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Functionality and avidity of norovirus-specific antibodies and T cells induced by GII.4 virus-like particles alone or co-administered with different genotypes

Maria Malm, Kirsi Tamminen, Suvi Heinimäki, Timo Vesikari, Vesna Blazevic *

Vaccine Research Center, University of Tampere, Biokatu 10, 33520 Tampere, Finland
University of Tampere, Faculty of Medicine and Life Sciences, Tampere, Finland

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ABSTRACT

Norovirus (NoV) is the main cause of acute gastroenteritis worldwide across all age groups. Current NoV vaccine candidates are based on non-infectious highly immunogenic virus-like particles (VLPs) produced in cell cultures in vitro. As NoVs infecting human population are highly divergent, it is proposed that the vaccine should contain at least two different NoV genotypes, potentially affecting the immunogenicity of each other. We investigated the immunogenicity of NoV GII.4 VLPs administered by intramuscular (IM) or intradermal (ID) injections to BALB/c mice either alone or co-delivered with genogroup I (GI) and other genogroup GII VLPs. Serum NoV-specific IgG binding antibody titers and antibody functionality in terms of avidity and blocking potential were assessed. Furthermore, the specificity and functional avidity of $CD4^+$ and $CD8^+$ T cell responses were analyzed using synthetic peptides previously identified to contain NoV VP1 P2 domain-specific H-2^d epitopes. The results showed that IM and ID immunization induced comparable GII.4-specific antibodies and T cell responses. Similar magnitude and functionality of antibodies and interferon-gamma producing T cells were developed using monovalent GII.4 VLPs or different genotype combinations. For the first time, degranulation assay using multicolor flow cytometry showed that NoV GII.4-specific $CD8^+$ T cells had cytotoxic T lymphocyte phenotype. To conclude, our results demonstrate that there is no immunological interference even if up to five different NoV VLP genotypes were co-administered at the same time. Furthermore, no inhibition of NoV-specific antibody functionality or the magnitude, specificity and affinity of T cell responses was observed in any of the immunized animals, observations relevant for the development of a multivalent NoV VLP vaccine.

1. Introduction

Acute gastroenteritis (GE) caused by noroviruses (NoVs) affects people of all ages worldwide with severe outcomes especially in young children and the elderly [1,2]. Highly contagious NoVs are genetically diverse with continuously emerging new variants that leads to fast spreading in human population through immune escape. NoVs are members of the *Caliciviridae* family with more than 30 recognized genotypes infecting humans that belong to genogroups (G) I, II, and IV [3]. The icosahedral viral particle of NoV, with ~38 nm diameter, is composed of 180 copies of major capsid protein, viral protein 1 (VP1). NoV virus-like particles (VLPs), formed by spontaneous self-assembly of VP1 in vitro, are morphologically and antigenically identical to the virus. VLPs has contributed significantly to the NoV research, as they are successfully used for structural and immunogenicity studies, and as promising NoV vaccine candidates. Due to the high genetic variability of NoVs and a lack of cross-protective immunity between GI and GII NoVs, it is believed that a vaccine should contain a minimum of one VLP from each genogroup. Our group has recently proposed a NoV candidate vaccine consisting of GI.3 and GII.4 VLPs in a combination with rotavirus (RV) VP6, to prevent two major causes of childhood viral GE worldwide [4,5].

Although cell culture systems for growth of NoV in vitro have recently been established, a traditional neutralization assay is still not in use [6,7]. Instead, a subset of NoV-specific antibodies that block binding of NoV VLPs to their cellular carbohydrate ligands, histo-blood group antigens (HBGA), are regarded as correlates of protection from infection [8–11]. The role of NoV-specific T cell responses in protection from NoV infection [12–16] has not yet

* Corresponding author at: Vaccine Research Center, University of Tampere, Biokatu 10, FI-33520 Tampere, Finland.
E-mail address: vesna.blazevic@uta.fi (V. Blazevic).

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been established and requires further research. Using matrix peptide pools, we have recently identified seven immunodominant H-2^d restricted NoV-specific T cell epitopes, including a highly conserved universal GII NoV-specific $CD8^+$ epitope (peptide 99-45, NNYDPTEEIPAPLGTPDF) and GII.4-1999 variant-specific $CD4^+$ T cell epitope (peptide 99-50, TRAHKATVSTGSVHFTPK) [17].

In the present study we investigated NoV GII.4-specific antibody functionality (avidity and blocking activity) and T cell responses in mice immunized with GII.4-1999 VLPs alone or co-delivered with different VLPs belonging to GI and GII NoVs, with the primary focus on mutual inhibition or immunological interference. In addition, NoV-specific cytotoxic T lymphocyte (CTL) responses were investigated for the first time.

2. Material and methods

2.1. Recombinant proteins and synthetic peptides

Five different NoV capsid VLPs derived from GII.4-1999 (reference strain accession no: AF080551), GII.4-2009 New Orleans (NO; reference strain accession no: GU445325), GII.12 (reference strain accession no:AJ277618), GI.1 (accession no: AY502016.1), and GI.3 (reference strain accession no: AF414403) VLPs were produced in baculovirus-insect cell system and purified by sucrose gradient ultracentrifugation as described in details earlier [4,18]. The purity, identity and morphology of VLPs were determined by SDS-PAGE, immunoblotting, and electron microscopy using previously described procedures [19].

