLPs. The presence of ASCs at day 0 indicates the presence of long-lived plasma cells. The difference in the number of cells between day 0 and day 4 indicates the memory B cell response.

Figure 18 shows the ELISPOT assay results of mesenteric lymph node cells harvested from rabbits immunized intranasally with a Norwalk VLP vaccine formulation. **Panel A** shows IgG positive antibody secreting cells (ASCs) specific for Norwalk VLPs. **Panel B** shows IgA positive ASCs specific for Norwalk VLPs. The left panels show the number of Norwalk VLP-specific ASCs at day 0 (day of tissue harvest), while the right panels illustrate the number of Norwalk VLP-specific ASCs after 4 days in culture with Norwalk VLPs. The presence of ASCs at day 0 indicates the presence of long-lived plasma cells. The difference in the number of cells between day 0 and day 4 indicates the memory B cell response.

Figure 19 illustrates *in vitro* antigen-specific proliferation assay of splenocytes following *in vivo* intranasal immunization in rabbits. The left panel shows T cell proliferation upon restimulation with Norwalk VLPs in unfractionated splenocytes, while the right panel shows CD4+ T cell proliferation upon restimulation with Norwalk VLPs.

DETAILED DESCRIPTION DISCLOSURE

[0016] The present disclosure relates to Norovirus antigenic and vaccine compositions and methods of preparing the compositions. In particular, the present disclosure provides a composition that comprises a Norovirus antigen and at least one adjuvant. Additionally or alternatively, the composition may further comprise at least one delivery agent. The disclosure also provides methods of administering the composition to an animal to produce an immune response or generate antibodies to Norovirus antigens.

Norovirus Antigens

[0017] The disclosure provides a composition comprising one or more Norovirus antigens. By "Norovirus," "Norovirus (NOR)," "norovirus," and grammatical equivalents herein, are meant members of the genus Norovirus of the family Caliciviridae. In some embodiments, a Norovirus can include a group of related, positive-sense single-stranded RNA, nonenveloped viruses that can be infectious to human or non-human mammalian species. In some embodiments, a Norovirus can cause acute gastroenteritis in humans. Noroviruses also can be referred to as small round structured viruses (SRSVs) having a defined surface structure or ragged edge when viewed by electron microscopy. Included within the Noroviruses are at least four genogroups (GI-IV) defined by nucleic acid and amino acid sequences, which comprise 15 genetic clusters. The major genogroups are GI and GII. GIII and GIV are proposed but generally accepted. Representative of GIII is the bovine, Jena strain. GIV contains one virus, Alphatron, at this time. For a further description of Noroviruses see Vinje et al. J. Clin. Micro. 41:1423-1433 (2003). By "Norovirus" also herein is meant recombinant Norovirus virus-like particles (rNOR VLPs). In some embodiments, the recombinant Norovirus VLPs are produced in an expression system using a Norovirus nucleic acid sequence, which encodes at least one capsid protein or fragment thereof. In other embodiments, recombinant expression of at least the Norovirus capsid protein encoded by ORF2 in cells, e.g., from a baculovirus vector in Sf9 cells, can result in spontaneous self-assembly of the capsid protein into VLPs. In yet other embodiments, recombinant expression of at least the Norovirus proteins encoded by ORF1 and ORF2 in cells, e.g., from a baculovirus vector in Sf9 cells, can result in spontaneous self-assembly of the capsid protein into VLPs. The Norovirus nucleic acid sequence may also be a consensus sequence comprising various Norovirus strains or a synthetic construct modified to enhance yields or stability, or improve antigenic or immunogenic properties of the encoded antigen. VLPs are structurally similar to Noroviruses but lack the viral RNA genome and therefore are not infectious. Accordingly, "Norovirus" includes virions that can be infectious or non-infectious particles, which include defective particles.

[0018] ! Non-limiting examples of Noroviruses include Norwalk virus (NV, GenBank M87661, NP_{056821}), Southampton virus (SHV, GenBank L07418), Desert Shield virus (DSV, U04469), Hesse virus (HSV), Chiba virus (CHV, GenBank AB042808), Hawaii virus (HV, GenBank U07611), Snow Mountain virus (SMV, GenBank U70059), Toronto virus (TV, Leite et al., Arch. Virol. 141:865-875), Bristol virus (BV), Jena virus (JV, AJ01099), Maryland virus (MV, AY032605), Seto virus (SV, GenBank AB031013), Camberwell (CV, AF145896), Lordsdale virus (LV, GenBank X86557), Grimsby virus (GrV, AJ004864), Mexico virus (MXV, GenBank U22498), Boxer (AF538679), C59 (AF435807), VA115 (AY038598), BUDS (AY660568), Houston virus (HoV), Minerva strain (EF126963.1), Laurens strain (EF126966.1), MOH (AF397156), Parris Island (PiV; AY652979), VA387 (AY038600), VA207 (AY038599), and Operation Iraqi Freedom (OIF, AY675554). The nucleic acid and corresponding amino acid sequences of each are all incorporated by reference in their entirety. In some embodiments, a cryptogram can be used for identification purposes and is organized: host species from which the virus was isolated/genus abbreviation/species abbreviation/strain name/year of occurrence/country of origin. *(*Green et al., Human Caliciviruses, in Fields Virology Vol. 1 841-874 (Knipe and Howley, editors-in-chief, 4th ed., Lippincott Williams & Wilkins 2001)). Use of a combination of Norovirus genogroups such as a genogroup I.1 (Norwalk virus) and II.4 (Houston virus) or other commonly circulating strains, or synthetic constructs representing combinations or portions thereof are preferred in some embodiments. New strains of Noroviruses are routinely identified (Centers for Disease Control, Morbidity and Mortality Weekly Report, 56(33):842-846 (2007)) and consensus sequences of two or more viral strains may also be used to express Norovirus antigens.

[0019] The Norovirus antigen may be in the form of peptides, proteins, or virus-like particles (VLPs). In a preferred embodiment, the Norovirus antigen comprises VLPs. As used herein, "virus-like particle(s) or VLPs" refer to a virus-like particle(s), fragment(s), aggregates, or portion(s) thereof produced from the capsid protein coding sequence of Norovirus and comprising antigenic characteristic(s) similar to those of infectious Norovirus particles. Norovirus antigens may also be in the form of capsid monomers, capsid multimers, protein or peptide fragments of VLPs, or aggregates or mixtures thereof. The Norovirus antigenic proteins or peptides may also be in a denatured form, produced using methods known in the art.

[0020] Norovirus antigens may also include variants of the said capsid proteins or fragments thereof expressed on or in the VLPs of the invention. The variants may contain alterations in the amino acid sequences of the constituent proteins. The term "variant" with respect to a polypeptide refers to an amino acid sequence that is altered by one or more amino acids with respect to a reference sequence. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. Alternatively, a variant can have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Analogous minor variations can also include amino acid deletion or insertion, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software.

[0021] General texts which describe molecular biological techniques, which are applicable to the present disclosure, such as cloning, mutation, 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 of capsid proteins of Norovirus. Thus, the disclosure 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 VLPs of the invention. Various types of mutagenesis can be used to produce and/or isolate variant nucleic acids including concensus sequences that encode for protein molecules and/or to further modify/mutate the proteins in or on the VLPs 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.

[0022] The VLPs of the present disclosure can be formed from either the full length Norovirus capsid protein such as VP1 and/or

VP2 proteins or certain VP1 or VP2 derivatives using standard methods in the art. Alternatively, the capsid protein used to form the VLP is a truncated capsid protein. In some embodiments, for example, at least one of the VLPs comprises a truncated VP1 protein. In other embodiments, all the VLPs comprise truncated VP1 proteins. The truncation may be an N- or C-terminal truncation. Truncated capsid proteins are suitably functional capsid protein derivatives. Functional capsid protein derivatives are capable of raising an immune response (if necessary, when suitably adjuvanted) in the same way as the immune response is raised by a VLP consisting of the full length capsid protein.

[0023] VLPs may contain major VP1 proteins and/or minor VP2 proteins. Preferably each VLP contains VP1 and/or VP2 protein from only one Norovirus genogroup giving rise to a monovalent VLP. As used herein, the term "monovalent" means the antigenic proteins are derived from a single Norovirus genogroup. For example, the VLPs contain VP1 and/or VP2 from a virus strain of genogroup I (e.g., VP1 and VP2 from Norwalk virus). Preferably the VLP is comprised of predominantly VP1 proteins. In one embodiment of the disclosure, the antigen is a mixture of monovalent VLPs wherein the composition includes VLPs comprised of VP1 and/or VP2 from a single Norovirus genogroup mixed with VLPs comprised of VP1 and/or VP2 from a different Norovirus genogroup taken from multiple viral strains (e.g. Norwalk virus and Houston virus). Purely by way of example the composition can contain monovalent VLPs from one or more strains of Norovirus genogroup I together with monovalent VLPs from one or more strains of Norovirus genogroup II. Preferably, the Norovirus VLP mixture is composed of the strains of Norwalk and Houston Noroviruses.

[0024] However, in an alternative embodiment of the disclosure, the VLPs may be multivalent VLPs that comprise, for example, VP1 and/or VP2 proteins from one Norovirus genogroup intermixed with VP1 and/or VP2 proteins from a second Norovirus genogroup, wherein the different VP1 and VP2 proteins are not chimeric VP1 and VP2 proteins, but associate together within the same capsid structure to form immunogenic VLPs. As used herein, the term "multivalent" means that the antigenic proteins are derived from two or more Norovirus genogroups. Multivalent VLPs may contain VLP antigens taken from two or more viral strains. Purely by way of example the composition can contain multivalent VLPs comprised of capsid monomers or multimers from one or more strains of Norovirus genogroup I together with capsid monomers or multimers from one or more strains of Norovirus genogroup II. Preferably, the multivalent VLPs contain capsid proteins from the strains of Norwalk and Houston Noroviruses.

[0025] The combination of monovalent or multivalent VLPs within the composition preferably would not block the immunogenicity of each VLP type. In particular it is preferred that there is no interference between Norovirus VLPs in the combination of the invention, such that the combined VLP composition of the invention is able to elicit immunity against infection by each Norovirus genotype represented in the vaccine. Suitably the immune response against a given VLP type in the combination is at least 50% of the immune response of that same VLP type when measured individually, preferably 100% or substantially 100%. The immune response may suitably be measured, for example, by antibody responses, as illustrated in the examples herein.

[0026] Multivalent VLPs may be produced by separate expression of the individual capsid proteins followed by combination to form VLPs. Alternatively multiple capsid proteins may be expressed within the same cell, from one or more DNA constructs. For example, multiple DNA constructs may be transformed or transfected into host cells, each vector encoding a different capsid protein. Alternatively a single vector having multiple capsid genes, controlled by a shared promoter or multiple individual promoters, may be used. IRES elements may also be incorporated into the vector, where appropriate. Using such expression strategies, the co-expressed capsid proteins may be co-purified for subsequent VLP formation, or may spontaneously form multivalent VLPs which can then be purified.

[0027] A preferred process for multivalent VLP production comprises preparation of VLP capsid proteins or derivatives, such as VP1 and/or VP2 proteins, from different Norovirus genotypes, mixing the proteins, and assembly of the proteins to produce multivalent VLPs. The capsid proteins may be in the form of a crude extract, be partially purified or purified prior to mixing. Assembled monovalent VLPs of different genogroups may be disassembled, mixed together and reassembled into multivalent VLPs. Preferably the proteins or VLPs are at least partially purified before being combined. Optionally, further purification of the multivalent VLPs may be carried out after assembly.

[0028] Suitably the VLPs of the disclosure are made by disassembly and reassembly of VLPs, to provide homogenous and pure VLPs. In one embodiment multivalent VLPs may be made by disassembly of two or more VLPs, followed by combination of the disassembled VLP components at any suitable point prior to reassembly. This approach is suitable when VLPs spontaneously form from expressed VP1 protein, as occurs for example, in some yeast strains. Where the expression of the VP1 protein does not lead to spontaneous VLP formation, preparations of VP1 proteins or capsomers may be combined before assembly into VLPs.

[0029] Where multivalent VLPs are used, preferably the components of the VLPs are mixed in the proportions in which they are desired in the final mixed VLP. For example, a mixture of the same amount of a partially purified VP1 protein from Norwalk and Houston viruses (or other Norovirus strains) provides a multivalent VLP with approximately equal amounts of each protein.

[0030] Compositions comprising multivalent VLPs may be stabilized by solutions known in the art, such as those of WO 98/44944, WO0045841.

