Immunogenicity and Specificity of Norovirus Consensus GII.4 Virus-like Particles in Monovalent and Bivalent Vaccine Formulations

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Abstract

Noroviruses, a major cause of acute gastroenteritis worldwide, present antigenic diversity that must be considered for the development of an effective vaccine. In this study, we explored approaches to increase the broad reactivity of virus-like particle (VLP) norovirus vaccine candidates. The immunogenicity of a GII.4 “Consensus” VLP that was engineered from sequences of three genetically distinct naturally-occurring GII.4 strains was examined for its ability to induce cross-reactive immune responses against different clusters of GII.4 noroviruses. Rabbits immunized with GII.4 Consensus VLPs developed high serum antibody titers against VLPs derived from a number of distinct wild-type GII.4 viruses, including some that have been circulating over 30 years. Because the sera exhibited low cross-reactivity with antigenically-distinct GI norovirus strains, we investigated the serum antibody response to a bivalent vaccine formulation containing GI.1 (Norwalk virus) and GII.4 Consensus VLPs that was administered to animals under varying conditions. In these studies, the highest homologous and heterologous antibody titers to the bivalent vaccine were elicited following immunization of animals by the intramuscular route using Alhydrogel (Al(OH)₃) as adjuvant. Our data indicate that the use of both genetically-engineered norovirus VLPs that incorporate relevant epitopes from multiple strains and multivalent vaccine formulations increase the breadth of the immune response to diverse variants within a genotype and, thus, prove helpful in the rational design of VLP-based vaccines against human noroviruses.

Keywords

Noroviruses; GII.4 noroviruses; Consensus virus like-particles; gastroenteritis
1. INTRODUCTION

Human noroviruses are the leading cause of epidemic non-bacterial gastroenteritis worldwide that is often associated with outbreaks in settings such as schools, hospitals, restaurants, hotels, cruise ships, nursing homes, and military facilities [1]. Recent estimates have indicated that noroviruses may be responsible up to 200,000 deaths in children under 5 years of age each year, mainly in the developing world [2].

Noroviruses are non-enveloped viruses with a single-stranded, positive-sense RNA genome that is organized into three open reading frames encoding nonstructural and structural proteins [1, 3]. The expression of VP1 (major capsid protein) results in the formation of virus-like particles (VLPs) that have been shown to be morphologically and antigenically similar to native virions [4]. Because the development of vaccines or antivirals against human noroviruses has been hampered by the lack of an in vitro cell culture system or small animal disease model, the generation of norovirus VLPs has been particularly important for studies of the virus structure, antigenic diversity, epitope mapping, and vaccine development [5-7].

Based on the amino acid sequence of the VP1 capsid protein, noroviruses have been classified into six Genogroups (GI-GVI) and multiple genotypes; however, only noroviruses from GI, GII and GIV have been shown to infect humans [8, 9]. Molecular epidemiological studies have shown that strains from genotype GII.4 are the most prevalent in humans, causing more than 60% of norovirus outbreaks worldwide [10, 11]. The chronological emergence of new variants of GII.4 noroviruses correlates with an increase in the number of reported global epidemics, leading to the proposal that GII.4 strains may evolve similarly to Influenza A virus, in which old variants are periodically replaced [12-14].

Phase I clinical studies have shown that norovirus VLPs are safe and induce humoral and mucosal immune responses in adults when administered by the oral route [6, 15, 16]. Recently, clinical trials using monovalent, adjuvanted GI.1 Norwalk VLP formulations administered intranasally have shown an increase (up to 9.1-fold) in preexisting serum norovirus-specific IgA and IgG titers after 2 doses (100 μg/dose) of VLPs [17]. While norovirus intranasal vaccination prevented illness in vaccinees challenged with homologous GI.1 viral pools (41), it is not yet known whether these titers correlate with protection against heterologous strains.

Early cross-challenge studies in adult volunteers with Norwalk virus (NV) and Hawaii (HV) [GI and GII prototype strains, respectively], demonstrated the absence of heterotypic immunity between these two genogroups [18]. Consistent with this, chimpanzees vaccinated with GI.1 NV VLPs were protected from infection when challenged with live GI.1 NV; while GII.4 VLP vaccinated-chimpanzees were not [19]. Although the GII.4 VLPs induced high homologous antibody titers in these animals, little or no cross-reactive antibodies to NV could be detected. To overcome the lack of GI/GII heterotypic protection and to broaden the immune responses against human noroviruses, the use of multivalent vaccine formulations has been suggested [20].

