

Characterization of Blockade Antibody Responses in GII.2.1976 Snow Mountain Virus-Infected Subjects

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Snow Mountain virus (GII.2.1976) is the prototype strain of GII.2 noroviruses (NoVs), which cause an estimated 8% of norovirus outbreaks, yet little is known about the immunobiology of these viruses. To define the human immune response induced by SMV infection and the antigenic relationship between different GII.2 strains that have circulated between 1976 and 2010, we developed a panel of four GII.2 variant virus-like particles (VLPs) and compared their antigenicities by enzyme immunoassay (EIA) and surrogate antibody neutralization (blockade) assays. Volunteers infected with GII.2.1976 developed a mean 167-fold increase in blockade response against the homotypic VLP by day 8 postchallenge. Blockade extended cross-genotype activity in some individuals but not cross-genogroup activity. Polyclonal sera from GII.2.1976-infected volunteers blocked GII.2.1976 significantly better than they blocked GII.2.2002, GII.2.2008, and GII.2.2010, suggesting that blockade epitopes within the GII.2 strains have evolved in the past decade. To potentially map these epitope changes, we developed mouse monoclonal antibodies (MAbs) against GII.2.1976 VLPs and compared their reactivities to a panel of norovirus VLPs. One MAb had broad cross-genogroup EIA reactivity to a nonblockade, linear, conserved epitope. Six MAbs recognized conformational epitopes exclusive to the GII.2 strains. Two MAbs recognized GII.2 blockade epitopes, and both blocked the entire panel of GII.2 variants. These data indicate that the GII.2 strains, unlike the predominant GII.4 strains, have undergone only a limited amount of evolution in blockade epitopes between 1976 and 2010 and indicate that the GII.2-protective component of a multivalent norovirus vaccine may not require frequent reformulation.

Noroviruses (NoVs) are the leading cause of viral acute gastroenteritis globally, resulting in over 20 million cases annually in the United States alone. Worldwide, an estimated 200,000 deaths occur each year in developing countries among children under age 5 (1, 2). Young children, the elderly, and the immunocompromised are the most susceptible populations to complications arising from norovirus infection (3–5). With an infectious dose of as few as 20 viral particles, large quantities of viral shedding, and resistance to many chemical disinfectants, noroviruses have been referred to as “the perfect human pathogen” (6). The onset of symptoms occurs 10 to 24 h following exposure, and the duration of severe gastroenteritis resolves after 24 to 48 h in most cases (7), but there are instances of chronic infection, primarily in immunocompromised individuals (8–10). Prevention of transmission is complicated by the fact that not all infected individuals are symptomatic, and viral shedding can occur for weeks after symptoms have resolved (1).

Members of the family *Caliciviridae*, noroviruses are ~7.5-kb positive-sense, single-stranded RNA viruses that are structurally composed of three open reading frames (ORFs). ORF1 encodes the nonstructural proteins, including the RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 encode the major (VP1) and minor (VP2) structural proteins, respectively (11, 12). The sequence of ORF2 is used to classify noroviruses; sequence identity of >85% corresponds to the strain, 56.2 to 85% to the genotype, and 38.6 to 55% to the genogroup (12). Genogroup I (GI) and GII strains cause almost all human NoV infections. Within GI and GII, there are currently 31 different genotypes (13), although enhanced outbreak investigation continues to expand the number of identified NoV genotypes.

Structurally, the major capsid protein is composed of the shell (S) domain, and the protruding (P) domain, which is further di-

vided into the P1 and P2 subdomains. Ninety copies of dimerized S domain make up the icosahedral shell. The P1 subdomain forms a stalk rising from the shell, while the P2 subdomain forms the tip of the protrusion of the capsomere and is responsible for histo-blood group antigen (HBGA) binding and a majority of identified antibody recognition sites (14–17). The P2 subdomain of GII.4 NoVs is under selective pressure and subsequently is hypervariable. Changes in the P2 subdomain result in loss of VLP reactivity with potentially neutralizing “blockade” antibodies and correlate with the emergence of new, antigenically distinct, GII.4 strains. Additionally, P2 amino acid changes have been shown to influence HBGA binding of GII.4 strains, potentially modulating population susceptibility. While immune escape and variable HBGA binding are well documented for GII.4 NoV strains, the impact of viral evolution on other NoV genotypes is not well characterized.