[0031] Compositions of the disclosure may comprise other proteins or protein fragments in addition to VP1 and VP2 proteins or derivatives. Other proteins or peptides may also be co-administered with the composition of the invention. Optionally the composition may also be formulated or co-administered with non-Norovirus antigens. Suitably these antigens can provide protection against other diseases.

[0032] The VP1 protein or functional protein derivative is suitably able to form a VLP, and VLP formation can be assessed by standard techniques such as, for example, electron microscopy and dynamic laser light scattering.

Antigen Preparation

[0033] The antigenic molecules of the present disclosure can be prepared by isolation and purification from the organisms in which they occur naturally, or they may be prepared by recombinant techniques. Preferably the Norovirus VLP antigens are prepared from insect cells such as Sf9 or H5 cells, although any suitable cells such as *E. coli* or yeast cells, for example, S. *cerevisiae, S. pombe, Pichia pastori* or other *Pichia* expression systems, mammalian cell expression such as CHO or HEK systems may also be used. When prepared by a recombinant method or by synthesis, one or more insertions, deletions, inversions or substitutions of the amino acids constituting the peptide may be made. Each of the aforementioned antigens is preferably used in the substantially pure state.

[0034] The procedures of production of norovirus VLPs in insect cell culture have been previously disclosed in U.S. Patent No. 6,942,865. Briefly, a cDNA from the 3' end of the genome containing the viral capsid gene (ORF2) and a minor structural gene (ORF3) were cloned. The recombinant baculoviruses carrying the viral capsid genes were constructed from the cloned cDNAs. Norovirus VLPs were produced in Sf9 or H5 insect cell cultures.

Adjuvants

[0035] The disclosure provides a composition comprising adjuvants for use with the Norovirus antigen. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as Bordatella pertussis or Mycobacterium tuberculosis derived proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Pifco Laboratories, Detroit, Mich.); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; and Quil A.

[0036] Suitable adjuvants also include, but are not limited to, toll-like receptor (TLR) agonists, monophosphoryl lipid A (MPL), synthetic lipid A, lipid A mimetics or analogs, aluminum salts, cytokines, saponins, muramyl dipeptide (MDP) derivatives, CpG oligos, lipopolysaccharide (LPS) of gram-negative bacteria, polyphosphazenes, emulsions, virosomes, cochleates, poly(lactide-co-glycolides) (PLG) microparticles, poloxamer particles, microparticles, and liposomes. Preferably, the adjuvants are not bacterially-derived exotoxins. Preferred adjuvants are those which stimulate a Th1 type response such as 3DMPL or QS21.

[0037] Monophosphoryl Lipid A (MPL), a non-toxic derivative of lipid A from Salmonella, is a potent TLR-4 agonist that has been developed as a vaccine adjuvant (Evans *et al.* 2003). In preclinical murine studies intranasal MPL has been shown to enhance secretory, as well as systemic, humoral responses (Baldridge *et al.* 2000; Yang *et al.* 2002). It has also been proven to be safe and effective as a vaccine adjuvant in clinical studies of greater than 120,000 patients (Baldrick *et al.,* 2002; 2004). MPL stimulates the induction of innate immunity through the TLR-4 receptor and is thus capable of eliciting nonspecific immune responses against a wide range of infectious pathogens, including both gram negative and gram positive bacteria, viruses, and parasites (Baldrick *et al.* 2004; Persing *et al.* 2002). Inclusion of MPL in intranasal formulations should provide rapid induction of innate responses, eliciting nonspecific immune responses from viral challenge while enhancing the specific responses generated by the antigenic components of the vaccine.

[0038] Accordingly, in one embodiment, the present disclosure provides a composition comprising monophosphoryl lipid A (MPL®) or 3 De-O-acylated monophosphoryl lipid A (3D-MPL®) as an enhancer of adaptive and innate immunity. Chemically 3D-

MPL® is a mixture of 3 De-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred form of 3 De-O-acylated monophosphoryl lipid A is disclosed in European Patent 0 689 454 B1 (SmithKline Beecham Biologicals SA). In another embodiment, the present disclosure provides a composition comprising synthetic lipid A, lipid A mimetics or analogs, such as BioMira's PET Lipid A, or synthetic derivatives designed to function like TLR-4 agonists.

[0039] The term "effective adjuvant amount" or "effective amount of adjuvant" will be well understood by those skilled in the art, and includes an amount of one or more adjuvants which is capable of stimulating the immune response to an administered antigen, *i.e.,* an amount that increases the immune response of an administered antigen composition, as measured in terms of the IgA levels in the nasal washings, serum IgG or IgM levels, or B and T-Cell proliferation. Suitably effective increases in immunoglobulin levels include by more than 5%, preferably by more than 25%, and in particular by more than 50%, as compared to the same antigen composition without any adjuvant.

Delivery Agent

[0040] The disclosure also provides a composition comprising a delivery agent which functions to enhance antigen uptake based upon, but not restricted to, increased fluid viscosity due to the single or combined effect of partial dehydration of host mucopolysaccharides, the physical properties of the delivery agent, or through ionic interactions between the delivery agent and host tissues at the site of exposure, which provides a depot effect. Alternatively, the delivery agent can increase antigen retention time at the site of delivery (e.g., delay expulsion of the antigen). Such a delivery agent may be a bioadhesive agent. In particular, the bioadhesive may be a mucoadhesive agent selected from the group consisting of glycosaminoglycans (e.g., chondroitin sulfate, dermatan sulfate chondroitin, keratan sulfate, heparin, heparan sulfate, hyaluronan), carbohydrate polymers (e.g., pectin, alginate, glycogen, amylase, amylopectin, cellulose, chitin, stachyose, unulin, dextrin, dextran), cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrollidone, polysaccharides (including mucin and other mucopolysaccharides) cellulose derivatives (e.g., hydroxypropyl methylcellulose, carboxymethylcellulose), proteins (e.g. lectins, fimbrial proteins), and deoxyribonucleic acid. Preferably, the mucoadhesive agent is a polysaccharide, such as chitosan, a chitosan salt, or chitosan base (e.g. chitosan glutamate).

[0041] Chitosan, a positively charged linear polysaccharide derived from chitin in the shells of crustaceans, is a bioadhesive for epithelial cells and their overlaying mucus layer. Formulation of antigens with chitosan increases their contact time with the nasal membrane, thus increasing uptake by virtue of a depot effect (Illum *et al.* 2001; 2003; Davis *et al.* 1999; Bacon *et al.* 2000; van der Lubben *et al.* 2001; 2001; Lim *et al.* 2001). Chitosan has been tested as a nasal delivery system for several vaccines, including influenza, pertussis and diphtheria, in both animal models and humans (Illum *et al.* 2001; 2003; Bacon *et al.* 2000; Jabbal-Gill *et al.* 1998; Mills *et al.* 2003; McNeela *et al.* 2004). In these trials, chitosan was shown to enhance systemic immune responses to levels equivalent to parenteral vaccination. In addition, significant antigen-specific IgA levels were also measured in mucosal secretions. Thus, chitosan can greatly enhance a nasal vaccine's effectiveness. Moreover, due to its physical characteristics, chitosan is particularly well suited to intranasal vaccines formulated as powders (van der Lubben *et al.* 2001; Mikszta *et al.* 2005; *Huang et al.* 2004).

[0042] Accordingly, in one embodiment, the present disclosure provides an antigenic or vaccine composition adapted for intranasal administration, wherein the composition includes antigen and optionally an effective amount of adjuvant. In preferred embodiments, the disclosure provides an antigenic or vaccine composition comprising Norovirus antigen such as Norovirus VLP, in combination with at least one delivery agent, such as chitosan, and at least one adjuvant, such as MPL®, CPGs, imiquimod, gardiquimod, or synthetic lipid A or lipid A mimetics or analogs.

[0043] The molecular weight of the chitosan may be between 10 kDa and 800 kDa, preferably between 100 kDa and 700 kDa and more preferably between 200 kDa and 600 kDa. The concentration of chitosan in the composition will typically be up to about 80% (w/w), for example, 5%, 10%, 30%, 50%, 70% or 80%. The chitosan is one which is preferably at least 75% deacetylated, for example 80-90%, more preferably 82-88% deacetylated, particular examples being 83%, 84%, 85%, 86% and 87% deacetylation.

Vaccine and Antigenic Formulations

[0044] The compositions of the disclosure can be formulated for administration as vaccines or antigenic formulations. As used herein, the term "vaccine" refers to a formulation which contains Norovirus VLPs or other Norovirus antigens of the present disclosure as described above, which is in a form that is capable of being administered to a vertebrate and which induces an immune response sufficient to induce a therapeutic immunity to ameliorate an infection and/or to reduce at least one symptom of an infection and/or to enhance the efficacy of another dose of VLPs or antigen. As used herein, the term "antigenic formulation" or "antigenic composition" refers to a preparation which, when administered to a vertebrate, e.g. a mammal, will induce an immune response. As used herein, the term "immune response" refers to both the humoral immune response and the cell-mediated immune response. The humoral immune response involves the stimulation of the production of antibodies by B lymphocytes that, for example, neutralize infectious agents, block infectious agents from entering cells, block replication of said infectious agents, and/or protect host cells from infection and destruction. The cell-mediated immune response refers to an immune response that is mediated by T-lymphocytes and/or other cells, such as macrophages, against an infectious agent, exhibited by a vertebrate (*e.g.*, a human), that prevents or ameliorates infection or reduces at least one symptom thereof. Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M. F. & Newman M. J.) (1995) Plenum Press New York). The compositions of the present disclosure can be formulated, for example, for delivery to one or more of the oral, gastro-intestinal, and respiratory (e.g. nasal) mucosa.

[0045] Where the composition is intended for delivery to the respiratory (e.g. nasal) mucosa, typically it is formulated as an aqueous solution for administration as an aerosol or nasal drops, or alternatively, as a dry powder, e.g. for rapid deposition within the nasal passage. Compositions for administration as nasal drops may contain one or more excipients of the type usually included in such compositions, for example preservatives, viscosity adjusting agents, tonicity adjusting agents, buffering agents, and the like. Viscosity agents can be microcrystalline cellulose, chitosan, starches, polysaccharides, and the like. Compositions for administration as dry powder may also contain one or more excipients usually included in such compositions, for example, mucoadhesive agents, bulking agents, and agents to deliver appropriate powder flow and size characteristics. Bulking and powder flow and size agents may include mannitol, sucrose, trehalose, and xylitol.

[0046] In one embodiment, the Norovirus vaccine or antigenic formulation of the present disclosure may be formulated as a dry powder containing one or more Norovirus genogroup antigen(s) as the immunogen, an adjuvant such as MPL®, a biopolymer such as chitosan to promote adhesion to mucosal surfaces, and bulking agents such as mannitol and sucrose. For example, the Norovirus vaccine may be formulated as 10 mg of a dry powder containing one or more Norovirus genogroup antigen(s) (e.g., Norwalk virus, Houston virus, Snow Mountain virus), MPL® adjuvant, chitosan mucoadhesive, and mannitol and sucrose as bulking agents and to provide proper flow characteristics. The formulation may comprise about 7.0 mg (25 to 90% w/w range) chitosan, about 1.5 mg mannitol (0 to 50% w/w range), about 1.5 mg sucrose (0 to 50% w/w range), about 25 µg MPL® (0.1 to 5% w/w range), and about 100 µg Norovirus antigen (0.05 to 5% w/w range).

[0047] Norovirus antigen may be present in a concentration of from about 0.01% (w/w) to about 80% (w/w). In one embodiment, Norovirus antigens can be formulated at dosages of about 5 µg, about 15 µg, and about 50 µg per 10 mg dry powder formulation (0.025, 0.075 and 0.25% w/w) for administration into both nostrils or about 10 µg, about 30 µg, and about 100 µg (0.1, 0.3 and 1.0% w/w) for administration into one nostril. The formulation may be given in one or both nostrils during each administration. There may be a booster administration 1 to 12 weeks after the first administration to improve the immune response. The content of the Norovirus antigens in the vaccine and antigenic formulations may be in the range of 1 µg to 100 mg, preferably in the range 1-500 µg, more preferably 5-200 µg, most typically in the range 10-100 µg. Total Norovirus antigen administered at each dose will be either about 10 µg, about 30 µg, or about 100 µg in a total of 20 mg dry powder when administered to both nostrils or 10 mg dry powder when administered to one nostril. Dry powder characteristics are such that less than 10% of the particles are less than 10 µm in diameter. Mean particle sizes range from 10 to 500 µm in diameter.

[0048] In another embodiment, the antigenic and vaccine compositions can be formulated as a liquid for subsequent administration to a subject. A liquid formulation intended for intranasal administration would comprise Norovirus genogroup antigen(s), adjuvant, and a delivery agent such as chitosan. Liquid formulations for intramuscular (i.m.) or oral administration would comprise Norovirus genogroup antigen(s), adjuvant, and a buffer, without a delivery agent (e.g., chitosan).

