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Intranasal delivery of Norwalk virus-like particles formulated in an in situ gelling, dry powder vaccine

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ABSTRACT

The development of a vaccine to prevent norovirus infections has been focused on immunization at a mucosal surface, but has been limited by the low immunogenicity of self-assembling Norwalk virus-like particles (NV VLPs) delivered enterically or at nasal surfaces. Nasal immunization, which offers the advantage of ease of immunization, faces obstacles imposed by the normal process of mucociliary clearance, which limits residence time of applied antigens. Herein, we describe the use of a dry powder formulation (GelVac) of an inert in situ gelling polysaccharide (GelSite) extracted from *Aloe vera* for nasal delivery of NV VLP antigen. Powder formulations, with or without NV VLP antigen, were similar in structure in dry form or when rehydrated in simulated nasal fluids. Immunogenicity of the dry powder VLP formulation was compared to equivalent antigen/adjuvant liquid formulations in animals. For the GelVac powder, we observed superior NV-specific serum and mucosal (aerodigestive and reproductive tracts) antibody responses relative to liquid formulations. Incorporation of the TLR7 agonist gardiquimod in dry powder formulations did not enhance antibody responses, although its inclusion in liquid formulations did enhance VLP immunogenicity irrespective of the presence or absence of GelSite. We interpret these data as showing that GelSite-based dry powder formulations (1) stabilize the immunogenic structural properties of VLPs and (2) induce systemic and mucosal antibody titers which are equal or greater than those achieved by VLPs plus adjuvant in a liquid formulation. We conclude that in situ gelation of the GelVac dry powder formulation at nasal mucosal surfaces delays mucociliary clearance and thereby prolongs VLP antigen exposure to immune effector sites.

1. Introduction

Viruses belonging to the genera *Norovirus* are responsible for over 90% of all non-bacterial gastroenteritis epidemics [1] and a leading cause of global diarrhea [2]. The high prevalence of norovirus infections has led investigators to develop vaccine candidates to prevent disease [3]. Norwalk virus (NV) is the prototype virus of the genera *Norovirus* and extensive preclinical studies in mice have shown that NV virus-like particles (VLPs) administered parenterally, orally, or intranasally are immunogenic [3–9]. In clinical trials, NV VLPs administered orally or intranasally have been shown to be well tolerated and modestly immunogenic [10–12]. Despite promising results, many challenges to developing a norovirus vaccine remain. A key obstacle has been the incomplete understanding of the immune correlates of protection [3,9,13], although a recent publication by Reeck et al. showed that antibodies that block histoblood group antigen binding to NV VLPs correlate with protection against clinical NV gastroenteritis [14].

The most effective means to prevent infectious diseases like norovirus is through vaccination strategies that initiate immune responses at the natural site of infection, the mucosa [15]. The majority of currently licensed vaccines are administered parenterally, even though these vaccines have the disadvantages of patient reluctance to tolerate needle sticks and lack of mucosal immune induction [16]. Previous studies have evaluated the immunogenic

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potential of oral, nasal, rectal, and vaginal routes of vaccine administration [17–28]. The nasal cavity is a promising site for vaccine delivery because it is easy to access, is highly vascularized, has a relatively large surface area, has low proteolytic activity, and is able to induce systemic immunity as well as both local and distal mucosal immunity via the Common Mucosal Immune System (CMIS) [16,29–32]. An intranasal influenza vaccine has been approved for clinical use by the U.S. Food and Drug Administration (FDA) [33–35] and intranasal vaccines for hepatitis B virus (HBV), measles, anthrax, bacterial meningitis, and others are being evaluated [18,36]. Additional VLP-based, nasal vaccines have been shown to induce distal mucosal and systemic immunity in mice [37,38]. The nasal route has also been shown to be superior to parenteral administration for VLP-based vaccines at eliciting IgA at distal mucosal sites [39].

Nasally administered vaccines initiate an immune response through the nasal-associated lymphoid tissue (NALT) [32,40]. The NALT is composed of an assembly of antigen-reactive cells including B cells, T cells, and antigen presenting cells (APCs). Upon nasal vaccine administration, antigens can be taken up by specialized epithelial cells called microfold cells (M cells), or by macrophages and dendritic cells, which in turn leads to the activation of T and B cells [40,41]. A drawback to nasal immunization is the limited time available for antigen absorption due to the rapid mucociliary clearance of foreign particles from the nasal cavity. Beginning in the 1980s the concept of mucosal adhesives, or mucoadhesives, has been explored to improve nasal drug delivery [42]. Various synthetic or natural polymers have been studied for their ability to interact with the mucus layer covering the epithelial surface. Mucoadhesives are thought to improve drug bioavailability by increasing contact time and localization at nasal surfaces and possibly modifying epithelial permeability. These properties increase antigen uptake by M cells and other APCs, and enhance the immune response [32,43,44]. In addition, dry powder formulations offer chemical and physical stability for antigens and other vaccine components, in comparison to liquid formulations [32].