GII.4 strains, which cause a majority (70 to 80%) of NoV outbreaks, have undergone an ~9% amino acid substitution rate in the capsid protein sequence over 12 years (18–23). In comparison, GII.2 strains cause about 8% of NoV infections and have only ~2.6% amino acid diversity in the GII.2 capsid over a similar time frame (4). Harris et al. (4) have proposed that the evolution in the capsid gene of the GII.2 variants is due to selective pressure causing genetic drift, similar to the emergence of new GII.4 variants

Received 24 September 2013 Accepted 23 October 2013

Published ahead of print 30 October 2013

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doi:10.1128/JVI.02793-13

(20, 21, 25, 26). Importantly, this suggestion was based solely on predictive biology. To date, there have been no empirical data demonstrating that the natural variation in the capsid gene of GII.2 strains results in viruses of altered antigenicity or receptor binding properties.

Due to a lack of a cell culture or small animal model for human noroviruses, *in vitro* assays have been used to characterize evolution and immunity. Samples collected during human challenge studies and outbreak investigations allow characterization of immune responses using *in vitro* assays. Various studies have found that HBGAs, found in mucosal secretions as well as on the surface of epithelial cells, are norovirus cell attachment factors (27–30). The capacity of human polyclonal sera to inhibit NoV VLP binding to HBGAs has been shown to correlate with protection from infection (31, 32). While enzyme immunoassays (EIAs) measure the presence or absence of reactive antibody, neutralization assays measure the potential protective activity of an antibody (20, 33, 34). Our group has pioneered the development of NoV surrogate neutralization assays (22, 35, 36), based on antibody “blockade” of VLP-carbohydrate ligand interactions.

What little is known about GII.2 susceptibility and immunity comes from studying naturally and experimentally infected humans. Utilizing a human challenge model, we challenged 15 volunteers with 10, 10³, or 10⁵ reverse transcription-PCR-detectable units of GII.2.1976 Snow Mountain virus (SMV) and evaluated potential susceptibility markers and immune responses in the subjects (37). Contrary to GI.1.1968 NoV, GII.2.1976 infection was not dependent on secretor or Lewis phenotypes in this small trial. *In vitro*, GII.2.1976 VLPs bound exclusively to type B saliva and not to synthetic biotinylated di- or trivalent saccharides (33, 38). However, subjects with O, A, and secretor-negative phenotypes became infected in this study, identifying the same disparity in GII.2.1976 virus infection and *in vitro* VLP-ligand binding that was evident in a much-larger-scale human challenge study with GI.1.1968 NoV. Although GI.1.1968 did not bind to type B saliva or biotinylated synthetic carbohydrate, 43% (28) and 75% (27) of B-positive subjects became infected when challenged with live GI.1.1968 virus. These studies suggest that *in vitro* binding profiles of norovirus VLPs correlate with the relative risk of norovirus infection but do not define absolute susceptibility profiles, especially at high virus doses (27). This discrepancy may be due to subtle differences between carbohydrate expression in saliva and that in the gut. Furthermore, GII.2.1976 infection induced a significant increase in homotypic serum IgG by day 8 postchallenge, as measured by EIA (37). The SMV-induced IgG cross-reacted with another GII VLP, but not with a GI VLP. Similar cross-reactivity results were characterized for CD4⁺ T cell responses. To date, nothing is known about the neutralization potential of IgG induced by GII.2 infection.

In this follow up study, we developed a surrogate neutralization assay for GII.2 NoVs based on VLP binding to B saliva. We used this assay to evaluate the timing of blockade antibody development after GII.2.1976 challenge and then compared the cross-reactive blockade of both human challenge sera and monoclonal antibodies (MAbs) to a panel of time-ordered GII.2 VLPs to assess the extent of antigenic evolution in this NoV genotype. Both human challenge sera and MAbs suggest that the GII.2 lineage of NoV is under limited pressure from herd immunity and has remained relatively antigenically static over the past 35 years. These results may explain why GII.2 NoVs cause only limited numbers

of outbreaks and provide support for a single GII.2 component in an NoV multivalent vaccine.