In this study, we describe the antigenic characterization of a novel GII.4 VLP (designated as Consensus VLP) derived by combination of sequences from three naturally-occurring GII.4 capsid protein sequences. Using different vaccine formulations (monovalent and bivalent) and routes of immunization (intranasal and intramuscular), we tested the ability of the Consensus VLPs to induce humoral responses in rabbits. GII.4 Consensus VLPs induced high antibody titers against a panel of GII.4 VLPs representing several distinct variants that circulated over a period of thirty years. Moreover, the intramuscular route of immunization
with a bivalent VLP formulation (GI.1/GII.4) induced slightly higher antibody titers than the intranasal route of delivery under the conditions examined in this study.

2. MATERIALS AND METHODS

2.1 Expression and purification of virus-like particles

The GII.4 Consensus norovirus VLP amino acid sequence was designed by aligning the following human norovirus GII.4 capsid protein sequences and determining the “Consensus” amino acid residues at each position: Houston/TCH186/2002/US (ABY27560), DenHaag89/2006/NL (ABL74395), and Yerseke38/2006/NL (ABL74391). At those amino acid positions where a different residue was found in each sequence, the amino acid residue found in the Yerseke38 sequence was chosen because fewer substitutions were needed to achieve consensus among the three strains. A synthetic DNA fragment encoding the Consensus GII.4 sequence with codon optimization for Spodoptera frugiperda Sf9 cells was synthesized by GeneArt (Regensburg, Germany) and engineered into a recombinant baculovirus for expression of VLPs. For production of GI.1 Norwalk and GII.4 Consensus VLP vaccine antigens used in the bivalent formulations, Sf9 cells were infected at low MOI and supernatant harvested ~ 5 days post infection. Following bioreactor production, highly-purified VLPs were generated using multiple orthogonal chromatography and UF/DF unit operations (LigoCyte Pharmaceuticals, manuscript in preparation). The expression and purification of the VLPs representing GI.1 (NV), GI.3 (Desert Shield virus, DSV), GII.1 (HV), GII.3 (Toronto virus, TV), GIV.1 (Saint Cloud virus, SCV) and the various GII.4 viruses has been described elsewhere [14, 21-24, Parra et al. manuscript in preparation].

2.2 Guinea pig hyperimmune sera production

A panel of hyperimmune reference sera against GI.1, GI.3, GII.1, GII.3, GII.4 and GIV.1 VLPs was produced in guinea pigs as described (Parra et al. manuscript in preparation). Briefly, the first subcutaneous injection was performed with 125 μg of purified VLPs in Freund’s complete adjuvant. After 2 weeks, the animals received two boosting doses (separated by 2 weeks) with incomplete Freund’s adjuvant. The animals were bled 4 weeks after the final boost, and sera were verified for the presence of high antibody titers against the immunizing VLP by ELISA. Reference serum was aliquoted and stored at −20°C. All guinea pig studies were conducted at the NIH, Bethesda, Maryland under an animal protocol (LID 73) approved by the NIAID Division of Intramural Research Animal Care and Use Committee.

2.3 Immunization of rabbits with NV and GII.4 Consensus VLPs

Varying norovirus vaccine formulations and routes of administration were evaluated in rabbits. Groups of female New Zealand White rabbits (Myrtle’s Rabbitry, Thompson Station, TN), approximately 10-12 weeks of age, were immunized with monovalent or bivalent formulations of norovirus VLPs in the presence of different adjuvants (Table 1). Each animal was immunized on day 0 and 21 with serum collected following day 35. The following adjuvants were evaluated: i. Alhydrogel 2% (Al(OH)₃) (Brenntag Biosector, Denmark); ii. Alhydrogel 2% plus Monophosphoryl lipid A (MPL; GlaxoSmithKline), a Toll-like receptor 4 (TLR-4) agonist; and iii. Chitosan glutamate (ChiSys®; Archimedes Development Ltd), a mucoadhesive, plus MPL. For intramuscular vaccinations, the components of each vaccine were mixed prior to immunization as described in Table 1. A dry powder was prepared by admixture of components, lyophilization, and milling. The powder vaccine was administered intranasally using a nasal hand filled Unit Dose Powder (UDP) device (Pfeiffer, Radolfzell, Germany) that contained a nominal 10 mg of powder. All rabbit studies were conducted at Warodon Antibodies, Inc., Belgrade, Montana, with protocols approved by the company’s Institutional Animal Care and Use Committee.
2.4 Enzyme-linked immunosorbent assay (ELISA)