GelSite® is an *Aloe vera* L.-derived polysaccharide (polygalacturonic acid) polymer with mucoadhesive properties. The GelSite polymer, which exists in liquid form or a dry powder formulation called GelVac™, is uniquely capable of in situ gelation, turning into a gel whether in liquid or powder form upon contact with body fluids at the site of administration [45]. This in situ gelation property thereby extends the mucosal residence time. An inactivated H5N1 influenza vaccine based on the GelVac nasal powder formulation has been approved for human testing by the FDA, and a phase I clinical study is currently underway (http://clinicaltrials.gov/ct2/show/NCT01258062?term=GelVac&rank=1).

Previously, our research group showed that liquid formulations of plant-derived NV VLPs elicit humoral and mucosal immune responses when delivered via the enteric or intranasal route of immunization in mice [4,6,46,47]. The intranasal route may be preferable for a commercial vaccine because it is easy to access, is highly vascularized, has a large surface area, has low proteolytic activity, and can induce both systemic and distal mucosal immune responses [29,31,32,44]. In addition, NV VLP immunogenicity was shown to be enhanced by codelivery with the imidazoquinoline-based, TLR7 agonist, gardiquimod (GARD) [4]. While the GARD-containing, NV VLP liquid vaccine was effective, we hypothesized that a mucoadhesive-containing, dry powder vaccine might prolong the residence time on the mucosa, thereby increasing antigen uptake and enhancing the immune response [32,43]. A dry powder formulation may also be preferable for a commercial vaccine because it offers higher sterility and stability, thus facilitating mass production and vaccination in both developed and developing countries [32].

2. Materials and methods

2.1. Preparation of vaccine formulations

Recombinant NV VLPs were expressed in *Nicotiana benthamiana* by Kentucky Bioprocessing (Owensboro, KY) following previously described protocols [47]. Clarified leaf extracts were filtered through a 0.2 μm capsule filter and concentrated using a 100 kD polyethersulfone (PES) tangential flow filtration (TFF) membrane (Pall Corporation, Port Washington, NY). A diethylaminoethyl (DEAE) sepharose column was used to collect a colorless fraction that allowed recovery of the VLPs in >98% protein purity. Endotoxins and remaining small molecules were removed by Q Column fractionation. The resulting concentrated VLPs as a liquid solution in PBS were diluted to 10 or 25 μg NV VLPs in sterile PBS with or without 10 μg GARD (InvivoGen, San Diego, CA).

GelSite liquid formulations were prepared by mixing sterile stock solutions of 0.4% GelSite (DelSite Biotechnologies, Inc., Irving, TX) with PBS liquid formulations containing NV VLP with or without GARD at a 1:1 dilution in a biological hood. GelVac powder formulations (DelSite Biotechnologies, Inc.) were prepared by spray drying the liquid formulations using a Buchi B-290 Mini spray dryer (Buchi laboratories, Switzerland) in a temperature and moisture-controlled class 1000 clean room. The following formulations were prepared: GelVac alone powder, GelVac NV VLP powder, GelVac GARD powder, or GelVac NV VLP + GARD powder. GelVac powder formulations had a GelSite polymer content of 0.25% (w/w). The particle size of the powder formulations was measured using a laser diffraction particle size analyzer (Beckman Coulter LS 230, Brea, CA) and the mean particle size was ~20 μm. The powders were transferred to tight-sealed tubes and packaged in moisture and light resistant aluminum foil bags (3M™, Minneapolis, MN) with a desiccant pack and stored at room temperature until use.

2.2. GelVac NV VLP structural characterization

2.2.1. Light microscopy

Micrographs of GelVac alone powder particles were collected using a Nikon epifluorescent microscope (Nikon, Melville, NY). Powder particles were examined either as dry samples, or when rehydrated in simulated nasal fluid as previously described without the addition of bovine serum albumin [48]. Rehydrated particles were stained with 0.01 mg/ml of toluidine blue dye (EMS, Hatfield, PA).

2.2.2. Scanning electron microscopy

GelVac alone or GelVac NV VLP dry powder formulations were prepared by dispersing each powder on a metal disk and the particles were held in place using double sided sticky carbon tape. Each powder was sputter coated with gold/palladium for 5 min using a Technis Hummer II sputtering device (Technis, Alexandria, VA). Micrographs of each powder were collected using a Philips XL30 environmental scanning electron microscope (ESEM).

2.3. GelVac NV VLP quantification

NV VLP stability and concentration in the GelVac powder formulations was determined by sucrose gradient sedimentation and ELISA, as previously described [47]. Briefly, a 6-layer gradient was created in Beckman SW55 Ti tubes (Beckman Coulter, Fullerton, CA) by layering equal volumes of 60, 50, 40, 30, 20 and 10% sucrose dissolved in modified phosphate buffer (25 mM sodium phosphate, 100 mM NaCl). Following incubation at 4 °C for 2 h, GelVac NV VLP or insect cell-derived NV VLP standard (Invitrogen, Carlsbad, CA) were loaded onto the gradient and centrifuged at 90,000 × *g* for 3 h at 4 °C. Fractions were removed from the top