MATERIALS AND METHODS

VLPs. GII.2 ORF2 genes (GII.2.1976 SMV [GenBank accession no. AY134748.1], GII.2.2002 Ina, [accession no. AB195225.1], GII.2.2008 Osaka City [accession no. AB662868.1], and GII.2.2010 Osaka City [accession no. AB662899.1]) were inserted into the VEE pVR21 replicon vector to create virus replicon particles (VRPs) and virus-like particles (VLPs) assembled in VRP-infected BHK-21 cells, as formerly described by our group (20, 39–41). Briefly, VLPs were purified by velocity sedimentation, the sucrose was diluted out with phosphate-buffered saline (PBS), and the VLPs were concentrated in an Amicon Ultra 100-kDa molecular mass cutoff centrifugal unit (Millipore, Billerica, MA). The purity of VLP preparations was determined by Coomassie blue-stained SDS-PAGE gels. Negative staining with uranyl acetate, followed by imaging on a LEO EM-910 transmission electron microscope, was done to evaluate the structural integrity of the particles. A bicinchoninic acid (BCA) protein assay (Thermo-Fisher) was performed to determine the protein concentration.

Study design and subjects. Archival serum samples from our previous SMV human challenge study conducted in 2001 were used in this study (37). Of the 15 original challenge study volunteers, serum samples from 13 subjects were available for analysis of blockade titer. All study protocols and methods were approved by the University of North Carolina School of Medicine Committee for Protection of Human Subjects. All volunteers participated after providing informed consent.

EIAs. Antibody binding was detected by enzyme immunoassays (EIAs), performed as previously described (22). Briefly, EIA high-binding, easy-wash plates were coated with 0.5 µg/ml VLP in phosphate-buffered saline (PBS) before addition of 2-fold serial dilutions of mouse monoclonal antibody (MAb) or polyclonal serum. The primary antibody incubation was followed by 1:10,000 horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare) and development with 3,3',5,5'-tetramethylbenzidine (TMB) Ultra (Thermo Scientific). Plates were washed with PBS–0.05% Tween 20 between each step, and all antibody dilutions were performed in 5% Blotto in PBS–0.05% Tween 20. Plates were read at the optical density at 450 nm (OD₄₅₀), and 50% effective concentration (EC₅₀) values were calculated using a sigmoidal dose-response curve and GraphPad Prism version 6.02 for Windows, GraphPad Software, La Jolla, CA. All data points represent an average of at least three replicates from a minimum of 2 independent runs. Antibodies were considered positive for reactivity if the mean optical density of the maximum binding, after subtraction of the background, was at least 0.2 (42). If the mean optical density was less than 0.2, the antibody was determined to be nonreactive and assigned a value of 2-fold the limit of detection, and the EC₅₀ was represented on graphs by a data marker above the limit of detection (dashed lines).

Carbohydrate-binding and blockade assay. EIA high-binding, easy-wash plates were coated with 50 µl per well of processed saliva (28, 37) diluted 1/500 in PBS and blocked with 5% Blotto in PBS–0.05% Tween 20 before the addition of serial dilutions of VLP. VLP binding was detected with 1/500 rabbit anti-GII.2.1976 polyclonal sera, followed by 1:10,000 HRP-conjugated goat anti-rabbit secondary antibody (GE Healthcare). All antibody incubations were performed at room temperature in 5% Blotto in PBS–0.05% Tween 20. Plates were washed with PBS-Tween 20 three times, with 15- to 60-s soaks between each step. TMB Ultra was added to each well, the mixture was incubated for 10 min, color development was stopped by adding 50 µl 2 M sulfuric acid, plates were read at OD₄₅₀ and half-maximal binding was calculated.

For blockade assays, 0.5 µg/ml VLP, either alone or pretreated with serial dilutions of serum or MAb for 30 min, was added to saliva-coated plates, and the mixture was incubated at room temperature for 90 min. The percentage of control binding was defined as the amount of VLP bound to the carbohydrate-coated plate in the presence of antibody pre-

treatment divided by the amount of VLP bound in the absence of antibody pretreatment multiplied by 100. EC_{50} values were calculated by using the reciprocal of the dilution of serum or $\mu\text{g/ml}$ of purified antibody. All experiments were performed at least three times in duplicate. The criteria used to determine if an antibody was a blockade antibody were (i) blockade of at least 50% of the VLP-carbohydrate interaction within the dilution series tested and (ii) a positive dose response between antibody concentration and mean percentage of control binding, as determined by a Hill slope absolute value greater than 0.8.