Serum antibody titers were measured by ELISA. Briefly, 96-well polystyrene microtiter plates (Thermo, Milford, MA) were coated with 100 μL of purified VLPs at a concentration of 0.5 μg/mL in phosphate-buffered saline (PBS), pH 7.4. Coated plates were washed with PBS containing 0.1% Tween-20 and blocked with 5% nonfat dry milk in PBS for 1 h at room temperature (RT). Serial 10-fold dilutions of each serum were made beginning at 1:100 and adsorbed for 2 h at RT. The binding of antibodies to the VLP antigen was detected with anti-guinea pig or anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (1:2,000 dilution) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, KPL, Gaithersburg, MD). ELISA titers were calculated using the reciprocal of the highest serum dilution that yielded an absorbance value above the average of the background absorbance value plus two standard deviations. Titers below the starting dilution (1:100) were assigned a value of 1 in the analysis. Reactivity with monoclonal antibodies was examined using a similar protocol and detected with anti-mouse IgG conjugated with horseradish peroxidase (1:2,000 dilution; KPL).

2.5 Binding of GII.4 Consensus VLPs to synthetic ABH histoblood group antigens (HBGA)

GII.4 Consensus VLPs were screened for binding to a panel of HBGA-associated carbohydrates (A, B, H1, H2, H3, Leα, Leβ, Leγ, Leδ) as described elsewhere [14]. Briefly, 96-well NeutriAvidin-coated plates (Pierce, Rockford, IL) were treated with 1 mg/ml of each biotinylated carbohydrate (Glycotech, Gaithersburg, MD) for 2 h and washed with PBS and 0.05% Tween-20 and 0.1% bovine serum albumin (BSA) before the 1 h incubation with 1.25 mg/ml of GII.4 Consensus VLPs in PBS. The binding of captured GII.4 Consensus VLPs was determined by incubation with guinea pig GII.4 (2004)-hyperimmune serum (1:2,000 dilution), followed by incubation with a peroxidase-conjugated goat anti-guinea pig IgG (1:2,000 dilution; KPL, Gaithersburg, MD), and peroxidase substrate ABTS (KPL). All incubations were performed at room temperature.

2.6 Statistical analysis

Data were analyzed with the GraphPad Prism version 5.0d, GraphPad Software (San Diego, CA). Groups were compared and analyzed by unpaired t-test. Differences were considered significant if p < 0.05.

3. RESULTS

3.1 Generation, characterization and antigenicity of GII.4 Consensus VLPs

The Consensus VP1 amino acid sequence was designed as a composite of three genetically distinct GII.4 Norovirus strains (Houston 2002, Den Haag 2006, Yerseke 2006). The amino acid alignment shows that 36 substitutions were present among the three GII.4 strains and the Consensus VP1 (5 located in the S domain and 31 in the P domain, Fig 1A). Importantly, all the substitutions on the P domain were located in the surface, and most of them (26/31) in loops (Supplementary Fig 1). Following production and downstream processing, the presence of properly assembled VLPs was confirmed by both size-exclusion chromatography (manuscript in preparation) and transmission electron microscopy (Fig 1B) which revealed the anticipated density of 38 nm particles that were morphologically similar to those described for NV [4].