[0049] Preferably the antigenic and vaccine compositions hereinbefore described are lyophilized and stored anhydrous until they are ready to be used, at which point they are reconstituted with diluent, if used in a liquid formulation. Alternatively, different components of the composition may be stored separately in a kit or device (any or all components being lyophilized). The components may remain in lyophilized form for dry formulation or be reconstituted for liquid formulations, and either mixed prior to use or administered separately to the patient. For dry powder administration the vaccine or antigenic formulation may be preloaded into an intranasal delivery device or topical (e.g., dermal) delivery patch and stored until used. Preferably, such delivery device and associated packaging would protect and ensure the stability of its contents.

[0050] The lyophilization of antigenic formulations and vaccines is well known in the art. Typically the liquid antigen is freeze dried in the presence of agents to protect the antigen during the lyophilization process and to yield powders with desirable characteristics. Sugars such as sucrose, mannitol, trehalose, or lactose (present at an initial concentration of 10-200 mg/mL) are commonly used for cryoprotection and lyoprotection of protein antigens and to yield lyophilized cake or powders with desirable characteristics. Lyophilized compositions are theoretically more stable. Other drying technologies, such as spray drying or spray freeze drying may also be used. While the goal of most formulation processes is to minimize protein aggregation and degradation, the inventors have demonstrated that the presence of aggregated antigen enhances the immune response to Norovirus VLPs (see Examples 3 and 4 in animal models). Therefore, the inventors have developed methods by which the percentage of aggregation of the antigen can be controlled during the lyophilization process to produce an optimal ratio of aggregated antigen to intact antigen to induce a maximal immune response in animal models.

[0051] Thus, the disclosure also encompasses a method of making Norovirus antigen formulations comprising (a) preparing a pre-lyophilization solution comprising Norovirus antigen, sucrose, and chitosan, wherein the ratios of sucrose to chitosan are from about 0:1 to about 10:1; (b) freezing the solution; and (c) lyophilizing the frozen solution for 30-72 hours, wherein the final lyophilized product contains a percentage of said Norovirus antigen in aggregated form. The lyophilization may occur at ambient temperature, reduced temperature, or proceed in cycles at various temperatures. For illustration purposes only, lyophilization may occur over a series of steps, for instance a cycle starting at -69 °C, gradually adjusting to -24 °C over 3 hours, then retaining this temperature for 18 hours, then gradually adjusting to -16 °C over 1 hour, then retaining this temperature for 6 hours, then gradually adjusting to +34 °C over 3 hours, and finally retaining this temperature over 9 hours In one embodiment, the pre-lyophilization solution further comprises a bulking agent. In another embodiment, said bulking agent is mannitol.

[0052] Appropriate ratios of sucrose and chitosan to yield desired percentages of aggregation can be determined by the following guidelines. A pre-lyophilization mixture containing mass ratios of sucrose to chitosan in a range from about 2:1 to about 10:1 will yield a range of about 50% to 100% intact Norovirus antigen (i.e. 0% to 50% aggregated antigen) post-lyophilization depending on pre-lyophilization solution concentrations (see Example 13). Mass ratios of 0:1 sucrose to chitosan will produce less than 30% of intact Norovirus antigen (i.e. greater than 70% aggregated antigen). Omission of both sucrose and chitosan and use of only a bulking agent, such as mannitol, will produce less than 10% intact antigen (i.e. greater than 90% aggregated antigen depending on pre-lyophilization solution concentrations). Using these guidelines, the skilled artisan could adjust the sucrose to chitosan mass ratios and concentrations in the pre-lyophilization mixture to obtain the desired amount of aggregation necessary to produce an optimal immune response.

[0053] In addition, the inclusion of sucrose, chitosan, and mannitol in the pre-lyophilization solution has no negative effect on the stability of the intact Norovirus antigen over time, *i.e.* the ratio of aggregated antigen/intact antigen in the formulation does not increase when stored as a dry powder for a period of about 12 months or greater (see Example 10). Thus, this lyophilization procedure ensures stable formulations with predictable and controllable ratios of aggregated to intact Norovirus antigen.

Methods of Stimulating an Immune Response

[0054] The amount of antigen in each antigenic or vaccine formulation dose is selected as an amount which induces a robust immune response without significant, adverse side effects. Such amount will vary depending upon which specific antigen(s) is employed, route of administration, and adjuvants used. In general, the dose administered to a patient, in the context of the present disclosure should be sufficient to effect a beneficial therapeutic response in the patient over time, or to induce the production of antigen-specific antibodies. Thus, the composition is administered to a patient in an amount sufficient to elicit an immune response to the specific antigens and/or to alleviate, reduce, or cure symptoms and/or complications from the disease or infection. An amount adequate to accomplish this is defined as a "therapeutically effective dose."

[0055] For a substantially pure form of the Norovirus antigen, it is expected that each dose will comprise about 1 µg to 10 mg, preferably about 2-50 µg for each Norovirus antigen in the formulation. In a typical immunization regime employing the antigenic preparations of the present invention, the formulations may be administered in several doses (*e.g.* 1-4), each dose containing 1-100 µg of each antigen. The dose will be determined by the immunological activity the composition produced and the condition of the patient, as well as the body weight or surface areas of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side effects that may accompany the administration of a particular composition in a particular patient.

[0056] The antigenic and vaccine formulations of the present disclosure may be administered via a non-mucosal or mucosal route. These administrations may include *in vivo* administration via parenteral injection (e.g. intravenous, subcutaneous, and intramuscular) or other traditional direct routes, such as buccal/sublingual, rectal, oral, nasal, topical (such as transdermal and ophthalmic), vaginal, pulmonary, intraarterial, intraperitoneal, intraocular, or intranasal routes or directly into a specific tissue.

Alternatively, the vaccines of the disclosure may be administered by any of a variety of routes such as oral, topical, subcutaneous, mucosal, intravenous, intramuscular, intranasal, sublingual, transcutaneous, subdermal, intradermal and via suppository. Administration may be accomplished simply by direct administration using a patch, needle, catheter or related device, at a single time point or at multiple time points.

[0057] In a preferred embodiment, the antigenic and vaccine formulations of the present disclosure are administered to a mucosal surface. Immunization via the mucosal surfaces offers numerous potential advantages over other routes of immunization. The most obvious benefits are 1) mucosal immunization does not require needles or highly-trained personnel for administration, and 2) immune responses are raised at the site(s) of pathogen entry, as well as systemically (Isaka *et al.* 1999; Kozlowski *et al.* 1997; Mestecky *et al.* 1997; Wu *et al.* 1997).

[0058] In a further aspect, the disclosure provides a method of eliciting an IgA mucosal immune response and an IgG systemic immune response by administering (preferably intranasally or orally) to a mucosal surface of the patient an antigenic or vaccine composition comprising one or more Norovirus antigens, at least one effective adjuvant and /or at least one delivery agent.

[0059] The present disclosure also contemplates the provision of means for dispensing intranasal formulations of Norovirus antigens hereinbefore defined, and at least one adjuvant or at least one delivery agent as hereinbefore defined. A dispensing device may, for example, take the form of an aerosol delivery system, and may be arranged to dispense only a single dose, or a multiplicity of doses. Such a device would deliver a metered dose of the vaccine or antigenic formulation to the nasal passage. Other examples of appropriate devices include, but are not limited to, droppers, swabs, aerosolizers, insufflators (e.g. Valois Monopowder Nasal Administration Device, Bespak UniDose DP), nebulizers, and inhalers. The devices may deliver the antigenic or vaccine formulation by passive means requiring the subject to inhale the formulation into the nasal cavity. Alternatively, the device may actively deliver the formulation by pumping or spraying a dose into the nasal cavity. The antigenic formulation or vaccine may be delivered into one or both nostrils by one or more such devices. Administration could include two devices per subject (one device per nostril). Actual dose of active ingredient (Norovirus antigen) may be about 5-1000 µg. In a preferred embodiment, the antigenic or vaccine formulation is administered to the nasal mucosa by rapid deposition within the nasal passage from a device containing the formulation held close to or inserted into the nasal passageway.

[0060] The disclosure also provides a method of generating antibodies to one or more Norovirus antigens, said method comprising administration of a vaccine or antigenic formulation of the invention as described above to a subject. These antibodies can be isolated and purified by routine methods in the art. The isolated antibodies specific for Norovirus antigens can be used in the development of diagnostic immunological assays. These assays could be employed to detect a Norovirus in clinical samples and identify the particular virus causing the infection (e.g. Norwalk, Houston, Snow Mountain, etc.). Alternatively, the isolated antibodies can be administered to subjects susceptible to Norovirus infection to confer passive or short-term immunity.

[0061] As mentioned above, the vaccine formulations of the disclosure may be administered to a subject to treat symptoms of a Norovirus infection. Symptoms of Norovirus infection are well known in the art and include nausea, vomiting, diarrhea, and stomach cramping. Additionally, a patient with a Norovirus infection may have a low-grade fever, headache, chills, muscle aches, and fatigue. The disclosure encompasses a method of inducing an immune response in a subject experiencing a Norovirus infection by administering to the subject a vaccine formulation of the invention such that at least one symptom associated with the Norovirus infection is alleviated and/or reduced. A reduction in a symptom may be determined subjectively or objectively, e.g., self assessment by a subject, by a clinician's assessment or by conducting an appropriate assay or measurement (*e.g.* body temperature), including, *e.g.*, a quality of life assessment, a slowed progression of a Norovirus infection or additional symptoms, a reduced severity of Norovirus symptoms or suitable assays (e.g. antibody titer and/or T-cell activation assay). The objective assessment comprises both animal and human assessments.

Examples

[0062] The invention will now be illustrated in greater detail by reference to the specific embodiments described in the following examples. The examples are intended to be purely illustrative of the invention and are not intended to limit its scope in any way.

Example 1. Investigations into Immune Responses to Different Norovirus Antigen Forms

[0063] To investigate the efficacy of the vaccine formulations, mice were immunized intranasally (i.n.) with liquid suspension vaccine formulation by micropipette. Mice received only a single vaccine dose (prime).

[0064] For the experiment, three vaccine formulations were prepared. The first, referred to as 100% aggregate, was prepared by lyophilization of VLPs under conditions that disrupt the native structure of the VLP and induce aggregation. The second, 100% intact, was prepared with rehydrated lyophilized placebo, spiked with 100% native monodisperse VLPs from non-lyophilized VLP stock. The third formulation, 50/50 Mix, is made either by mixing the previous two formulations at a ratio of 1:1, or by lyophilizing under conditions that yield ~ 50% intact and 50% aggregated VLPs. The structural state and concentration of the intact native VLP was assayed by size exclusion high performance liquid chromatography (SE-HPLC) and ultraviolet (UV) absorbance. The total protein concentration (which includes the aggregate) of the formulations was determined by quantitative staining of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) -resolved proteins. Percent aggregated/intact was calculated as the ratio of intact native VLP to total protein.

Table 1. Mixtures shown below were prepared for Experiment 605.125, mouse i.n. liquid vaccination.

Group number	Chitosan (mg/mL)	Mannitol (mg/mL)	Sucrose (mg/mL)	MPL (mg/mL)	Norwalk VLP (mg/mL)
1	3.5	0.750	0.750	1.0	1.0
2	3.5	0.750	0.750	1.0	1.0
3	3.5	0.750	0.750	1.0	1.0
4	3.5	0.750	0.750	1.0	0
Table 1. Prime for exp 605.125 (mouse i.n.) Values indicate final concentrations of the formulations.					
Dose: 20 µL per mouse, 10 µL per nare. Group 1, 100% Agg: rehydrated 100% aggregated VLP Group 2, 100% Intact: rehydrated lyophilized placebo, spiked with 100% intact VLPs from non-lyophilized VLP stock. Group 3, 50/50 mix: 1:1 mixture of solutions from Groups 1 and 2. Group 4, Naïve: rehydrated lyophilized placebo					

[0065] This experiment measures the immune response in mice to different Norovirus VLP formulations. Groups of mice (5 per group) were vaccinated intranasally (i.n.) once with rehydrated dry powder formulations shown in Table 1. Animals vaccinated with VLP-containing formulations received the same amount of total protein. 100% Agg (100% aggregated VLP protein); 100% Intact (100% native, monodisperse VLPs); 50/50 Mix (1:1 mixture of monodisperse and aggregated VLP); Naive (no VLP protein). On day 14 following i.n. immunization, mice were euthanized, the cervical lymph nodes and spleens were harvested, and a single cell suspension was prepared for *in vitro* antigen-specific cell proliferation assays. In these assays the response of cervical lymph node cells or splenocytes were assessed to determine immunogenic responses against the antigen following *in vivo* immunization. Cervical lymph node cells or splenocytes were restimulated with either native monodisperse VLPs (native VLP, black bars) or heat-denatured VLP protein (ΔVLP, white bars) and the extent of cellular proliferation from each antigen form (100% Agg, 100% Intact, 50/50 Mix, or naïve) was measured by tritiated thymidine incorporation as indicated on the ordinate axis (CPM) (Figure 1, cervical lymph node cells; Figure 2, splenocytes).