Production of mouse monoclonal antibodies. Swiss Webster mice were immunized intraperitoneally (i.p.) on days 0, 20, and 40 with 50 μg of VLP in PBS plus 50 μl Gerbu adjuvant (Thermo Fisher). On day 80, mice were boosted before splenocytes were chemically fused with mouse myeloma line P3X63-AG8.653 on day 84. Aminopterin was added to the media to select for fused cells, the resulting hybridomas were screened by EIA, and clonal colonies were isolated by limiting dilution subcloning. The resulting antibodies were isotyped (Roche) and purified by protein G chromatography (GE Healthcare).

Western blots. VLPs were suspended in Laemmli loading buffer and loaded at 9 μg of protein in a prep-well 7.5% SDS-polyacrylamide gel (Bio-Rad). Electrophoresed proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and blocked. Immobilized proteins were probed with 1 or 5 $\mu\text{g/ml}$ MAb at 37°C, which was detected with HRP-conjugated goat anti-mouse IgG secondary antibody (GE Healthcare). The ECL enhanced chemiluminescence Western blotting detection kit (GE Healthcare) was used to visualize antibody-reactive protein. Each step was followed by multiple washings with PBS–0.05% Tween 20, and reagents were diluted in 5% Blotto in PBS–0.05% Tween 20.

Structural models of GII.2 P domains. The amino acid sequences of GII.2.1976, GII.2.2002, GII.2.2008, and GII.2.2010 capsids were individually aligned to the VA387 P domain sequence using ClustalX1.86, and the GII.4.2002 P domain dimer X-ray crystal structure (PDB accession no. 2OBT) was used as a template for generation of homology models. Homology models were generated using the program Modeller available via the Max Planck Institute Bioinformatics Toolkit (<http://toolkit.tuebingen.mpg.de/>). The structural models were analyzed and compared, and figures were generated using Mac Pymol (Delano Scientific).

Statistical analysis. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was performed when three or more values were compared; direct comparisons between two values were measured using the unpaired *t* test. For statistical significance, $P < 0.05$ was considered significant. All statistics were done using GraphPad Prism Software.

RESULTS

SMV infection induces a strong intragenotype blockade response. The sequence of the P2 domain of GII.2 strains has evolved over time, although less extensively than has been reported for GII.4 NoV strains (24). To determine if these GII.2 residue changes led to antigenic differences between the GII.2 NoV strains, we performed a multiple sequence alignment with 10 GII.2 capsid sequences from GenBank and identified 16 amino acid substitutions that occurred over time (Fig. 1A). We selected four sequences that represented the diversity of sequence identity, and when the amino acid differences between the four GII.2 reference sequences were mapped onto a GII.4.2002 P dimer model, the model showed that the differences mapped to the most-surface-exposed ridges of the GII.2 capsid (Fig. 1B). To determine if these surface changes resulted in antigenicity changes, we synthesized four time-ordered GII.2 VLPs representing strains that circulated between 1976 and 2010 for comparison. Structural integrity of the new GII.2 VLPs was verified by transmission electron microscopy (TEM) visualization of ~40-nm particles and confir-

mation of carbohydrate binding to saliva (Fig. 1C and D). GII.2.1976 and GII.2.2002 VLPs bound preferentially to type B saliva, requiring significantly more VLP to reach half-maximum binding to type A saliva (33, 38). In comparison, the more contemporary GII.2 strain VLPs, GII.2.2008 and GII.2.2010, bound to type B and A saliva similarly (Fig. 1D), suggesting that residue changes in the P2 domain of GII.2.2008 and 2010 may have influenced VLP affinity for an expanded set of histo-blood group antigens, as has been reported for GII.4 NoVs.