Since the GII.4 Consensus VLPs represent a composite sequence, they could possess certain structural modifications not present in VLPs derived from wild-type strains. To provide a more detailed characterization of Consensus VLPs, we performed experiments to determine their carbohydrate and antibody binding profiles. The Consensus GII.4 VLPs were examined for binding to a panel of synthetic oligosaccharides representing nine HBGAs that have been
associated with recognition by noroviruses in previous studies [25]. Although the Consensus VLPs showed some degree of reactivity with all the carbohydrates, the strongest reactivity was observed with the H3, B, and Le x oligosaccharides (Fig 1C). The Consensus GII.4 VLPs reacted with four of six MAbs that bind to conformational epitopes on GII.4 strains from the 2004 Farmington Hills cluster (Fig 1D). Three of these MAbs block carbohydrate binding interactions (data not shown) and all three blocking MAbs retain reactivity with the GII.4 Consensus VLP. The reactivity pattern of the GII.4 Consensus VLPs with the GII.4 MAbs was consistent with available mapping data for the epitopes. For example, VP1 amino acid residues Ala294 and Asp295 were shown to be critical in the binding of MAbs A6 and A10 to VLPs from the 2004 GII.4 cluster (Parra et al. submitted manuscript). The substitution of Gly295 in the Consensus GII.4 VP1 (Fig 1A) (and in several other circulating GII.4 strains) likely resulted in its loss of reactivity with MAbs A6 and A10. Taken together, the GII.4 Consensus VLPs retained two fundamental, physical properties (carbohydrate binding and expected reactivity with certain conformational MAbs) that have been described for other GII.4 VLPs [12, 14, 26].

To determine the antigenic relationship between GII.4 Consensus VLPs and wild-type prototype strains, guinea pig hyperimmune reference sera raised against a panel of VLPs from prototypic strains (Fig 2A) were tested against the GII.4 Consensus VLPs by ELISA. The titers indicate that the GII.4 Consensus VLPs are most closely related to VLPs from GII.4 viruses, especially those isolated more recently (Geometric mean titer [GMT]=1.7 $10^5$, Fig 2A-B). Even though the GII.4 Consensus VLPs were cross-reactive with anti-GI and anti-GIV.1 sera, the titers ranged from $10^2$ to $10^4$ (GMT= 630), which correlates with the low percentage of amino acid similarity (<55%) with strains from these genogroups (Table 2). Interestingly, although the GII.4 Consensus VP1 presented a 67% similarity with HV (GII.1) and >94% with GII.4 strains (Table 2), the guinea pig anti-GII.1 sera reacted with titers comparable to that of the GII.4 strains (Fig 2B).

We next evaluated the immunogenicity and specificity of the GII.4 Consensus VLPs in rabbits, which are the preferred experimental animal model for evaluation of the UDP intranasal delivery device. Initially, rabbits were immunized intramuscularly with two doses of 50 μg of VLPs with Al(OH)₃ (Table 1) and the sera were tested against a panel of VLPs. Rabbits immunized with GII.4 Consensus VLPs showed titers ranging from $10^3$ to $10^5$ (GMT= $10^4$) against GII.4 VLPs, and $≤10^3$ (GMT= $10^2$) against non-GII.4 VLPs (p value from pooled data = 0.0002; Fig 2C and Supplementary Fig 2). Taken together, these data show that the GII.4 Consensus VLPs are structurally and antigenically similar to other GII.4 strains and are able to elicit a strong humoral immune response in immunized animals.

### 3.2 Immunogenicity of a bivalent formulation of norovirus vaccine

Because sera from rabbits immunized intramuscularly with 50 μg of GII.4 Consensus VLPs presented low levels of cross-reactivity with GI VLPs (i.e. GI.1 and GI.3, Fig 2C), we explored whether bivalent formulations of VLPs (i.e. GI [NV] and GII [GII.4 Consensus]) would increase the breadth of cross-reactivity against VLPs from different genotypes. The sera from animals immunized with the bivalent formulation presented similar levels of homologous GII.4 antibody titers when compared with those from animals immunized with GII.4 Consensus VLPs alone (GMT= $10^4$ and $1.2×10^4$, respectively). In contrast, animals immunized with the bivalent formulation developed lower GI.1 antibody titers (GMT= $10^2$) compared to animals receiving NV VLPs alone (GMT= $3.7×10^4$, Fig 3). Importantly, the heterologous titers (VLPs: GI.3, GI.1, GII.3, and GIV.1) from the bivalent formulation increased the GMT more than four-fold when compared with monovalent formulations (GI.1, GMT=111; GII.4 Consensus, GMT=100; Bivalent, GMT=484, Fig 3).
We next tested whether bivalent formulations of VLPs administered by different routes of immunization and with varying adjuvant formulations would improve vaccine performance (Table 1). Sera from rabbits immunized intranasally with Chitosan formulations or intramuscularly with Al(OH)$_3$ alone or Al(OH)$_3$+MPL formulations were examined by ELISA for homologous (VLPs: GII.4 and GI.1) and heterologous (VLPs: GI.3, GI.1, GII.3, and GIV.1) titers. The sera from rabbits immunized intramuscularly in the presence of Al(OH)$_3$ presented slightly higher homologous titers (GMT=1.3×10$^4$) than those immunized with Al(OH)$_3$+MPL (GMT=10$^4$) or intranasally with Chitosan (GMT=3500) formulations. This difference was also observed in the heterologous titers elicited by intramuscular (IM) immunization when compared with intranasal (IN) immunization (Fig 4). It has been shown that higher doses of VLPs increase the humoral homologous responses; therefore, we explored whether a higher dose of VLPs would increase the heterologous response. An overall increase in the heterologous serum titers was detected in rabbits immunized with 150 $\mu$g of each VLP (50 $\mu$g, GMT= 768; range 10$^2$ - 10$^4$; 150 $\mu$g, GMT= 5011; range 10$^2$ - 10$^5$) (Fig. 5). In addition, it was noteworthy that the homologous responses were significantly higher when administering 150 $\mu$g (GMT= 2.10$^6$ vs. 1.10$^4$), reaching titers up to 10$^9$ (Fig. 5).