Example 2. *In vitro* Antigen-Specific Proliferation Assay

[0066] To further investigate the potency of the vaccine formulations, mice were immunized intraperitoneally (i.p.) with liquid suspension vaccine formulation. Mice received only a single vaccine dose (prime).

[0067] Similar to Example 1, groups of mice (5 per group) were vaccinated, but this time intraperitoneally (i.p.), once with rehydrated dry powder formulations shown in Table 2. Again, animals vaccinated with VLP-containing formulations received the same amount of total protein. 100% Agg (100% aggregated VLP protein); 100% Intact (100% native, intact VLPs); 50/50 Mix (1:1 mixture of intact and aggregated VLP); Naïve (no VLP protein).

Table 2. Mixtures shown below were prepared for 605.127, mouse i.p. liquid immunization.

Group number	Chitosan (mg/mL)	Mannitol (mg/mL)	Sucrose (mg/mL)	MPL (mg/mL)	Norwalk VLP (mg/mL)
1	7	1.475	1.475	0.025	0.025
2	7	1.475	1.475	0.025	0.025
3	7	1.475	1.475	0.025	0.025
4	7	1.475	1.475	0.025	0

Group number	Chitosan (mg/mL)	Mannitol (mg/mL)	Sucrose (mg/mL)	MPL (mg/mL)	Norwalk VLP (mg/mL)
Prime for exp 605.127 (mouse i.p.) Values indicate final concentrations of the formulations and are equivalent to a single 10 mg delivery device.					
Dose: 1 mL per mouse i.p. Group 1, 100% Agg: rehydrated 100% aggregated VLP Group 2, 100% Intact: rehydrated lyophilized placebo, spiked with 100% intact VLPs from non-lyophilized VLP stock. Group 3, 50/50 mix: rehydrated from lyophilized 50/50 intact VLP/Aggregate Group 4, Naive: rehydrated lyophilized placebo					

[0068] In this assay, response of different murine cells to VLPs following *in vivo* immunization was measured. On day 14 following immunization, mice were euthanized, the spleens were harvested, and a single cell suspension was prepared. Splenocytes were restimulated with either intact, native VLPs (native VLP, dotted bars) or heat-denatured VLP protein (ΔVLP, white bars) and the extent of cellular proliferation from each antigen form (100% Agg, 100% Intact, 50/50 Mix, or naïve) was measured by tritiated thymidine incorporation as indicated on the ordinate axis (CPM) (Figure 3). These data indicate that different biophysical forms of the VLPs prepared in the vaccine formulations elicit comparable T cell responses.

Example 3. VLP-Specific ELISPOT Assay

[0069] VLP-specific antibody-secreting cell (ASC) responses were measured from mice immunized intraperitoneally with different NV-VLP formulations described in Example 2. Groups of mice (5 per group) were vaccinated i.p. once with rehydrated dry powder formulations shown in Table 2 (Example 2). Animals vaccinated with VLP-containing formulations received the same amount of total protein. 100% Agg (100% aggregated VLP protein); 100% Intact (100% native, intact VLPs); 50/50 Mix (1:1 mixture of intact and aggregated VLP); Naïve (no VLP protein). On day 14, the mice were euthanized and the cervical lymph nodes were harvested. The cervical lymph node cells were cultured overnight on native, intact VLP-coated ELISPOT plates and were developed for either IgG or IgA-specific ELISPOTS using the appropriate HRP-conjugated secondary antibodies (Figure 4). These data show that the three VLP antigen formulations all elicit an antigen-specific B cell response. The group immunized with 100% Agg VLPs exhibited the greatest immune response.

Example 4. VLP-Specific ELISA

[0070] Serum IgG levels were measured from mice immunized i.p. with different NV-VLP formulations. Groups of mice (5 per group) were vaccinated i.p. once with rehydrated dry powder formulations shown in Table 2 (Example 2). Animals vaccinated with VLP-containing formulations received the same amount of total protein. 100% Agg (100% aggregated VLP protein); 100% Intact (100% native, intact VLPs); 50/50 Mix (1:1 mixture of intact and aggregated VLP); Naive (no VLP protein). On day 14, serum was collected and assayed by ELISA for anti-VLP-specific serum IgG (Figure 5). These data correlate with the results shown in Example 3, indicating that the three VLP antigen formulations all elicit an antigen-specific B cell response. Again, the group immunized with 100% Agg VLPs showed the greatest immune response.

Example 5. Vaccine Formulations in Rabbits.

[0071] Formulations were administered intranasally (i.n.) in rabbits using the Valois Monopowder Nasal Administration Device. The dry powder formulations are shown in Tables 3 and 4.

Table 3. Formulations described below were prepared for 605.129, rabbit i.n. dry powder (DP) vaccination.

Prime formulations for exp 605.129 (Rabbit i.n.) (final amounts for DP vaccines).					
Group number	Chitosan (mg/10mg DP)	Mannitol (mg/10mg)	Sucrose (mg/10mg)	MPL (mg/10mg)	Norwalk VLP (mg/10mg)
1	7	1.475	1.475	0.025	0.025
2	7	1.475	1.475	0.025	0.025
3	7	1.475	1.475	0.025	0.025

Prime formulations for exp 605.129 (Rabbit i.n.) (final amounts for DP vaccines).					
Group number	Chitosan (mg/10mg DP)	Mannitol (mg/10mg)	Sucrose (mg/10mg)	MPL (mg/10mg)	Norwalk VLP (mg/10mg)
4	7	1.475	1.475	0.025	0
Values indicate final concentrations of the formulations based on a single device (10 mg DP) which is ½ total dose.					
Dose: 20 mg DP per animal, 10 mg per nare. Group 1, 100% Agg: 100% aggregated lyophilized VLP Group 2, 100% Intact: 100% intact lyophilized VLP Group 3, 50/50 mix: 50/50 intact/aggregate lyophilized VLP (not a mixture of 1 & 2) Group 4, Naïve: placebo					

Table 4. Formulations shown below were prepared for 605.129, rabbit i.n. dry powder (DP) vaccination.

Boost formulations for exp 605.129 (Rabbit i.n.) (final amounts for DP vaccines).					
Group number	Chitosan (mg/10mg DP)	Mannitol (mg/10mg)	Sucrose (mg/10mg)	MPL (mg/10mg)	Norwalk VLP (mg/10mg)
1	7	1.475	1.475	0.025	0.025
2	7	0	2.95	0.025	0.025
3	7	1.475	1.475	0.025	0.025
4	7	1.475	1.475	0.025	0
Values indicate final concentrations of the formulations based on a single device (10 mg DP) which is ½ total dose.					
Dose: 20 mg DP per animal, 10 mg per nare. Group 1, 100% Agg: 100% lyophilized aggregated VLP Group 2, 100% Intact: 100% intact** VLP* Group 3, 50/50 mix: 50/50 intact /aggregate lyophilized VLP (not a mixture of 1 & 2) Group 4, Naïve: lyophilized placebo *Formulated without mannitol to increase amount of intact VLP post lyophilization. **Preparation yielded only ~80% intact VLP.					

Example 6. Potency Assay of Norovirus Vaccine Formulation in Mice

[0072] Female C57B16 mice were immunized intraperitoneally (i.p.) on day 0 with different dilutions of a reconstituted Norwalk VLP dry powder vaccine (containing Norwalk VLP, MPL and chitosan). Each animal was injected with 100 µL of the formulations indicated. Serum was collected weekly and serum anti-VLP IgG measured by ELISA. Values for serum collected 3 weeks following immunization are shown in Figure 6.

[0073] The value for each individual mouse is represented, with bars indicating the group mean. Serum anti-VLP IgG values correlated with the dose of vaccine indicated. This experimental design has been refined and developed as a potency assay required for the release of GMP manufactured vaccines for human clinical trials (Figure 6).

Example 7. Potency of Liquid vs. Reconstituted Norovirus Formulations in Mice

[0074] Female C57B16 mice were immunized i.p. on day 0 with formulations that contained chitosan, mannitol, MPL, and various concentrations of Norwalk VLP (Table 5) in a volume of 100 µL. An internal standard curve was generated (groups 1-5) by solubilizing 10 mg/mL of dry powder matrix (mannitol, MPL, and chitosan) in purified water and adding the specified amounts of liquid Norwalk VLP. In contrast, the GMP VLP lots were previously lyophilized and then solubilized in 1.0 ml of purified water (groups 6-8). Serum was collected from mice on days 14, 21 and 30, and serum anti-Norwalk VLP IgG was measured by ELISA.

Table 5. Liquid and Reconstituted Norwalk Formulations used to immunize mice (i.p.).

			Calculated Potency		
Group	**Treatment**	**95% CI**	**Potency**	**Min**	**Max**
1	5 µg VLP in Placebo	0.173	58.0	39.0	86.3
2	2.5 µg VLP in Placebo	0.192	23.3	15.0	36.3

			Calculated Potency		
Group	Treatment	95% CI	Potency	Min	Max
3	1.25 µg VLP in Placebo	0.182	11.2	7.4	17.0
4	0.63 µg VLP in Placebo	0.287	5.4	2.8	10.4
5	0.31 µg VLP in Placebo	0.114	3.8	2.9	4.9
6	2.5 µg GMP lot	0.276	11.3	6.0	21.3
7	7.5 µg GMP lot	0.221	96.8	58.2	161.0
8	25 µg GMP lot	0.147	113.6	80.9	159.5

[0075] The relative potency for each formulation was calculated using the following formula: Inv Log (Ave.- Y intercept/slope). Potency is plotted against VLP concentration in the formulations and reported in relation to the standard curve generated using known amounts of VLP spiked into the matrix background (Figure 7). The results shown are representative of 3 separate serum collection time points. These data indicate that the Norwalk VLP formulation reconstituted from dry powder has an overall higher potency than the liquid formulations.

Example 8. Potency of Dry Powder Formulation in Rabbits

[0076] Forty-three female New Zealand White rabbits were intranasally (i.n.) immunized using the Valois Monopowder Nasal Administration Device with either 5 µg (Low) or 25 µg (Hi) of Norwalk VLPs ± MPL and ± chitosan formulated into dry powders. One group received the Hi dose of VLPs and MPL formulated as a liquid and administered intramuscularly (i.m.). Rabbits were vaccinated on days 0 and 21. MPL, when used, was used at the same dose as the VLPs (*i.e.,* 5 µg Norwalk VLPs and 5 µg MPL). Chitosan, when used, was 7 mg/dose.

[0077] Serum IgG specific for the Norwalk VLPs (as determined by ELISA) is shown in Figure 8. Mean values for each treatment groups are shown for day 21 (left panel, collected just prior to administration of the booster immunization) and day 42 (right panel). Values are reported in U/mL of VLP-specific IgG, with 1 U approximating 1 µg. Standard deviations are indicated by bars. All treatment groups had 6 animals, except the negative control group (3 rabbits) and the intramuscularly immunized group (4 animals). These data show that generally the higher VLP dose results in greater serum anti-VLP IgG levels. Chitosan, in particular, enhances responses to intranasal vaccines. The i.m. immunized group showed the greatest responses. However, VLP-specific IgG levels in the intranasally immunized groups were also quite robust.

Example 9. Potency of Liquid vs. Dry Norovirus Formulations given Intranasally in Rabbits

[0078] Female New Zealand White rabbits were intranasally immunized using the Valois Monopowder Nasal Administration Device with 50 µg of Norwalk VLPs + 50 µg MPL + 14 mg chitosan formulated into either a dry powder or a liquid. The vaccine content was identical, except for the physical state. Immunizations were on days 0 and 21 (weeks 0 and 3), with serum collected prior to the boost at 3 weeks, and again at 6 weeks following the initial vaccination. Serum IgG specific for Norwalk VLPs was measured by ELISA, and the results are shown in Figure 9.

[0079] Group means are indicated, with the bars representing standard deviations. The dry powder immunization group had 6 rabbits, and the liquid immunization group had 10 rabbits. Eight negative control rabbits are represented. Little difference was seen between the liquid and dry powder immunization groups at 3 weeks; however, at 6 weeks following the initial immunization, rabbits immunized with the dry powder formulation had superior serum anti-VLP IgG responses compared to the liquid immunization group.