Using B saliva as the carbohydrate binding ligand for GII.2 (43, 44), we evaluated archived serum samples from our original SMV human challenge study for the capacity to block homotypic VLP-ligand interactions. We observed two homotypic blockade response phenotypes. As a group, infected subjects did not have any detectable blockade activity at day 0 (EC_{50} titer below the limit of detection) but progressed to a significantly increased blockade response by day 8 postchallenge EC_{50} of 4,178 (95% confidence interval [CI], 3,327 to 5,247) (Fig. 2A), representing an estimated 167-fold increase in blockade titer. Titers remained high through day 21, the final day of sample collection. As a group, uninfected subjects had low but detectable SMV blockade titers at day 0, and these titers did not vary significantly over the course of sample collection (Fig. 2B). At day 0, the GII.2.1976 blockade titer was 116.3 (95% CI, 101.0 to 133.8), with 5 of the 6 uninfected individuals having detectable preexisting blockade titers. At day 8 postchallenge, mean titers remained consistent at 151.7 (95% CI, 124.7 to 184.6). The results from these blockade assays parallel the homotypic, human polyclonal IgG serum responses measured by EIA, in which infected individuals had a significant increase in SMV-specific antibodies postinfection (37), and provide the first evidence that SMV infection induces an antibody response targeting potentially neutralizing epitopes.

Changes in the sequence of the capsid gene of GII.4 noroviruses have been suggested as a mechanism to escape herd immunity, resulting in the emergence of new antigenically distinct pandemic strains every few years (18–20, 22, 25, 34). To test if the GII.2 strains are undergoing a similar antigenic drift, we determined the capacity of polyclonal sera from SMV-challenged subjects to block other GII.2 VLPs (Fig. 3). As a group, the infected subjects had no detectable blockade titer at day 0 and developed increased blockade titers to the entire panel of GII.2 VLPs by day 8, although significantly more serum was needed to block GII.2.2002, -2008, and -2010 than GII.2.1976. Blockade of GII.2.2002 required 1.5-fold more sera (EC_{50} , 2,765; 95% CI, 2,287 to 3,342), GII.2.2008 required 2.5-fold more serum (EC_{50} , 1,552; 95% CI, 983.5 to 2,451), and GII.2.2010 required 5-fold more serum (EC_{50} , 843.8; 95% CI, 693.7 to 1,026) to block 50% of VLP-ligand interaction (Fig. 3A). As a group, sera from the uninfected subjects had low blockade titers at day 0 (<200 for each VLP), and these titers remained consistent at day 8 (<200 for each VLP) (Fig. 3B). Of note, even in the uninfected subjects, significantly more serum was needed to block binding of GII.2.2010 (EC_{50} , 56.68; 95% CI, 53.54 to 60.01) than was needed to block GII.2.1976 (EC_{50} , 152; 95% CI, 125 to 185). While increasing amounts of SMV-induced polyclonal sera were required to block GII.2 VLP binding to saliva in a time-dependent pattern in infected subjects, none of the GII.2 variants tested changed antigenicity enough to completely escape antibody blockade, suggesting that GII.2 strains have undergone a limited degree of evolution

