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Prevailing Sydney like Norovirus GII.4 VLPs induce systemic and mucosal immune responses in mice

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ABSTRACT

The newly emerged Norovirus (NoV) Sydney 2012 strain has been sweeping all over the world, causing acute non-bacterial gastroenteritis in adults and children. Due to a lack of cell culture system, virus like particles (VLPs) has been assembled and used as vaccine candidates in preclinical and clinical studies. Expression of the major capsid protein of NoVs using recombinant baculovirus expression system in Sf9 cells leads to formation of VLPs that are morphologically and antigenically similar to true virions. In this study, VLPs were successfully produced using the VP1 of Sydney-2012-like strain and its immunogenicity was evaluated by different routes and its capability in inducing mucosal immune responses in the presence and absence of adjuvants in BALB/c mice. Administration of NoV VLPs in the presence of $Al(OH)_3$ or monophosphoryl lipid A (MPL-A) led to high titers of VLP-specific IgG antibodies. Administration of VLPs orally in the presence of cholera toxin subunit B (CTB) didn't enhance mucosal immune response as less fecal IgA positive mice were observed when compared with those given VLPs only. Our study represents the first immunogenicity study of VLPs derived from current pandemic Sydney 2012 strain and which might have implications in the development of NoVs vaccine in china.

1. Introduction

Noroviruses (NoVs) are the leading cause of acute non-bacterial gastroenteritis worldwide and infect people of all ages (Patel et al., 2008). It is estimated that more than 50% of viral gastroenteritis cases worldwide are caused by NoVs. In the United States of America, more than 21 million cases are caused by NoVs annually. NoVs, belonging to the family of Caliciviridae, are divided into 5 genogroups designated GI through to GV. They are further subdivided into 9 and 22 genotypes (Kroneman et al., 2013; Zheng et al., 2006). Genogroup II, genotype 4 of the human NoVs are most commonly detected in outbreaks, and they undergo rapid evolution so that the previous dominating strains are replaced by new variants every two or three years (Lee and Pang, 2013; Hasing et al., 2013; Bull et al., 2010). Our understanding of NoVs has been hampered by the lack of suitable cell culture systems and animal models. Several studies have reported successful in vitro replication of NoVs in cells generated by using reverse genetic systems or direct infection of B cells with isolated virions from fecal samples (Asanaka et al., 2005; Katayama et al., 2014; Jones et al., 2014). Although these progresses might be of importance in elucidating biological life and developing compounds for the treatment of NoVs, the low viral titers achieved have greatly restricted its use for vaccine development.

Major capsid protein VP1 of NoVs self-assembles into virus-like particles (VLPs) when expressed in eukaryotic cells. The VLPs have been extensively studied as vaccine candidates (Jiang et al., 1999; Bernstein et al., 2014; Atmar et al., 2011; Ball et al., 1999). Studies in animals and clinical trials have shown promising results. Administration of NoV VLPs in different routes in the presence or absence of adjuvants has induced strong humoral and mucosal immune responses.

Most previous animal immunization studies are conducted using Norwalk like virus particles as Norwalk-virus was the prototype norovirus. In this study, VLPs were successfully produced by expressing the VP1 of the current Sydney 2012-like Chinese strain isolated in our laboratory in April 2013, by using recombinant baculovirus expression system. In a BALB/c mouse model, its immunogenicity was investigated in different formulations and immunization routes. It was found that mucosal immune responses were induced via oral administration in the presence and absence of adjuvant CTB (Huo et al., 2014). Also intramuscular immunization in the presence of $Al(OH)_3$ and MPL-A induced high titers of serum

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IgG with ability to block the VLP binding to salivary histo-blood group antigens (HBGAs).

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in accordance with the guidelines of Chinese Council on Animal Care. The research protocol was approved by Animal Care and Use Committee of the Wuhan Institute of Biological Products (WIBP).

2.2. Recombinant VLP preparation and characterization

The major capsid protein coding sequence was codon-optimized based on the codon usage frequency of *Spodoptera* (*S.*) *frugiperda* cells. The synthesized VP1 gene (Shanghai Sangon Biotech) was cloned into transfer vector pVl1393 flanked by BamHI and NotI sites at its 5′ and 3′ ends, respectively. Baculovirus recombinant containing the VP1 gene in pVL1393 vector was obtained by homologous recombination between the transfer vector and linearized Baculovirus DNA (BD Biosciences, San Diego, CA). In brief, sf9 cells were transferred to a 6-well plate at a density of 2×10^5/ml and let adhere at room temperature (RT) for 2 h. Purified sterile pVL1393 vector (2 μg) containing target sequence and linearized Baculovirus DNA (0.2 μg) were mixed with equal volume of Lipofectin (Invitrogen, US). The mixture was vortexed and incubated at RT for 20 min before it was added to a 6-well plate. Cytopathic effects were generally observed within 3–5 days and further amplification of recombinant Baculovirus was performed by infecting more cells in T225 culture flask. The medium was harvested 5 to 7 days post infection and frozen at −80 °C as viral stocks.