4. DISCUSSION

The diversity and plasticity of RNA viruses has challenged the design of efficient antiviral drugs and vaccines. Multivalent vaccines have been used successfully for certain RNA viruses [27, 28], but the serotypic and antigenic diversity of many RNA viruses has impaired the development of effective vaccines [29-31]. To overcome this problem, the development of vaccines with engineered virus proteins that represent different variants and/or strains (e.g. artificial composite proteins) has been explored [32, 33]. In this study, we examined the use of genetically-engineered norovirus GII.4 Consensus VLPs as an immunogen to elicit broadly reactive immune responses against different clusters of GII.4 noroviruses. Our data show that GII.4 Consensus VLPs are antigenically similar to GII.4 strains that have been circulating for over 30 years in the human population. Moreover, GII.4 Consensus VLPs were recognized by GII.4-MAbs that bind to conformational epitopes located in different regions of the norovirus capsid. Of note, these MAbs were associated with amino acid residues shown to be linked with the emergence of new epidemic GII.4 strains (Parra et al. submitted manuscript), suggesting structural and antigenic similarities with wild-type GII.4 strains.

The GII.4 strains exhibit a complex pattern of reactivity with HBGA carbohydrates. It has been reported that GII.4 strains that circulated from the 1970s to 1990s have strong reactivity with H3 and Le$^V$ carbohydrates (Supplementary Table 1), while GII.4 strains that emerged after the Farmington Hills cluster lost such reactivity [12, 14, 26]. GII.4 Consensus VLPs recognized three carbohydrates (B, Le$^x$ and H3), showing the strongest reactivity with H3. Thus, although designed based on sequences from more recent clusters of GII.4, the HBGA binding pattern of GII.4 Consensus VLPs shares similarity with strains collected over 30 years ago. Taken together, the MAb recognition pattern and the reactivity with HBGA carbohydrates indicate that the engineered GII.4 Consensus VLPs are structurally similar to a wide range of wild-type noroviruses.

Human cross-challenge studies have shown evidence for low heterotypic responses between GI and GII noroviruses [34], as indicated by the lack of protection following cross-challenge [19]. Importantly, due to the limited number of norovirus challenge pools, little is known concerning the intra-genogroup or intra-genotype correlates of protection; making it difficult to determine the number of components required for an effective norovirus vaccine. To elicit a broader immune response against noroviruses, LoBue et al. 2006 immunized mice with
multivalent Venezuelan equine encephalitis virus replicon particle (VRP) vaccines that expressed VLPs from different human strains in vivo following administration. Following trivalent (GI.1, GII.1, GII.2) VRP vaccine administration, a high level of antibodies was detected against homologous and heterologous (i.e. GII.4) VLPs. However, a tetravalent (GI.1, GII.1, GII.2 and GII.4) VRP vaccine proved less efficient in inducing a broad antibody response to all the vaccine components; i.e. a possible slight immune interference was seen as measured by GI.1 and GII.2 antibody responses. In this study, we explored the use of a bivalent (GII.4 Consensus and NV VLPs) vaccine formulation administered by different routes and with varying adjuvants. Clinical trials have shown NV VLPs to be safe and immunogenic when administered by the IN route to human volunteers [17], but we explored whether an increase in heterotypic antibody titers could be achieved by modifying route, dose, and vaccine formulation. In general, the IM route resulted in higher GMT levels than IN immunization and Al(OH)₃ alone proved to be an effective adjuvant. Slight differences were observed in the animals immunized with Al(OH)₃ or Al(OH)₃+MPL as adjuvant, suggesting that the presence of MPL did not enhance the total humoral response in rabbits after immunization with norovirus VLPs. MPL is a TLR4 agonist that has been successfully used as an adjuvant to enhance humoral and memory B cellular immunity in the human papillomavirus (HPV) vaccine in mice, monkeys and humans [35]. The use of MPL with Al(OH)₃ has been shown to have varying effects depending on the immunogen, inoculation route, and animal model. For example, the use of MPL in HPV VLPs-based formulations enhanced the antibody response and B cell proliferation in IM-inoculated mice, while little effect was shown in mice immunized intraperitoneally with murine leukemia virus gag-based VLPs carrying influenza virus antigens [35, 36].