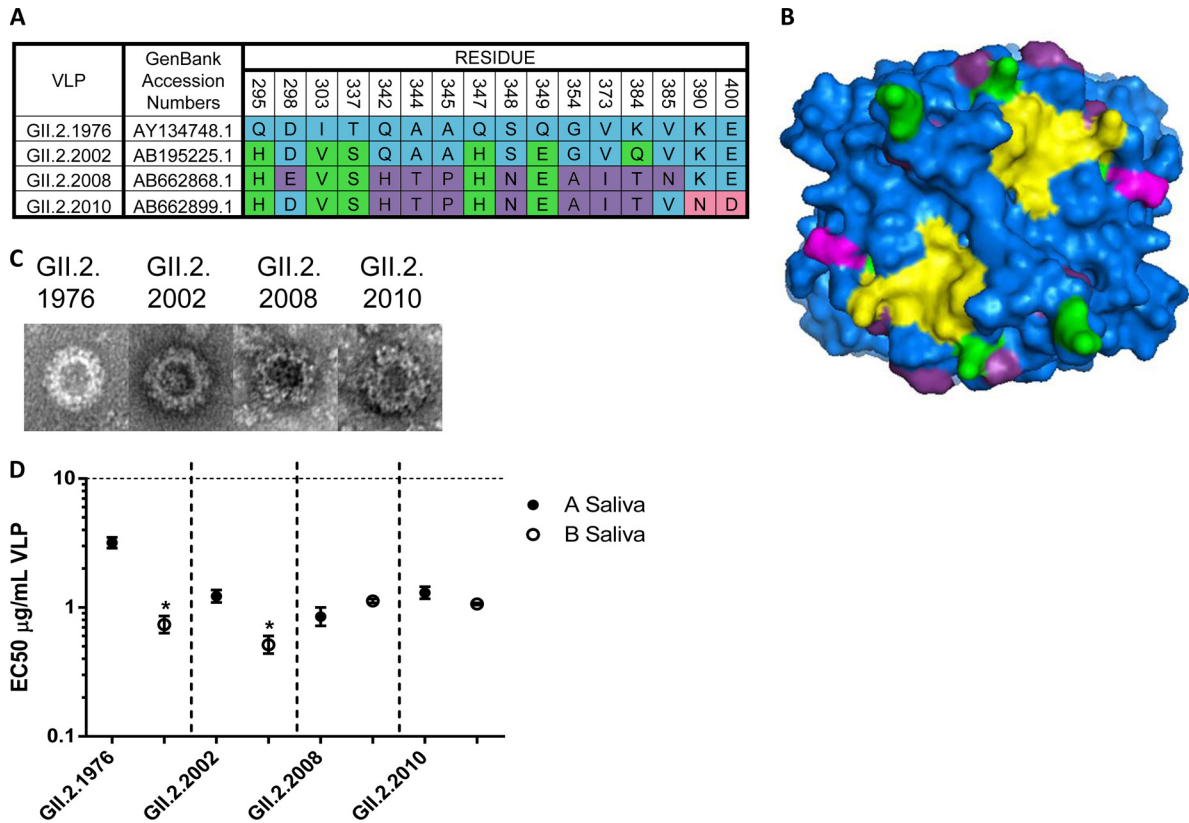


FIG 1 Characterization of GII.2 VLPs. (A) Variations in the P2 domain amino acids within the GII.2 strains are color coded to indicate in what strain the residue changes originated: GII.2.1976, blue; GII.2.2002, green; GII.2.2008, purple; GII.2.2010, pink. (B) The variation shown in panel A was mapped onto the P domain dimer, which is shown in two shades of blue representing the two P domain chains of the dimer. (C) Electron microscopy visualization of negative-stained VLPs: GII.2.1976 SMV, GII.2.2002 Ina, GII.2.2008, and GII.2.2010. (D) GII.2 VLPs were assayed for binding to type A or B saliva by EIA, and the half-maximal binding was calculated in $\mu\text{g}/\text{mL}$. The horizontal dashed line indicates the upper limit of detection. *, binding to type B saliva statistically different from binding to type A saliva.

in antibody blockade epitopes during the 34-year period examined.

SMV infection induces a modest cross-genotype blockade response in some subjects. To determine if antibodies produced in response to Snow Mountain virus infection not only recognize blockade epitopes conserved within the genotype but also recognize those within the genogroup, serum samples were examined for ligand interaction blockade of GII.3.1999 (Toronto), GII.4.1997 (the circulating GII.4 strain at the time of the SMV challenge study), and GI.1.1968 (Norwalk) VLPs. As a group, day 0 sera from infected volunteers did not have any blockade activity for any of the VLPs tested. At day 8, cross-genotype blockade titers had risen 3-fold for GII.3.1999 (EC_{50} , 81.98; 95% CI, 58.94 to 114.0). Day 8 sera did not block GII.4.1997 or GI.1.1968 (Fig. 4A). As a group, the sera from uninfected subjects did not have any blockade titer, and that titer remained below levels of detection at day 8 (Fig. 4B). Individually, two of the infected subjects had at least a 4-fold increase in blockade antibodies against GII.3.1999. This cross-reactive blockade potential extended to GII.4.1997 in one of these subjects, and another subject had at least a 4-fold increase in blockade antibodies against only GII.4.1997. None of the individual uninfected subjects had at least a 4-fold change in blockade titer to any of the cross-genotype VLPs tested (data not shown).