Monolayer sf9 cells were infected with recombinant baculovirus, and VLPs were purified as previously reported (Huo et al., 2015). In brief, medium of infected sf9 cell was harvested 5 to 7 days post infection. The harvested cell medium was clarified at 3000 × *g* to remove cell debris followed by ultracentrifugation at 141,000 × *g* for 3 h at 8 °C in a SW 28 rotor to pellet VLPs. Pellets were resuspended in PBS (pH 7.3) prefiltered with 0.22 μm membrane. VLPs in PBS were mixed with equal volume of CsCl (1.6 g/ml) and centrifuged at 288,000 g for 24 h at 4 °C in a Beckman SW 41 Ti rotor. Visible bands were collected and analyzed by western blotting using rabbit anti-GII.4 VP1 specific hyperimmune serum. Purity and concentration of VLP were determined by denatured SDS-PAGE analysis. Protein concentration was determined by using the BCA-protein assay according to the manufacturer's protocol (Thermo Scientific, US). The quality and integrity of VLPs were confirmed by negative staining and observed under electron microscopy.

2.3. Immunization and sample collection

Groups of 6-8 weeks old female BALB/c mice (5 per group) were immunized twice (Day 0 and day 21) by administering NoV VLPs intramuscularly (100 μl) in the presence and absence of $Al(OH)_3$ (0.1 mg/dose) and MPL-A (1 μg/dose), and orally by gavage (100 μl), using a stainless steel intubation needle. The amount of VLPs was 1 μg and 10 μg for $Al(OH)_3$ and MPL-A groups, and 5 μg and 50 μg for CTB (1 μg/dose) groups, with an extra group delivered by gavage of 50 μg of VLPs only. Control mice were given PBS, pH 7.4, PBS plus $Al(OH)_3$, PBS plus MPL-A, and PBS plus CTB, respectively. Pre-immunization serum and stool samples were collected before first immunization. Post-immunization serum and fecal samples were collected on days 21, 28 and 42, respectively.

Blood samples were collected by retro-orbital plexus puncture, and fecal samples were obtained from individual mouse by using a fecal collection cage. Fecal samples were extracted by making a 10% suspension (wt/vol), in PBS containing 0.1 mg of soybean trypsin inhibitor/ml, and 0.1 mg of merthiolate/ml. Each fecal sample suspension in an EP tube was sonicated for 15 s on ice and centrifuged for 10 min at 5000 g in a microcentrifuge. The clarified supernatant was transferred to a fresh new EP tube and stored at −20 °C.

2.4. Antibody enzyme-linked immunosorbent assays (ELISAs)

Polyvinyl chloride 96-well plates (Yunpeng, China) were coated with VLPs by adding 100 μl of VLPs per well (2 μg/ml for IgG and 3 μg/ml for IgA assay) and incubated at 4 °C overnight. Nonspecific protein binding was blocked overnight at 4 °C with 1% BSA in PBS containing 0.05% of Tween-20 (PBS-T) (150 μl/well).

(1) Serum IgG ELISA: NoV-specific IgG titers in serum were determined by testing individual samples on VLP-coated plates. Testing sera were serially diluted 2-fold in PBS-T containing 1% BSA in a microtiter plate and incubated for 1 h at 37 °C to permit antibody binding. Pre-immunization mouse sera were used as negative control and wells added with PBS-T + 1% BSA solution only was used to determine the background binding. The plates were washed five times with PBS-T and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Thermo Scientific, US) diluted at 1:4,000 in enzyme buffer (0.05 mM Tris, pH 7.5, containing 100 mM NaCl, 10% fetal bovine serum, 0.25% Triton X-100, 0.05% proclin 300) was added and incubated for 30 min at 37 °C. The reaction was developed with 50 μl of substrate A containing tetramethylbenzidine (TMB) and 50 μl of substrate B containing urea peroxide for 10 min, and color development was stopped by the addition of an equal volume of 2 M sulfuric acid. Absorbance was measured at 450 nm using a Multiskan MK3 plate reader (Thermo Scientific, US). End point titers were reported as the reciprocal of the highest dilution that had an absorbance value greater than or equal to 0.1 above the background (absorbance of the wells lacking antigen).
(2) Fecal IgA ELISA: To determine the level of NoV VLP-specific fecal IgA, the stool extracts were diluted at 1:3 with 1% BSA in PBS-T, added to VLPs-coated plates and incubated at 37 °C for 2 h. After washing for five times with PBS-T, HRP-conjugated goat anti-mouse IgA at a dilution of 1:10,000 was added and incubated at 37 °C for 1 h. The unbound HRP-conjugate was removed by washing five times with PBS-T. The optical density was read with a Multiscan plate reader at 450 nm after incubation with peroxidase substrate TMB and urea peroxide for 30 min.

2.5. Antibody avidity assay

An avidity assay to detect high avidity of NoV(s) antibodies was exploited. Briefly, the IgG ELISA was conducted as described in the previous section except that after the incubation of serum samples (1:4000 dilution) on VLP-coated microtitre plates the samples were aspirated and 8 M urea was added (250 μl/well). Two 5-min incubation steps with urea were followed to remove the low avidity antibodies. The avidity index was calculated as (OD with urea/OD without urea) ×100% and an index value equal to or greater than 50% was considered to be high avidity.

2.6. Saliva-VLPs binding blocking assay

Sera from final bleeding were used to test ability in blocking saliva-VLP binding. The blocking assay using human saliva was conducted as described by others with minor modification (Marionneau et al., 2005; Huang et al., 2003). Briefly, secretor positive human saliva with known ABO antigens was boiled for 10 min to inactivate native antibodies. The boiled saliva was diluted at 1:2000 in carbonate buffer, pH 9.6, added into 96-well plates

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