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Preclinical dose-ranging studies of a novel dry powder norovirus vaccine formulation

Q1 Michael J. Springer[a], Yawei Ni[a], Isaac Finger-Baker[a], Jordan P. Ball[a], Jessica Hahn[a], Ashley V. DiMarco[a], Dean Kobs[b], Bobbi Horne[c], James D. Talton[a], Ronald R. Cobb[a,*]

[a] Research and Development Department, Nanotherapeutics, Inc., Alachua, FL, United States
[b] Toxicology Department, Battelle Memorial Institute, West Jefferson, OH, United States
[c] Battelle Eastern Science and Technology Center, Aberdeen, MD, United States

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ABSTRACT

Norovirus is the primary cause of viral gastroenteritis in humans with multiple genotypes currently circulating worldwide. The development of a successful norovirus vaccine is contingent on its ability to induce both systemic and mucosal antibody responses against a wide range of norovirus genotypes. Norovirus virus-like particles (VLPs) are known to elicit systemic and mucosal immune responses when delivered intranasally. Incorporation of these VLPs into an intranasal powder vaccine offers the advantage of simplicity and induction of neutralizing systemic and mucosal antibodies. Nasal immunization, which provides the advantage of ease of administration and a mucosal delivery mechanism, faces the real issue of limited nasal residence time due to mucociliary clearance. Herein, we describe a novel dry powder (GelVac™) formulation of GI or GII.4 norovirus VLPs, two dominant circulating genotypes, to identify the optimal antigen dosages based on systemic and mucosal immune responses in guinea pigs. Systemic and mucosal immunogenicity of each of the VLPs was observed in a dose-dependent manner. In addition, a boosting effect was observed after the second dosing of each VLP antigen. With the GelVac™ formulation, a total antigen dose of ≥ 15 μg was determined to be the maximally immunogenic dose for both GI and GII.4 norovirus VLPs based on evaluation for 56 days. Taken together, these results indicate that norovirus VLPs could be used as potential vaccine candidates without using an immunostimulatory adjuvant and provide a basis for the development of a GelVac™ bivalent GI/GII.4 norovirus VLP vaccine.

1. Introduction

Q2 Norovirus, a single-stranded RNA virus in the *Caliciviridae* family, is the primary cause of nonbacterial gastroenteritis worldwide, accounting for 96% of all cases of viral gastroenteritis [1–5]. It is distributed among five different genogroups GI, GII, GIII, GIV, and GV [3,6,7]. Only genogroups I, II, and IV are infectious to humans, with GI and GII being most prevalent [8,9]. Recently, genogroup II has become the most prevalent, accounting for 81.4% of norovirus outbreaks worldwide [10]. Each genogroup is subdivided further into genoclusters. Full-length genomic sequencing of various norovirus strains indicates that norovirus can vary by 3–31% within genogroups and 44–49% between genogroups [11]. Due to this wide variation, development of a broadly effective vaccine remains a challenge as the antibodies from humans immunized against one genogroup do not cross react with noroviruses from other genogroups [12].

The success of virus-like particles (VLPs) as vaccine antigens has been demonstrated by the licensure of hepatitis B virus VLP and human papilloma-virus VLP vaccines. Extensive research has focused on the development of norovirus VLPs as vaccine antigens that can be delivered parenterally, orally, or mucosally [13,14]. Clinical evidence has demonstrated that norovirus VLPs administered orally or intranasally were well tolerated and were modestly immunogenic [15,16]. The lack of a clear immune correlate of protection has been an obstacle for the development of such vaccine candidates. A recent study has shown that antibodies that block the binding of norovirus VLPs to histo-blood group antigens correlate with clinical protection against norovirus-induced gastroenteritis [17]. Additional studies have employed recombinant expression techniques to create norovirus VLPs using baculovirus and tobacco mosaic virus demonstrating that VLPs produced by both production systems have similar structure and immunogenicity [18,19].

* Corresponding author at: Research and Development Department, Nanotherapeutics, Inc., 13859 Progress Blvd., Suite 300, Alachua, FL 32615, USA.
E-mail address: rcobb@nanotherapeutics.com (R.R. Cobb).

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Previous studies have shown that administration of a norovirus vaccine through the nasal cavity is able to induce systemic immunity as well as local and distal mucosal immunity [20,21]. Furthermore, the incorporation of VLP with GelVac™ nasal dry powder formulation elicits a greater immune response than antigen alone [21]. GelVac™ is the dry powder formulation with GelSite®, which is an *Aloe vera* L.-derived polysaccharide polymer with mucoadhesive properties. In the presence of divalent cations, GelVac™ is capable of *in-situ* gelation which improves mucosal residence time of intranasally administered vaccines [22]. Intranasal immunization of guinea pigs with the GelVac™ norovirus vaccine showed high levels of mucosal IgA antibodies along with high levels of serum IgG antibodies [21].

This study extends the previous work [21] and demonstrates GelVac™-formulated norovirus GI and GII.4 VLPs induce high levels of antigen-specific systemic and mucosal antibodies in a dose-dependent manner. The GelVac™ norovirus vaccine formulation also induced neutralizing antibodies that have been shown as a surrogate marker for efficacy in humans [17,18]. Based on the results presented herein, future studies are recommended to investigate a bivalent GelVac™ GI/GII.4 norovirus vaccine formulation. This bivalent vaccine could result in the prevention of norovirus-induced gastroenteritis in humans.