After oral immunization with NV VLPs, human volunteers showed a dose-dependent increase in virus-specific IgG titers in sera [15], and El-Kamary et al. [15] have recently shown that after IN immunization of NV VLPs with Chitosan and MPL, both serum IgG and IgA virus-specific titers were higher with increasing doses of the immunogen. In the present study, we explored whether higher doses increased heterologous responses, and observed that both homologous and heterologous titers were enhanced after higher doses of the bivalent VLP formulation were administered.

The use of consensus proteins for development of effective vaccines has been extensively studied for HIV [30, 37-40], and shown to elicit a broader immune response than polyvalent or monovalent vaccines [32]. Our data show that a genetically engineered norovirus GII.4 Consensus VLP can retain structural and functional domains present in wild-type strains and elicit broad immune responses against different clusters of GII.4 noroviruses. A Consensus VLP approach may assist in the development of norovirus vaccines that induce broad protection against multiple serotypes and variants.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

The nasal vaccine incorporates chitosan. This application of chitosan (ChiSys®) has been licensed from Archimedes Development Ltd. ChiSys is a trademark of Archimedes Development Ltd., and is registered as a CTM, as a US Registered Trademark and in certain other jurisdictions. This research was supported, in part, by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, U. S. Department of Health and Human Services.
REFERENCES


Research Highlights for Parra et al

1. A norovirus GII.4 Consensus virus-like particle was engineered for development as a vaccine candidate
2. Intramuscular immunization elicited a strong antibody responses in rabbits
3. Antibodies developed against the Consensus GII.4 VLPs cross-reacted with a panel of VLPs representing different GII.4 variants
4. A bivalent formulation of GI and GII.4 VLPs was shown to effectively induce heterotypic responses
Figure 1. Norovirus GII.4 Consensus virus-like particles (VLPs). (A) Amino acid sequence alignment of the Consensus and three representative GII.4 norovirus VP1 sequences were used to generate the composite. The S domain is highlighted with a dark line and the P domain with a grey line. (B) For EM analysis, VLPs were diluted to 0.2 mg/mL in 20 mM L-Histidine (pH 6.5), 150 mM Sodium Chloride, spotted on copper mesh grids and stained with 2% uranyl acetate (images collected at 25,000 x mag). (C) Histo-blood group antigens binding profile for GII.4 Consensus VLPs. (D) Reactivity of GII.4 VLPs with MAbs that recognize conformational epitopes (Parra et al. submitted manuscript). “-” non-reactive; “+” reactive; “+++” strong reactivity. ELISAs were performed as described in Materials and Methods.
Figure 2.
GII.4 Consensus VLPs are antigenically similar to GII.4 viruses that have been circulating for more than three decades. (A) Representative end-point titrations of guinea pig hyperimmune serum raised against GI.1, GI.3, GII.1, or GII.4 VLPs against the GII.4 Consensus VLPs as determined by ELISA. (B) ELISA titers of sera from guinea pigs immunized with various norovirus VLPs (x-axis) against the GII.4 Consensus VLPs. (C) ELISA sera titers specific for various norovirus VLPs (x-axis) from rabbits immunized intramuscularly with 50 μg GII.4 Consensus VLP + Al(OH)₃/MPL. Each animal is represented by a symbol. Bars represent the geometric mean titer. ELISAs were performed as described in Materials and Methods.
Figure 3.
Homologous and heterologous responses of rabbits immunized with the monovalent and bivalent formulations of norovirus VLPs (50ug) and Al(OH)\textsubscript{3} + MPL as adjuvants (x-axis). Statistical significance is denoted by * p ≤ 0.05; ** p ≤ 0.01. Symbols in pink correspond to homologous titers, and in orange to heterologous titers. Each animal is represented by a symbol. Bars represent the geometric mean titer.
Figure 4.
Homologous and heterologous responses of rabbits immunized with the bivalent formulation of norovirus VLPs (50µg) by different routes of immunization and adjuvants (x-axis). IN: Intranasal inoculation; IM: Intramuscular inoculation. Statistical significance is denoted by * p ≤0.05; ** p ≤0.01. Symbols in pink correspond to homologous titers, and in orange to heterologous titers. Each animal is represented by a symbol. Bars represent the geometric mean titer.
Figure 5.
Serum titers of rabbits immunized intramuscularly with two different concentrations of the bivalent formulation of norovirus VLPs. Statistical significance is denoted by ** $p \leq 0.01$; *** $p \leq 0.001$. Symbols in pink correspond to homologous titers, and in orange to heterologous titers. Each animal is represented by a symbol. Bars represent the geometric mean titer.
Table 1