Example 10. Stability of Norovirus Dy Powder Formulations

[0080] To investigate the stability of the dry powder VLP formulation, bulk drug product was prepared by mixing (per 10 mg drug product) 25 µg of a Genogroup I VLP in solution with 25 µg MPL, 700 µg chitosan glutamate, 1.475 mg mannitol, and 1.475 mg sucrose. The solution was lyophilized, blended with an additional 6.3 mg chitosan glutamate (per 10 mg drug product), filled into Bespak unidose devices at a nominal 10 mg of dry powder, and stored in sealed foil pouches with desiccant capsules. Total VLP content was measured using Imperial stained SDS-PAGE and scanning densitometry, while size exclusion chromatography (SEC) was used to quantify intact VLP content. These measurements indicated that, within experimental error, no change in either total or intact VLP was detectable over the 12 month period (Figure 10). Assuming that the lower VLP protein recovery by SEC, when compared to SDS-PAGE results, was due to aggregation, the calculated % aggregate did not increase with time but rather remained constant or decreased throughout the 12 months of storage. One of the more common stability issues with proteins is increased aggregation with storage. Based on the results in Figure 10, it can be concluded that the formulation results in a stable percentage of intact VLPs allowing the product to be manufactured and used over at least a one year period.

Example 11. Multiple Norovirus Antigens

[0081] Eight C57B1/6 mice (female, 9 weeks of age) were immunized intraperitoneally (IP) on days 0 and 14 with 2.5 µg Norwalk VLP formulated with 0.7 mg chitosan, 2.5 µg MPL and 0.3 mg mannitol brought to 0.1 mL with water. Two control mice were immunized with saline. On days 28 and 49, they were immunized again IP with 2.5 µg Norwalk VLP + 2.5 µg Houston VLP formulated with 0.7 mg chitosan, 2.5 µg MPL and 0.3 mg mannitol brought to 0.1 mL with water. The control mice again received saline. Serum samples were collected weekly beginning on week 5 (day 35) and analyzed by ELISA for reactivity with Norwalk VLPs or Houston VLPs. The time of boost with the Norwalk only mixtures are indicated by the thin arrows, and the Norwalk + Houston VLP mixtures are indicated by thick arrows. Individual serum IgG responses specific for Norwalk VLPs (top panel) or Houston VLPs (bottom panel) in U/mL (with 1 U approximating 1 µg of IgG) are shown. Means are indicated by bars. Note that the Y-axis scales are different, as the anti-Norwalk-responses were much more robust due to two previous immunizations on days 0 and 14 (weeks 0 and 2). However, the responses against Houston VLPs are quite robust, with a large increase appearing in the second week after the boost. These data demonstrate that specific immune responses can be generated against different antigenic strains of Norovirus VLPs in the same immunizing mixture. (Figure 11).

Example 12. Immune Response to Different Norovirus Antigens

[0082] Female C57B16 mice were immunized intraperitoneally (IP) on days 0 and 14 with 25 µg Norwalk VLP, 25 µg Houston VLP, or a combination of 25 µg of each Norwalk and Houston VLP. Serum was collected weekly and serum anti-VLP IgG measured by ELISA. Values for serum collected 4 weeks following immunization are shown in Figure 12.

[0083] VLP content of the immunizations is indicated on the X axis. The value for each individual mouse is represented, with bars indicating the group mean. Antibody levels are represented in U/mL, with 1 U approximating 1 µg of serum IgG. Values in the left panel were determined using Norwalk VLPs as the capture agent, while Houston VLPs were used to coat ELISA plates in order to measure the values on the right panel. These data show that immunization with Norwalk VLP does not lead to serum antibodies that are able to recognize Houston VLPs, or vice versa.

Example 13. Mixtures of Sucrose and Chitosan Preserve Norovirus VLP Structure in Dry Powder Formulations

[0084] The following experiments examined the effects of sucrose, chitosan, and mannitol, alone or in combination, in pre-lyophilization solutions on the native Norwalk VLP quaternary structure during lyophilization. Table 6 is a composite of several experiments showing pre-lyophilization solution concentrations of the constituents of interest, the total volume of the mixture, and the corresponding mass ratios. All solutions were manually swirled and gently vortexed to homogeneity, then shell frozen in liquid nitrogen and lyophilized external to the unit using side-arm vessels for times ranging from about 30 to 60 hours.
Table 6. Pre-lyophilization solution mixtures used for testing the effects of different concentrations and combinations of sucrose, chitosan glutamate (chitosan) and mannitol on the structure of the quaternary structure the Norwalk VLP.

Experiment and Sample	Solution concentrations of constituents pre-lyophilization (mg/mL)				Volume (mL)	Mass equivalents S = sucrose C = chitosan M = mannitol		
	Sucrose	Chitosan	Mannitol	VLP (protein)		S	C	M
LE1	0	0	100	0.83	0.30			
LE2	0	0	75.0	0.62	0.40			
LE3	0	0	50.0	0.42	0.60	0	0	1
LE4	0	0	25.0	0.21	1.20			
LE5	0	0	10.0	0.08	3.00			
LG1-LG3	0	7.83	0	0.20	1.28			
LG4-LG6	0	5.06	0	0.13	1.98	0	1	0
LG7-LG9	0	2.09	0	0.05	4.78			
LG10	19.32	1.93	0	0.05	5.18	10	1	
LG11	10.05	2.01	0	0.05	4.98	5	1	0
LG12	5.13	2.05	0	0.05	4.88	2.5	1	
LG13	9.52	0.00	0	0.09	2.63	1	0	
LJ1-LJ2	5.29	2.51	0	0.09	2.79			
LJ3-LJ4	4.17	1.98	0	0.07	3.54			
LJ5-LJ6	3.65	1.73	0	0.06	4.04			
LJ7-LJ8	2.93	1.39	0	0.05	5.04	2	1	0
LJ9-LJ10	5.25	2.49	0	0.09	2.81			
LJ11-LJ12	4.14	1.97	0	0.07	3.56			
LJ13-LJ14	3.63	1.72	0	0.06	4.06			
LIG1d - Sa	2.98	1.42	0.00	1.12	4.94	2	1	0
LIG1d - S1	12.89	6.12	0.00	1.12	2.29	2	1	0
LIG1d - S2	12.26	5.82	12.26	0.67	2.41	1	0.5	1
LIG1d - Sb	2.95	1.40	2.95	0.67	5.00	1	0.5	1
LIG1d - S3	29.32	0.00	29.32	0.83	1.01	0	0	1

[0085] Table 7 shows the results from size exclusion-high performance liquid chromatography (SE-HPLC) analysis of the lyophilized samples shown in Table 6. Lyophilized samples were reconstituted with water and analyzed by SE-HPLC. Unprocessed NV-VLPs; analyzed concurrently, were used as a reference standard to quantify the NV-VLP content of the reconstituted test samples. Both UV and fluorescence detectors were used for quantification (data shown are from the fluorescence detector). The SE-HPLC was conducted using a Superose™ 6 10-300 column, with mobile phase consisting of 10 mM sodium phosphate, 10 mM citric acid, pH 5, and 500 mM NaCl, at a flow rate of 0.5 mL/min. Protein concentrations were quantified using integrated areas of elution peaks. "VLP" is the peak that eluted at about 15 min from the column, and any preceding shoulder and/or peak tail within the approximate elution time window of the reference, standard NV-VLP analyzed concurrently. The VLP fragment that elutes from the column at around 32 min is a highly stable single species that results from destabilization and consequent disassembly of the VLP. Intermediate and smaller fragments were not observed.

[0086] The results show that combinations of sucrose and chitosan produced a wide range of native monodisperse NV-VLP recoveries including the highest (approximately 100% recovery) post-lyophilization (samples LG10-LG12). Moreover, the NV-VLP elution peak shapes from these samples were identical to the unprocessed NV-VLP reference standard indicating high preservation of native structure. Samples containing sucrose only exhibited peak shapes similar to the reference standard, though NV-VLP recoveries were lower (approx. 60% recovery (sample LG 13) Samples that contained only mannitol resulted in nearly completely aggregated VLPs (samples LE1-LE6 and LIG1d-S3). The deleterious effects of mannitol on NV-VLP structure were counteracted by the presence of chitosan and sucrose (samples LIG1d - S2 and LIG1d-SB).

Table 7. Experiment and sample identification, and results for testing the effect of sucrose, chitosan, and mannitol or combinations thereof on stability of NV-VLP structure during freezing and lyophilization.

Experiment and Sample	Theoretical VLP conc (mg/mL)	N	Measured SE-HPLC mean protein concentration and peak elution time		Mean percent values of recovered protein as percent of theoretical		Mass equivalents S = sucrose C = chitosan M = mannitol		
			"VLP" ~ 15 min (mg/mL)	VLP Fragment ~ 32 min (mg/mL)	Total protein (%)	"VLP" (%)	S	C	M
LE1-LE5	0.25	5	0.02	0.12	56.0	6.3	0	0	1
LG1-LG9	0.25	9	0.06	0.00	24.0	24.0	0	1	0
LG10	0.25	1	0.25	0.00	101	101	10	1	0
LG11	0.25	1	0.25	0.00	101	101	5	1	0
LG12	0.25	1	0.25	0.00	100	100	2.5	1	0
LG13	0.25	1	0.16	0.00	65	65	1	0	0
LJ1-LJ14	0.25	14	0.22	0	85.4	85.4	2	1	0
LIG1d-S1	0.25	1	0.21	0	88	88	2	1	0
LIG1d-Sa	0.25	1	0.12	0	50	50	2	1	0
LIG1d-S2	0.25	1	92	0	92	92	1	0.5	1
LIG1d-Sb	0.25	1	60	0	60	60	1	0.5	1
LIG1d-S3	0.25	1	<1	<1	<1	<1	0	0	1

Example 14. Induction of Norovirus-Specific Long-Lived Plasma Cells and Memory B Cells in Mice Immunized Intranasally

A. Norwalk VLP-specific long-lived plasma cells

[0087] BALB/c mice were immunized intranasally with Norovirus VLPs and an adjuvant. Naïve controls were administered the adjuvant alone. At 114 days after immunization, spleen, cervical lymph nodes, and bone marrow were harvested from both groups of mice. On the day of harvesting the tissues (day 0), cells were assayed using an ELISPOT assay for the presence of antigen-specific antibody-secreting cells (ASCs). The results are presented in Figure 13A-C for the different tissues. The detection of immunoglobulins (IgG, IgA, and IgM) in these tissues indicates the presence of Norovirus-specific long-lived plasma cells.

B. Norwalk VLP-specific memory B cells

[0088] An *in vitro* assay was developed to detect the presence of Norwalk VLP-specific memory B-cells from mice immunized intranasally with Norwalk VLPs. Various lymphoid tissues or whole blood (peripheral blood mononuclear cells, splenocytes, lymph node cells, etc.) can serve as the source of cells that can be assayed for the presence of memory B-cells using this assay.

[0089] In this experiment, the spleen was harvested and processed from immunized and naive animals (controls), and splenocytes were cultured for four days in the presence or absence (controls) of Norwalk VLPs (20 µg/ml). An initial VIP-specific ELISPOT assay was performed on the day of tissue harvest (day 0) to establish background levels of ASCs (see Section A above). After four days in culture the cells were harvested and assayed again in an ELISPOT assay to quantify the number of VLP-specific ASCs. The difference in VLP-specific ASC numbers between the day 0 and the day 4 assays represent the antigen-specific memory B-cell population. The results of this experiment are shown in Figure 14A and B.

Example 15. Norovirus Memory B Cell Responses in Rabbits

[0090] Two female New Zealand White rabbits were immunized intranasally with a dry powder formulation consisting of 25 µg Norwalk VLP, 25 µg MPL, 1.5 mg mannitol, 1.5 mg sucrose, and 7.0 mg chitosan per 10 mg of dry powder loaded into Valois Mark 4 intranasal delivery devices. The two rabbits received a total of three immunizations at 14 day intervals. For these experiments, a non-immunized female rabbit was used as a naïve control.

A. Collection and processing of rabbit tissues

[0091] Peripheral blood mononuclear cells (PBMCs): Whole blood (~50 mL) was obtained from rabbits in collection tubes containing EDTA to prevent coagulation. The whole blood was diluted 1:3 with sterile D-PBS and -35 mL of diluted whole blood was layered onto 15 mL of Lympholyte Separation Medium in a sterile 50-mL centrifuge tube. The tubes were centrifuged at 800 x g for 20 minutes at room temperature. The buffy coat layer containing the PBMCs was carefully removed using a sterile 5 mL pipette and the cells were washed twice with D-PBS. If necessary, contaminating red blood cells were removed by ACK lysis. The cells were resuspended in RPMI-1640-10% FBS (1640-C) and counted in a hemocytometer using a Trypan exclusion method.