Seventy-four synthetic peptides representing the entire 539 amino acid (aa) sequence of GII.4-1999 NoV VP1 were synthesized by Synpeptide Co., Ltd (Shanghai, China) as 18-mers overlapping by 11 aa. The purity was >70% as determined by high-pressure liquid chromatography. Each lyophilized peptide was dissolved in DMSO and further diluted in sterile PBS for use in the assays. In addition, all individual peptides were pooled (GII.4-99 pool) as recently described [15]. The GII.4-99 pool was used at a predetermined concentration of 2 μg/ml in all assays. Individual GII.4 peptides, 99-45 (^{309}NNYDPTEEIPAPLGTPDF^{326}) and 99-50 (^{344}TRAHKATVSTGSVHFTPK^{361}), a predicted [17] mouse H-2^d 9-mer epitope (^{318}PAPLGTPDF^{326}, included in the 99-45 sequence), and two negative control peptides, 10-mer (^{139}TMFPHIIVDV^{148}) and 17-mer (ovalbumin, OVA ^{323}ISQAVHAAHAEINEAGR^{339}), were synthetized as described above and used at a final concentration of 4 μg/ml.

2.2. Immunizations

BALB/c (*H-2^d*) mice were obtained from Envigo RMS BV (formerly Harlan Laboratories, Horst, Netherlands) and immunized at 7 weeks of age after a week of acclimatization. Forty mice were divided into 8 groups (5 mice/group) and mice were immunized either intramuscularly (IM) at the right caudal thigh muscle or intradermally (ID) at the base of the tail (Table 1). The dose of 10 μg or 50 μg of GII.4-1999 VLPs was administrated in a 50 μl volume/dose at week 0 and week 3, a standard procedure used by our laboratory [4,5]. A group of mice received a mixture of GII VLPs (GII.4-1999, GII.4 NO and GII.12, 20 μg each) by IM delivery according to the same schedule. Additional group of mice received a mixture of GII.4 VLPs (as above) and a mixture of GI VLPs (GI.1 and GI.3) simultaneously at the different sites. Two negative control groups of mice received a carrier only (phosphate-buffered saline, PBS) either IM or ID. No external adjuvants were used. Two weeks after the second immunization mice were sacrificed and serum and spleen cells were collected and prepared as previously described [4,5] for the analysis of NoV-specific antibodies and T cell responses. Mice welfare was monitored throughout the study and experiments were performed in accordance with the guidelines of the Finnish National Animal Experiment Board.

Table 1
Experimental and control groups of immunized mice. Mice were immunized intramuscularly (IM) or intradermally (ID) at day 0 and 21 with the indicated dose and terminated at day 35.

Group	Immunogen	Dose (μg)	Route
I	GII.4-1999 VLP	10	IM
II	GII.4-1999 VLP	10	ID
III	GII.4-1999 VLP	50	IM
IV	GII.4-1999 VLP	50	ID
V	GII-mix VLPs (GII.4-1999, GII.4 NO, GII.12)	60 (20 each)	IM
VI	GII-mix VLPs (GII.4-1999, GII.4 NO, GII.12)+ GI-mix VLPs (GI.1 and GI.3)	60 + 40 (20 each)	IM ID
VII	PBS	–	IM
VIII	PBS	–	ID

PBS, phosphate-buffered saline.

2.3. Serum IgG antibody titer and avidity

Serum GII.4-specific IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) as described elsewhere in detail [4,5]. In brief, twofold diluted serum samples were analyzed on NoV VLP-coated plates. IgG antibodies were detected with HRP-conjugated anti-mouse IgG (Sigma Aldrich, Saint Louis, MO) followed by o-Phenylenediamine dihydrochloride (OPD)-substrate (Sigma-Aldrich). The optical density (OD) was measured at 490 nm in a microplate reader (Victor^2 1420, Perkin Elmer, Waltham, MA). The mean end-point titers of the individual serum were determined as the reciprocal of the highest serum dilution giving an OD above the set cut-off value (mean OD of negative control mice serum wells + 3 × SD) and at least 0.100 OD.

Avidity assay was conducted as the IgG ELISA assay described above using 1:200 serum dilution, but additional two 8 M urea incubation steps were included to remove low avidity antibodies according to previously published method [20]. Avidity index was calculated as (OD with urea/OD without urea) × 100%.

2.4. Blocking assay

Blocking assay was used to detect antibodies preventing NoV GII.4-1999 VLP binding to the synthetic HBGAs as previously described in detail [11]. Briefly, synthetic biotinylated Le^d (H type 1)-PAA-Biotin (Glycotech, Gaithersburg, MD) was coated on NeutrAvidin plates (Pierce, Rockford, IL). After 1 h pre-incubation at 37 °C, a mixture of NoV VLPs (0.4 μg/ml) and serially twofold diluted serum samples were plated and incubated for 2 h at 4 °C. The bound VLPs were detected using NoV VLP type-specific human serum, followed by goat anti-human IgG (H + L)-HRP (Invitrogen, Carlsbad, CA) and OPD-substrate (Sigma-Aldrich). The OD was measured as described above. Maximum binding was determined by VLP lacking mouse sera. The results are expressed as the mean blocking index (%) calculated as 100% − [(OD wells with VLP − serum mix/OD maximum binding OD) × 100%]. Blocking titer 50 (BT50) was expressed as the reciprocal of the highest serum dilution blocking 50% of the maximum VLP binding.

2.5. IFN-γ ELISPOT assay

NoV-specific T cell responses were determined by the ability of short synthetic peptides to induce ex vivo IFN-γ production by splenocytes of immunized mice using interferon-gamma (IFN-γ) enzyme-linked immunospot (ELISPOT) assay [5]. Liquid nitrogen

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