Immunization protocols for norovirus VLPs co-adjuvanted with Al(OH)₃, MPL and chitosan

<table>
<thead>
<tr>
<th># Animals per immunization</th>
<th>Delivery Route*</th>
<th>Formulation (Adjuvant)</th>
<th>Amount of VLPs used**</th>
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<tbody>
<tr>
<td>7</td>
<td>IM</td>
<td>G1.1 (Al(OH)₃ + MPL)</td>
<td>50 µg</td>
</tr>
<tr>
<td>7</td>
<td>IM</td>
<td>G1.4 (Al(OH)₃ + MPL)</td>
<td>50 µg</td>
</tr>
<tr>
<td>7</td>
<td>IM</td>
<td>G1.1 + G1.4 (Al(OH)₃ + MPL)</td>
<td>50 µg</td>
</tr>
<tr>
<td>6</td>
<td>IM</td>
<td>G1.1 + G1.4 (Al(OH)₃)</td>
<td>50 µg</td>
</tr>
<tr>
<td>7</td>
<td>IM</td>
<td>G1.1 + G1.4 (Al(OH)₃)</td>
<td>150 µg</td>
</tr>
<tr>
<td>3</td>
<td>Naive</td>
<td>PBS</td>
<td>-</td>
</tr>
</tbody>
</table>

*IM, Intramuscular; IN, intranasal (Dry Powder). Two doses separated by 3 weeks were administered for each animal.

**The same amount of each VLPs was used for bivalent formulations.
### Table 2

Relationship of amino acid similarity of the major capsid protein and the cross-reactivity of VLP-specific hyperimmune sera against GII.4 Consensus VLPs

<table>
<thead>
<tr>
<th>VLPs*</th>
<th>GenBank</th>
<th>% Similarity</th>
<th>Sera titer**</th>
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</thead>
<tbody>
<tr>
<td>Hu/GI.1/Norwalk/1968/US</td>
<td>M87661</td>
<td>45.9</td>
<td>10^2</td>
</tr>
<tr>
<td>Hu/GI.3/DesertShield1993/US</td>
<td>U04469</td>
<td>45.9</td>
<td>10^3</td>
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<tr>
<td>Hu/GII.1/Hawaii/1971/US</td>
<td>U07611</td>
<td>67.0</td>
<td>10^5</td>
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<tr>
<td>Hu/GII.3/Toronto24/1991/CA</td>
<td>U02030</td>
<td>70.1</td>
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<tr>
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<td>FJ537134</td>
<td>94.1</td>
<td>10^5</td>
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<tr>
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<td>FJ537138</td>
<td>94.3</td>
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<tr>
<td>Hu/GII.4/MD145-12/1987/US</td>
<td>AY032605</td>
<td>94.9</td>
<td>10^5</td>
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<tr>
<td>Hu/GIV.1/SaintCloud624/1998/US</td>
<td>AF414427</td>
<td>54.3</td>
<td>10^2</td>
</tr>
</tbody>
</table>

* Cryptogram and GenBank accession numbers are indicated for each strain.

** Reciprocal of serum dilution from two animals. Serum from only one animal was available for GI.3 VLPs.