[0092] Mesenteric lymph node cells: The lymph nodes were aseptically collected from each rabbit separately following euthanasia. The tissues were maintained in a sterile plastic Petri dish containing ~10 mL of RPMI-1640-No Serum (1640-NS). The lymph nodes were pressed through a sterile mesh screen using a sterile pestle to disperse the tissue and obtain a single cell suspension of lymph node cells. The cells were collected, washed twice with 1640-NS, and finally filtered through a sterile 70 µm filter to remove clumps and debris. The cells were resuspended in 1640-C and counted in a hemocytometer using a Trypan blue exclusion method.

[0093] Splenocytes: Spleens were aseptically obtained from each rabbit following euthanasia. The spleens were placed in sterile Petri dishes containing approximately 10 mL of 1640-NS. Using a sterile 22-guage needle and syringe the media was repeatedly injected into the tissue to disrupt the splenic capsule and elaborate the cells. Sterile forceps were then used to tease apart the remaining tissue fragments. The contents of the Petri dish were transferred to a sterile centrifuge tube and the cell suspension and disrupted splenic tissue was allowed to sit for 6-8 minutes to allow for the settling of large tissue fragments. The single cell suspension was transferred to a second sterile centrifuge tube and the cells were washed once with 1640-NS. The red blood cells in the splenocyte prep were removed by an ACK lysis (8 mL ACK buffer, 8 minutes, room temperature) and the cells were washed one more time with 1640-NS and finally filtered through a sterile 70 µm filter to remove clumps and debris. The final cell pellet was resuspended in 1640-Complete and counted in a hemocytometer using a Trypan blue exclusion method.

[0094] Bone marrow cells: The tibia bones in the lower legs were removed from individual rabbits following euthanasia. To remove the bone marrow cells the ends of the bones were aseptically cut off using a bone saw and the contents of the bone were flushed out by repeated injections of 1640-NS medium. The bone marrow cells were pipetted up and down repeatedly to break up and disperse clumps of cells. The cells were washed once with 1640-NS; the red blood cells were lysed with ACK, and the cells were washed one more time with 1640-NS. Finally, the cells were filtered through a sterile 70 µm filter to remove clumps and debris. The final cell pellet was resuspended in 1640-Complete and counted in a hemocytometer using a Trypan blue exclusion method.

B. ELISPOT assays

[0095] Following pre-wetting and washing, 96-well Millipore PVDF filter plates were coated with a sterile solution of native Norwalk VLPs at a concentration of 40 µg/ml in a final volume of 50 µl/well. The plates were incubated overnight at 4 °C, washed with D-PBS, and blocked with the addition of 1640-C. Mesenteric lymph node cells, splenocytes, and bone marrow cells from the immunized rabbits and from the naive control rabbit were added to the wells at varying concentrations ($1x10^6$, $5x10^5$, $2x10^5$, and $1x10^5$ cells/well) and the plates were incubated overnight at 37 °C. The plates were washed thoroughly with PBS-Tween and secondary reagents specific for rabbit IgG and IgA were added to the wells and incubated for an additional 2 hours at room temperature. Following extensive washing the plates were developed with DAB chromagen/substrate and read in an ELISPOT plate reader. Spots appearing on wells from naïve control animals were subtracted from the experimental groups. The data is expressed as Norwalk VLP-specific antibody-secreting cells (ASCs) and is normalize per $1x10^6$ cells.

C. Norwalk VLP-Specific Memory B-cell Assay

[0096] Isolated lymphoid cells from the various tissues described above were resuspended in 1640-C medium in the presence of Norwalk VLPs (10 µg/mL) at a density of 5x10^6 cells per mL. The cells were incubated in 24-well plates in 1-mL volumes for four days at 37 °C. VLP-specific ELISPOT assays were performed on these cells at the time of culturing. After four days in culture the cells were harvested, washed twice with 1640-NS medium, resuspended in 1640-Complete, and counted in a hemocytometer using a Trypan blue exclusion method. The cells were tested once again in a Norwalk VLP-specific ELISPOT assay. The data obtained from the ELISPOT assays performed on the day of tissue harvest is referred to as day 0 (background) ASC activity. Any spots detected at the day 0 time point are assumed to be actively-secreting plasma cells or long-lived plasma cells (LLPCs).The data obtained from the ELISPOT assay performed on the 4-day cultured cells is referred to as day 4 ASC activity, and the memory B-cell activity is represented by the difference between day 4 ASC activity and day 0 ASC activity.

D. Norwalk VLP-Specific Memory B-Cells are Present in the Peripheral Blood of Intranasally Immunized Rabbits

[0097] Whole blood was obtained from two immunized rabbits (RB735, RB1411) 141 days following the last of three intranasal immunizations with a dry powder formulation vaccine containing Norwalk VLPs as described above. Blood was also obtained from an non-immunized, naïve rabbit. The blood was processed to obtain peripheral blood mononuclear cells (PBMCs) and the PBMCs were placed in a Norwalk VLP-Specific memory B-Cell assay (section C above). The results are shown in Figure 15. The left panel shows results of the initial ELISPOT assay at the time of tissue harvest (day 0 ASCs). The right panel shows the results of the ELISPOT assay after 4 days in culture with Norwalk VLPs (day 4 ASCs).

[0098] The day 0 ELISPOT results (Fig. 15, left panel) illustrate that there are no VLP-specific plasma cells remaining in the peripheral blood approximately 140 days after the last boost with Norwalk VLP dry powder vaccine. The right panel of Figure 15 shows the ELISPOT assay results from PBMCs cultured for four days *in vitro* with Norwalk VLPs. In the two immunized rabbits, a significant number of PBMCs, presumably a subpopulation of memory B-cells, have matured into active IgG-secreting Norwalk VLP-specific plasma cells. Although assays for IgA-secreting memory B-cells were conducted, only IgG-secreting memory B-cells were detected in the PBMC population. As expected, the naive animal showed no antigen-specific memory B-cells. Thus, VLP-specific memory B-cells were found in the peripheral circulation of rabbits 140+ days following the last of three intranasal immunizations.

E. Norwalk VLP-Specific Memory B-Cells are Present in the Spleen of Intranasally Immunized Rabbits

[0099] Splenocytes were obtained from the spleens of the two vaccine immunized rabbits and the non-immunized control rabbit. Norwalk VLP-specific memory B-cell assays (described above) were performed on these cells and the results are shown in Figure 16. As observed for the PBMC population the day 0 ELISPOT assay shows that there are no antigen-specific plasma cells present in the spleen (Fig. 16, left panel). However, following a four day *in vitro* incubation with Norwalk VLPs, IgG-secreting Norwalk VLP-specific memory B-cells are apparent in the splenocyte population. Thus, the spleen represents one site for the migration of memory B-cells following intranasal immunization.

F. A Population of Norwalk VLP-Specific Long-lived Plasma Cells is Found in the Bone Marrow but No Memory B-cells Are Present

[0100] Bone marrow cells were obtained from the tibias of the experimental rabbits and assayed for the presence of long-lived plasma cells and memory B-cells. The results are presented in Figure 17. The left panel of Figure 17 shows that rabbit 1411 still had a significant population of antigen-specific plasma cells in the bone marrow. Plasma cells that migrate to the bone marrow and reside there for a significant period of time following immunization are referred to as long-lived plasma cells (LLPCs). Rabbit 735 did not show a high number of LLPCs. No LLPCs were found in the bone marrow of the naive rabbit. The bone marrow cells were cultured in a memory B-cell assay and re-tested for the presence of memory B-cells. The right panel of Figure 17 shows that there are essentially no antigen-specific memory B-cells present in the bone marrow. Thus, long-lived plasma cells migrate to the bone marrow but no memory B-cells are found there.

G. Both IgG-Secreting and IgA-Secreting Norwalk VLP-Specific Memory B-Cells are Present in the Mesenteric Lymph Nodes of Intranasally Immunized Rabbits

[0101] The mesenteric lymph nodes were obtained from all of the experimental rabbits and the isolated cells were assayed for LLPCs and memory B-cells. The results from this assay are shown in Figure 18A. As with most of the lymphoid tissue analyzed, except bone marrow, no LLPCs (Fig. 18A left panels) were found in the mesenteric nodes. Following *in vitro* incubation with Norwalk VLPs, a very high number of IgG-secreting VLP-specific memory B-cells were evident in the mesenteric lymph node population (Fig. 18A, right panel). The numbers of memory B-cells observed in the mesenteric lymph nodes were significantly higher than those observed for the other lymphoid tissues assayed.

[0102] Numerous researchers have shown that immunization at a mucosal inductive site, such as the nasal passages or the gut, is capable of eliciting a so-called mucosal immune response. This response has generally been characterized by the presence of IgA+ B-cells and IgA-secreting plasma cells localized in the mucosal lymphoid tissue. For this reason the mesenteric lymph node cells were also assayed for the presence of IgA-secreting LLPCs or memory B-cells. The results from these assays are shown in Figure 18B. Once again, no IgA+ LLPCs were found in the mesenteric lymph node population (Figure 18B, left panel). However, IgA-secreting memory B-cells were detected in this tissue (Figure 18B, right panel). Thus, intranasal immunization with a dry powder Norwalk VLP vaccine formulation elicited a mucosal immune response that resulted in the migration of both IgG+ and IgA+ antigen-specific memory B-cells to the gut-associated lymphoid tissue. The production of antigen-specific memory B cells induced by immunization with the Norwalk vaccine formulation is a possible indicator of vaccine effectiveness. The presence of memory B cells is one marker of long-lasting immunity.

H. VLP-specific CD4+ Memory T cells

[0103] Splenocytes harvested from immunized rabbits were restimulated with intact Norwalk VLPs and the extent of cellular proliferation was measured by tritiated thymidine incorporation as indicated on the ordinate axis (CPM) (Figure 19). The left panel shows cellular proliferation of an unfractionated population of splenocytes, while the right panel shows cellular proliferation of CD4+ T cells.

[0104] The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein, will become apparent to those skilled in the art from the foregoing description and accompanying drawings using no more than routine experimentation. Such modifications and equivalents are intended to fall within the scope of the appended claims.

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[0105]

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Patentkrav

1. Sammensætning omfattende mindst ét Norovirus-antigen og mindst én adjuvans; hvor adjuvansen er valgt fra gruppen bestående af en adjuvans der er et aluminiumsalt og en adjuvans der er en toll-like receptor-(TLR)-agonist; hvor sammensætningen er formuleret som en væske; og hvor indholdet af det mindst ene Norovirus-antigen i sammensætningen er fra ca. 1 μg til ca. 200 μg pr. dosis.

2. Sammensætningen ifølge krav 1, hvor indholdet af det mindst ene Norovirus-antigen i sammensætningen er fra ca. 1 μg to 100 μg pr. dosis.

3. Sammensætningen ifølge krav 1 eller 2, hvor indholdet af det mindst ene Norovirus-antigen i sammensætningen er fra ca. 1 μg to 50 μg pr. dosis.

4. Sammensætningen ifølge et hvilket som helst af kravene 1 - 3, hvor Norovirus-antigenet omfatter peptider, proteiner, Norovirus virus-lignende partikler (VLP), denaturerede VLP, capsid-monomerer, capsid-multimerer, aggregater, eller blandinger deraf.

5. Sammensætningen ifølge krav 4, hvor Noroviruset er en Norovirus genogruppe I eller genogruppe II virusstamme.

6. Sammensætningen ifølge krav 4 eller krav 5, hvor Norovirus VLP omfatter et VP1 og/eller VP2-capsidprotein.

7. Sammensætningen ifølge krav 4, hvor VLP er monovalente VLP eller multivalente VLP.

8. Sammensætningen ifølge krav 4, hvor Norovirus VLP er fremstillet i et rekombinant cellullært ekspressionssystem eller et baculovirus-inficeret cellulært ekspressionssystem.

9. Sammensætningen ifølge krav 1, yderligere omfattende et andet Norovirus-antigen.

10. Sammensætningen ifølge krav 9, hvor første og andet Norovirus-antigen er monovalente VLP fra forskellige genogrupper.

11. Sammensætningen ifølge krav 10, hvor det første Norovirus-antigen er en monovalent VLP omfattende mindst ét capsidprotein fra en genogruppe I Norovirus og det andet Norovirus-antigen er en monovalent VLP omfattende mindst ét capsidprotein fra en genogruppe II Norovirus.