2. Materials and methods

2.1. GI and GII.4 vaccine formulation

Recombinant norovirus GI and GII.4 VLPs expressed in *Nicotiana benthamiana* were obtained from Kentucky Bioprocessing (Owensboro, KY) as previously described [23]. Endotoxins and remaining small molecules were removed by Q Column fractionation. Electron microscopy was performed to confirm the presence of VLPs (Supplementary Fig. 1). Stability evaluation of the VLPs was also conducted based on SYPRO Orange binding and antigenicity, which showed that the VLPs were stable up to 65 °C (Supplementary Figs. 2 and 3).

The GelVac™ vaccine powders were produced using a lyophilization-milling method. Liquid formulations were first prepared using a proprietary formulation comprising the recombinant VLP in a solution with GelSite ® polymer and then lyophilized. Following lyophilization, dried formulation contained 0.25%(w/w) GelSite® and 0–100 μg (based on enzyme-linked immunosorbent assay (ELISA) data) of VLP per 20 mg of formulation, depending on the desired dose. Each dried formulation was milled using a mortar and pestle under a controlled, low-humidity (< 10% RH) environment and passed through a 70 μm filter. Powders were stored in sealed containers under desiccation at room temperature until use.

2.2. Laser diffraction particle size distribution

A 50 mg sample of each powder was suspended in 100% isopropanol and particle size distribution, by volume, was determined using a laser diffraction particle size analyzer with a liquid module (Beckman Coulter LS13 320, Pasadena, CA). Performance of the instrument was verified using a 35 μm garnet reference standard.

2.3. Capture ELISA

Mouse monoclonal IgG2 anti-norovirus antibodies (Maine Biotech, MAB228 (GI); MAB227 (GII)) diluted 1:2000 in PBS were coated on Nunc MaxiSorp 96-well plates (Fisher Scientific, Pittsburgh, PA) overnight at 4 °C. The wells were washed 5 times with wash buffer, and then blocked for 1 h at room temperature in blocking buffer. Norovirus VLPs at indicated concentrations were diluted in blocking buffer, and allowed to incubate on the plate at room temperature for 1 h. The wells were washed 3 times with wash buffer, followed by incubation with corresponding mouse monoclonal IgG1 anti-norovirus antibodies (Millipore, MAB80143 (GI); Maine Biotech MAB226 (GII)) diluted 1:2000 in blocking buffer for 1 h at room temperature. The wells were washed 3 times with wash buffer, followed by incubation with a polyclonal anti-mouse IgG1:HRP (Abcam, Cambridge, MA) diluted 1:2000 in blocking buffer for 1 h at room temperature. Finally, the wells were washed 3 times with wash buffer and were developed using 1-step Ultra TMB according to manufacturer's protocol (Thermo Scientific, Waltham, MA). The OD at 450 nm was measured and plotted against known VLP concentrations.

Table 1
Animal experimental design.

Monovalent guinea pig studies		
Group #	*n*	Total antigen per vaccination(μg)*
1	4	100
2	4	50
3	4	15
4	4	5
5	4	1
6	4	0.1
7	4	0

* Animals were immunized with a total of 20 mg of powder via both nares. Each nare received 10 mg of powder or half of the total antigen dose.

2.4. Animal studies

All animal study protocols were approved by the Institutional Animal Care and Use Committee at Battelle Memorial Institute. General procedures for animal care and housing were in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) recommendations. Female (250 g) Hartley guinea pigs (Harlan Laboratories) were selected as the model animal for evaluation of dry power delivery. Two separate studies were conducted to examine the dose response of guinea pigs to both GelVac™ GI and GelVac™ GII.4 VLP vaccines independently (Table 1). Each study was designed identically with the exception of the antigen present during immunization.

Prior to immunization, animals were randomly distributed into study groups ($n = 4$) and allowed to acclimate for one week. Each study consisted of seven study groups, one for placebo powders and six for GelVac™ VLP vaccines at different antigen dose levels. Guinea pigs were anaesthetized with 5% isoflurane prior to immunization. Powder vaccines were administered intranasally on days 0 and 21 with 10 mg/nare of dry powder formulation. Total antigen exposure for each group is described in Table 1. For intranasal powder delivery, Aptar Unit Dose Spray (UDS) Devices (Aptar Pharma, Congers, NY) were each loaded with 10 mg of vaccine powder. Small animal adapters were attached to the end of each device for immunization. While under anesthesia, the end of the adapter was inserted into the nare and the device was actuated. Each animal was given two administrations of VLP powders, one per nare with half of the total antigen dose per nare (10 mg total powder per nare). The control group was administered the same amount of a placebo powder formulation.

Guinea pig serum and vaginal lavage samples were collected on days 0 (pre-immunization), 21, 42, and 56. Blood (1 ml/collection period) was collected from the superior vena cava and allowed to coagulate in serum separation tubes. Serum was collected as the supernatant after centrifugation for 10 min at 6000 rpm. Vaginal lavages were collected by lavaging 300 μL of PBS for 60 s in the vaginal tract with an oral feeding tube. On day 56, guinea pigs were maintained under 5% isoflurane and exsanguinated by

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