12. Sammensætningen ifølge krav 11, hvor genogruppe II Noroviruset er et GII.4 Norovirus.

13. Sammensætningen ifølge krav 1, hvor sammensætningen er formuleret til parenteral administration.

14. Sammensætningen ifølge krav 1, yderligere omfattende mineralolie.

15. Sammensætningen ifølge krav 1 til anvendelse i en fremgangsmåde til induktion af immunrespons mod Norovirus i et pattedyr.

DRAWINGS

Figure 1

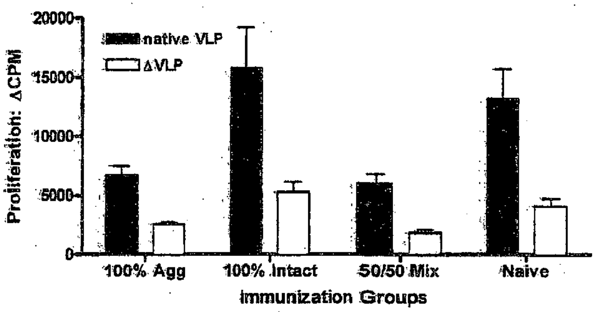
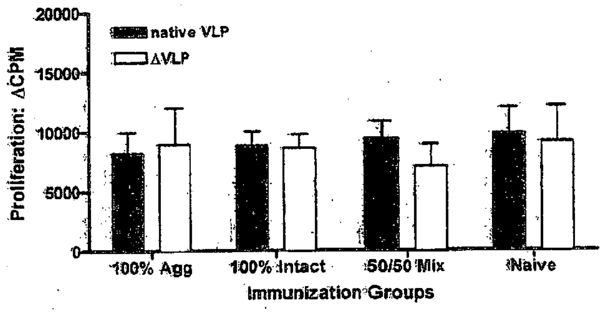


Figure 2



20000
15000
10000
5000
0
native VLP
ΔVLP
Proliferation: ΔCPM
100% Agg
100% Intact
50/50 Mix
Naive
Immunization Groups

Figure 3

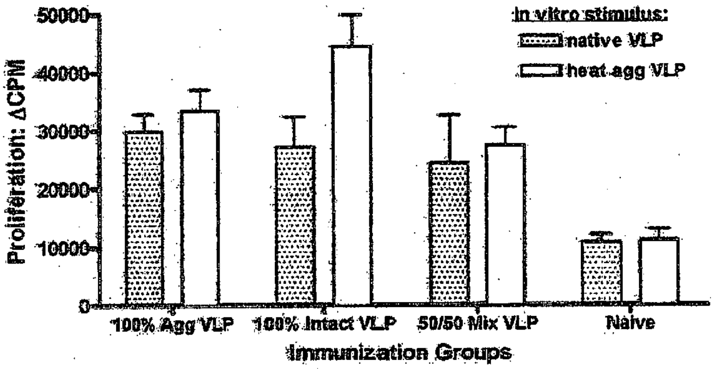

in vitro stimulus:
native VLP
heat agg VLP
Proliferation: ΔCPM
50000
40000
30000
20000
10000
0
100% Agg VLP
100% Intact VLP
50/50 Mix VLP
Naive
Immunization Groups

Figure 4

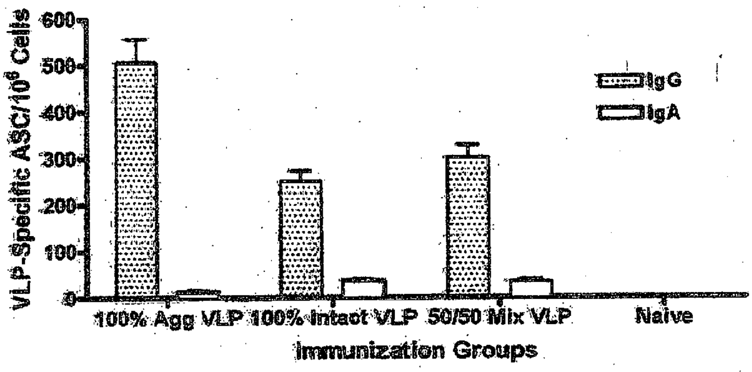

VLP-Specific ASC/10^6 Cells
600
500
400
300
200
100
0
IgG
IgA
100% Agg VLP
100% Intact VLP
50/50 Mix VLP
Naive
Immunization Groups

Figure 5

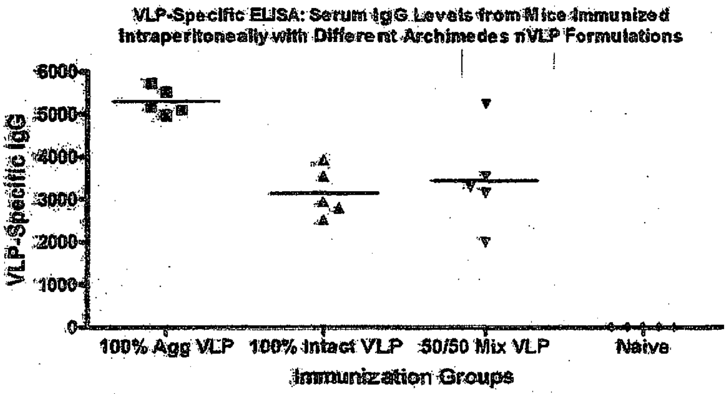

VLP-Specific ELISA: Serum IgG Levels from Mice Immunized Intraperitoneally with Different Archimedes nVLP Formulations
VLP-Specific IgG
6000
5000
4000
3000
2000
1000
0
100% Agg VLP
100% Intact VLP
50/50 Mix VLP
Naive
Immunization Groups

Figure 6

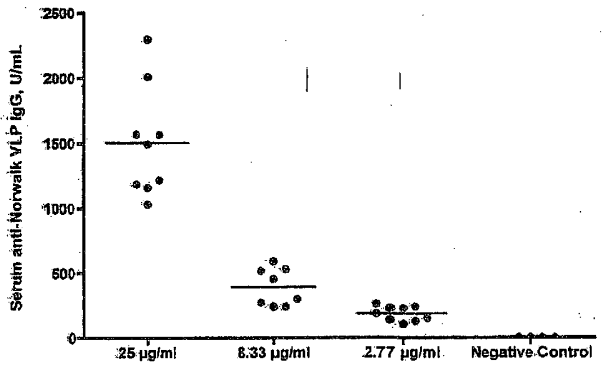
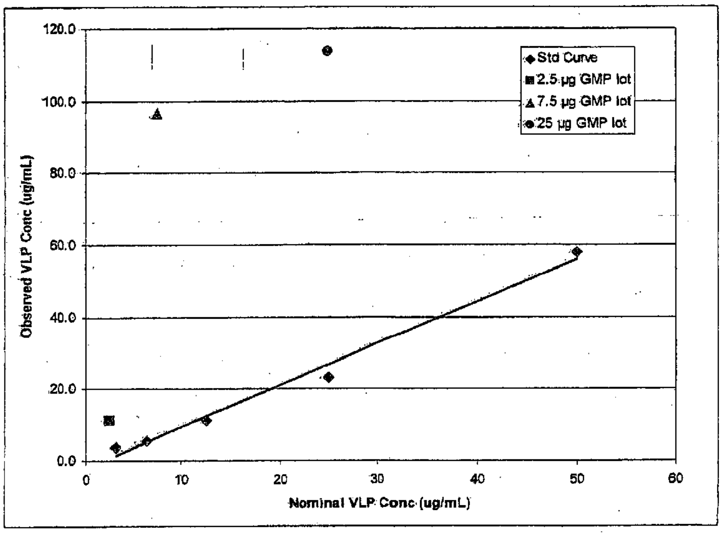

Serum anti-Norwalk VLP igG, U/mL
2500
2000
1500
1000
500
0
25 µg/ml
8.33 µg/ml
2.77 µg/ml
Negative Control

Figure 7



120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
10
20
30
40
50
60
Observed VLP Conc (ug/mL)
Nominal VLP Conc (ug/mL)
Std Curve
2.5 µg GMP lot
7.5 µg GMP lot
25 µg GMP lot

Figure 8

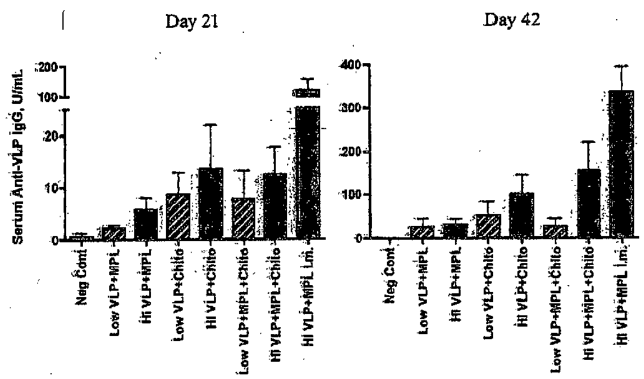

Day 21
Day 42
Serum Anti-VLP IgG, U/ml.
Neg Cont
Low VLP+MPL
Hi VLP+MPL
Low VLP+Chito
Hi VLP+Chito
Low VLP+MPL+Chito
Hi VLP+MPL+Chito
Hi VLP+MPL i.m.

Figure 9

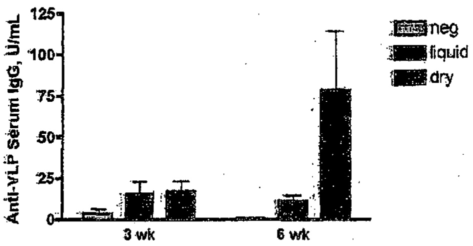


Figure 10

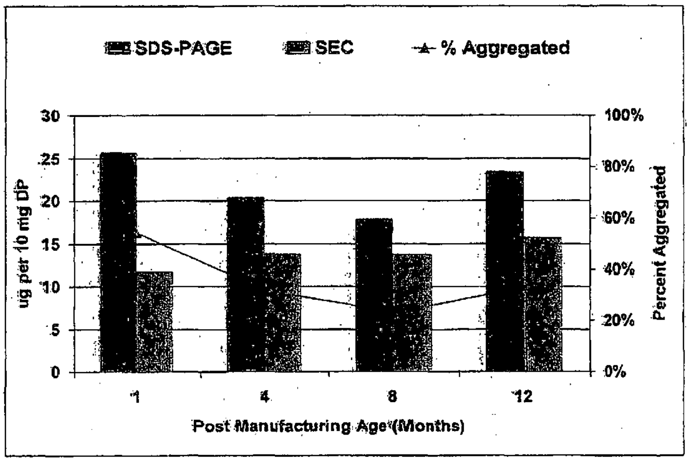

SDS-PAGE
SEC
% Aggregated
30
25
20
15
10
5
0
ug per 10 mg DP
100%
80%
60%
40%
20%
0%
Percent Aggregated
1
4
8
12
Post Manufacturing Age (Months)

Figure 11

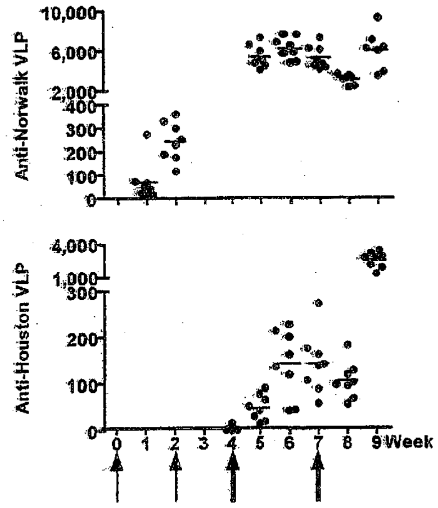

Anti-Norwalk VLP
10,000
6,000
2,000
400
300
200
100
0
Anti-Houston VLP
4,000
1,000
300
200
100
0
0
1
2
3
4
5
6
7
8
9 Week

Figure 12

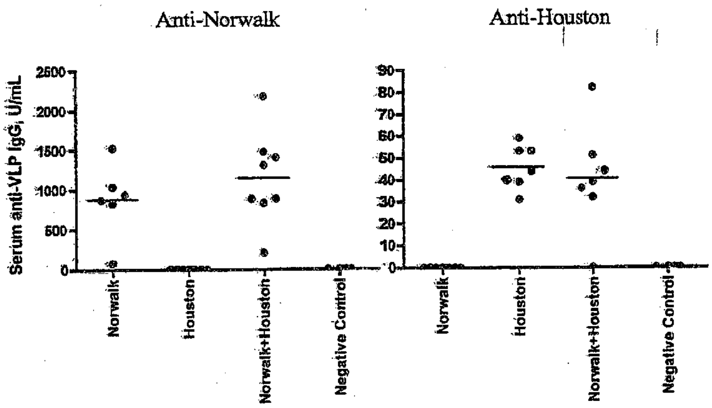

Anti-Norwalk
Anti-Houston
Serum anti-VLP IgG, U/mL
2500
2000
1500
1000
500
0
90
80
70
60
50
40
30
20
10
0
Norwalk
Houston
Norwalk+Houston
Negative Control

Figure 13

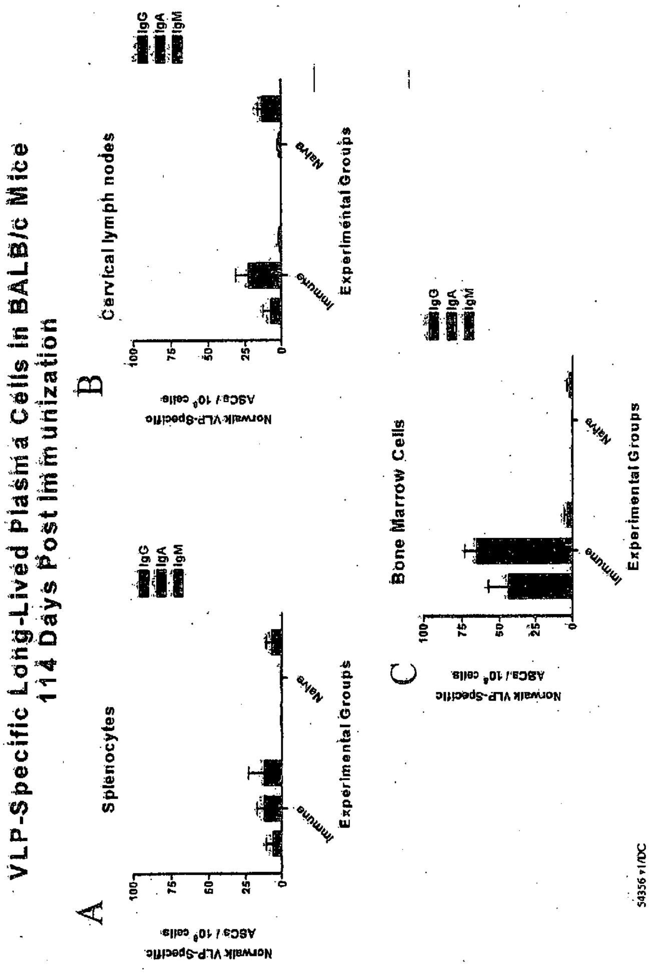

VLP-Specific Long-Lived Plasma Cells in BALB/c Mice
114 Days Post Immunization
A
Splenocytes
B
Cervical lymph nodes
C
Bone Marrow Cells
Norwalk VLP-Specific
ASCs / 10^6 cells
100
75
50
25
0
Immune
Naive
Experimental Groups
IgG
IgA
IgM
54356 v1/DC

Figure 14

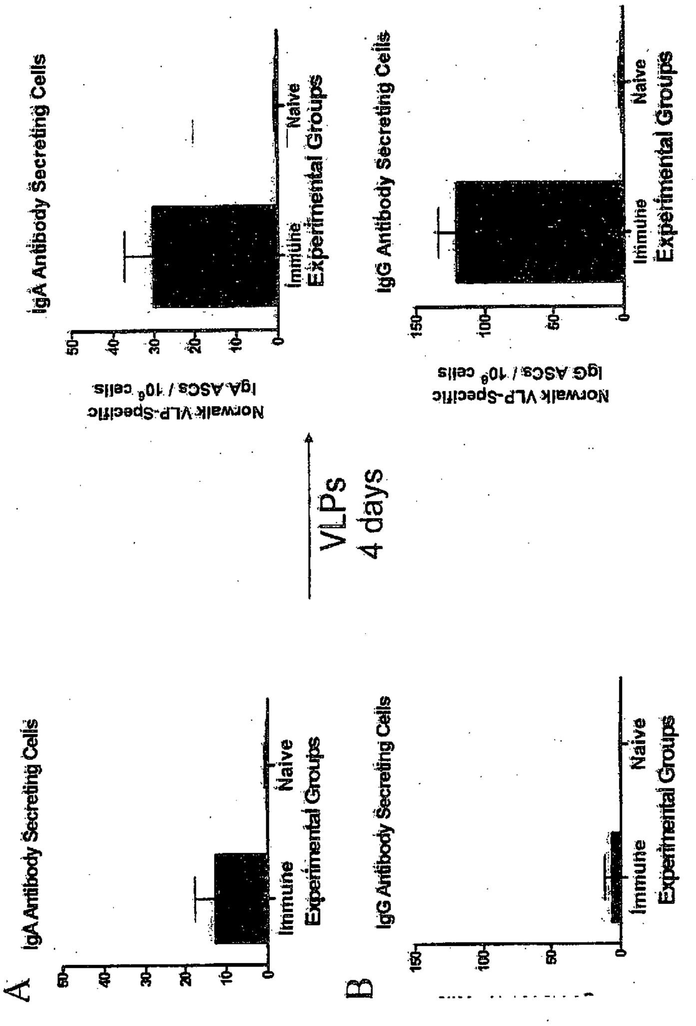

A
B
IgA Antibody Secreting Cells
IgG Antibody Secreting Cells
Norwalk VLP-Specific IgA ASCs / 10⁶ cells
Norwalk VLP-Specific IgG ASCs / 10⁶ cells
Immune
Naive
Experimental Groups
VLPs
4 days

Figure 15

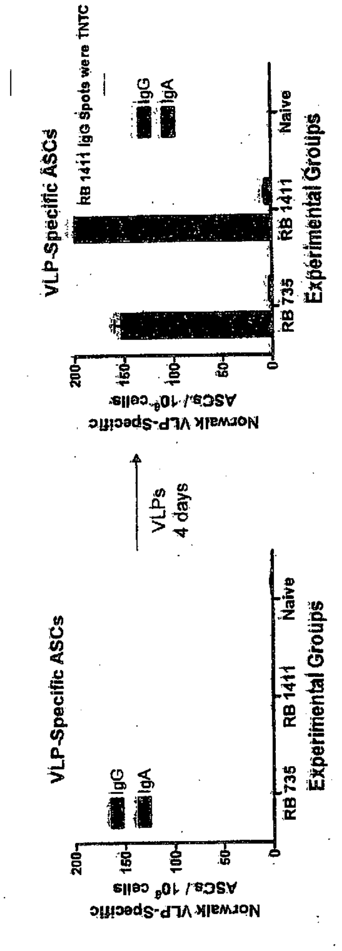

VLP-Specific ASCs
Norwalk VLP-Specific ASCs/ 10⁶ cells
IgG
IgA
RB 735
RB 1411
Naive
Experimental Groups
VLPs
4 days
VLP-Specific ASCs
RB 1411 IgG Spots were TNTC
IgG
IgA
RB 735
RB 1411
Naive
Experimental Groups

Figure 16

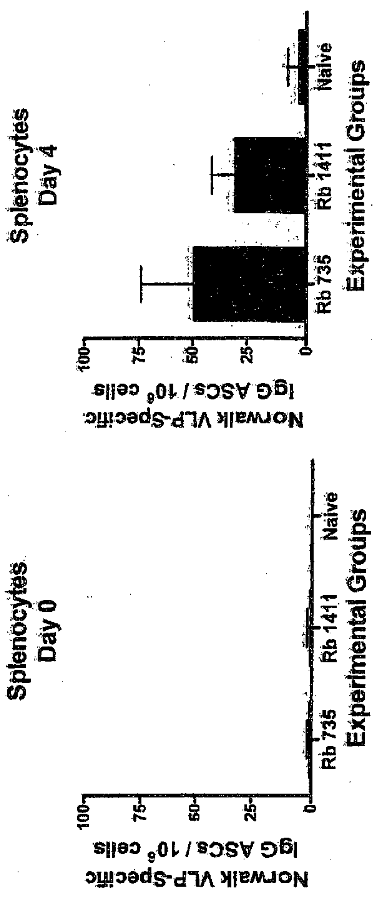

Splenocytes
Day 0
Norwalk VLP-Specific
IgG ASCs / 10⁶ cells
100
75
50
25
0
Rb 735
Rb 1411
Naive
Experimental Groups
Splenocytes
Day 4
Norwalk VLP-Specific
IgG ASCs / 10⁶ cells
100
75
50
25
0
Rb 735
Rb 1411
Naive
Experimental Groups

Figure 17

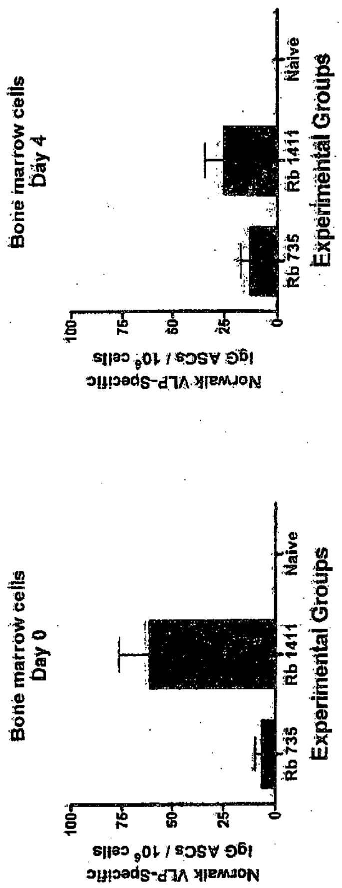

Bone marrow cells
Day 0
Norwalk VLP-Specific
IgG ASCs / 10⁶ cells
100
75
50
25
0
Rb 735
Rb 1411
Naive
Experimental Groups
Bone marrow cells
Day 4
Norwalk VLP-Specific
IgG ASCs / 10⁶ cells
100
75
50
25
0
Rb 735
Rb 1411
Naive
Experimental Groups

Figure 18

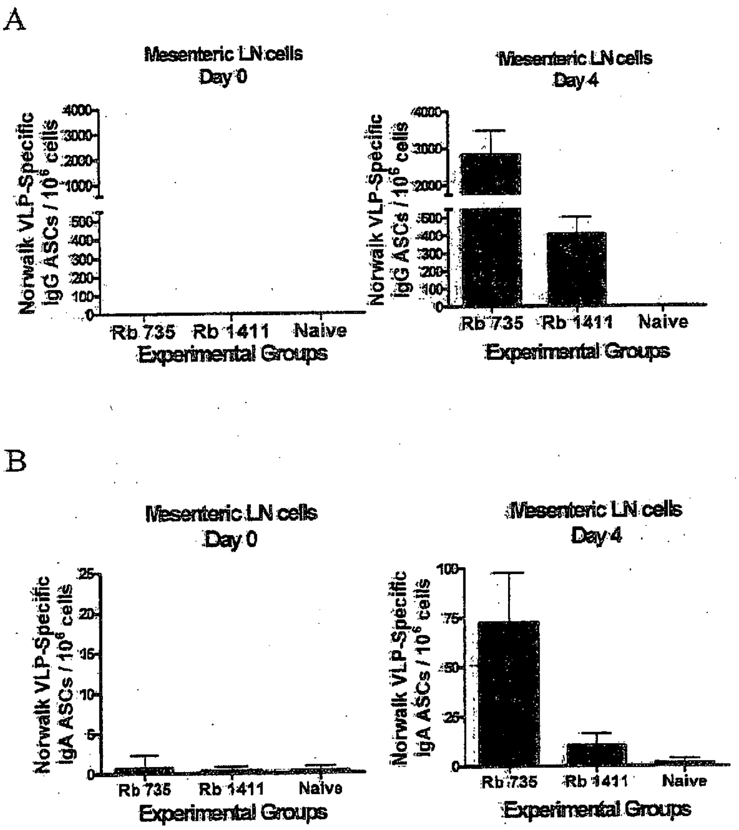

A
Mesenteric LN cells
Day 0
Mesenteric LN cells
Day 4
Norwalk VLP-Specific
IgG ASCs / 10⁶ cells
Rb 735
Rb 1411
Naive
Experimental Groups
B
Mesenteric LN cells
Day 0
Mesenteric LN cells
Day 4
Norwalk VLP-Specific
IgA ASCs / 10⁶ cells
Rb 735
Rb 1411
Naive
Experimental Groups

Figure 19

Unfractionated Splenocytes:
In Vitro VLP-Specific T-Cell Proliferation
ΔCPM
50000
40000
30000
20000
10000
0
Rb 1411
Rb 735
Naive
Experimental Groups
CD4+ T-Cells:
In Vitro VLP-Specific T-Cell Proliferation
ΔCPM
40000
30000
20000
10000
0
Rb 1411
Rb 735
Experimental Groups