Anti-GII.2.1976 SMV MAb characterization. The variances in blockade between GII.2 VLPs by polyclonal sera (Fig. 3) indicate the possibility that some blockade epitopes may be changing over time. To potentially map the GII.2 antigenic variation, we developed a panel of seven mouse MAbs against the GII.2.1976 VLP (18, 21, 41). The MAbs were purified, isotyped, and screened by Western blotting for reactivity to linear epitopes in the major capsid protein of GII.2.1976 (Table 1) and against a panel of GI and GII VLPs by EIA for reactivity to conformational epitopes (Fig. 5 and 6). All seven MAbs recognize the panel of GII.2 VLPs by EIA (Fig. 5). SMV37, SMV59, SMV114, and SMV129 bound more contemporary GII.2 strain VLPs at significantly lower EC_{50} titers than they bound to the homotypic GII.2.1976 VLP. One MAb, SMV130, bound all four GII.2 VLPs similarly, and 2 MAbs, SMV187 and SMV276, required more antibody to reach 50% maximum binding to GII.2.2002 than was needed to bind the other GII.2 strain VLPs. Uniquely, SMV59 reacted outside the GII.2 genocenter and bound to a diverse panel of NoV VLPs representing GI and GII strains (Fig. 6). The broadly reactive MAb SMV59 detected the major capsid protein by Western blotting, indicating that it binds to a linear epitope. The absence of SMV59 reactivity to GII.4.2002, GII.4.2006, and GII.4.2009 P particles by EIA and Western blotting indicates that the antibody likely targets a conserved region of the shell domain (data not shown). The

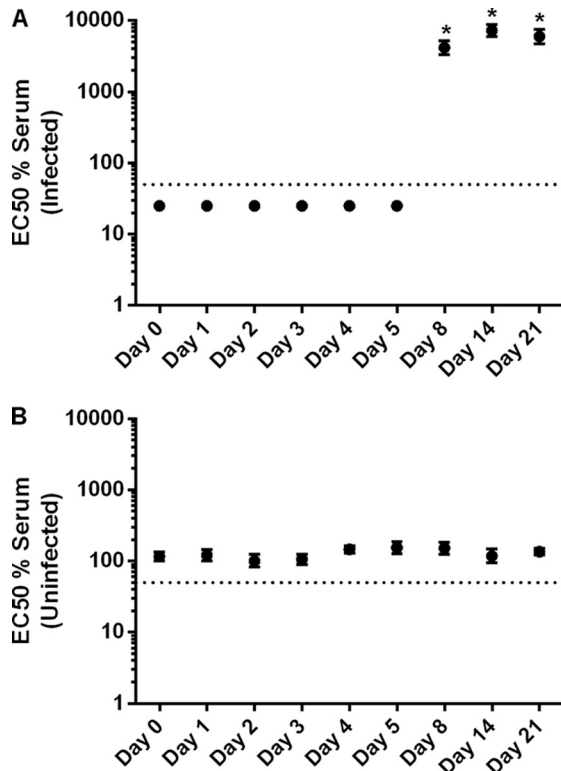


FIG 2 SMV infection induces a strong homotypic blockade response by day 8 postchallenge. Sera collected at days 0 to 5, day 8, day 14, and day 21 post-GII.2.1976 SMV challenge were assayed for the ability to block the GII.2.1976 SMV interaction with ligand in surrogate neutralization assays. Sigmoidal dose-response curves were fit to the mean percent control binding (percentage of VLP bound to ligand in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment), and the mean EC_{50} titers for sera from infected (A) and uninfected (B) volunteers were calculated. Error bars represent 95% confidence intervals. Nonblockade sera were assigned an EC_{50} of 25 for statistical analysis and are denoted by data markers on the graph below the lower limit of detection (dashed line) for visual comparison. *, sample days with titers at least 4-fold above and significantly different from day 0 titers.

remaining six genotype-specific antibodies recognize conformational epitopes, as they did not detect the major capsid protein by Western blot analysis (Table 1).

Each MAb was then evaluated for capacity to inhibit VLP-carbohydrate interactions, using our surrogate neutralization assay. The EIA EC_{50} titer did not correlate to the blockade titer. Of the seven MAbs developed against the GII.2.1976 VLP, only SMV129 and SMV187 blocked the homotypic VLP-ligand interaction, suggesting that these two MAbs recognize blockade epitopes (Fig. 7). We then examined the cross-reactive blockade capacity of the MAbs across the panel of GII.2 VLPs. None of the MAbs gained blockade activity against the heterotypic VLPs. Compared to homotypic GII.2.1976-ligand interaction blockade, significantly less SMV129 was needed to block GII.2.2002, significantly more MAb was needed to block GII.2.2008, and a similar amount of MAb was needed to block GII.2.2010. SMV187 blocked the ligand interaction of the GII.2.1976, -2002, and -2010 strains similarly but required significantly more antibody to block GII.2.2008. These data support findings with polyclonal sera indicating that GII.2 strains have undergone limited antigenic changes.

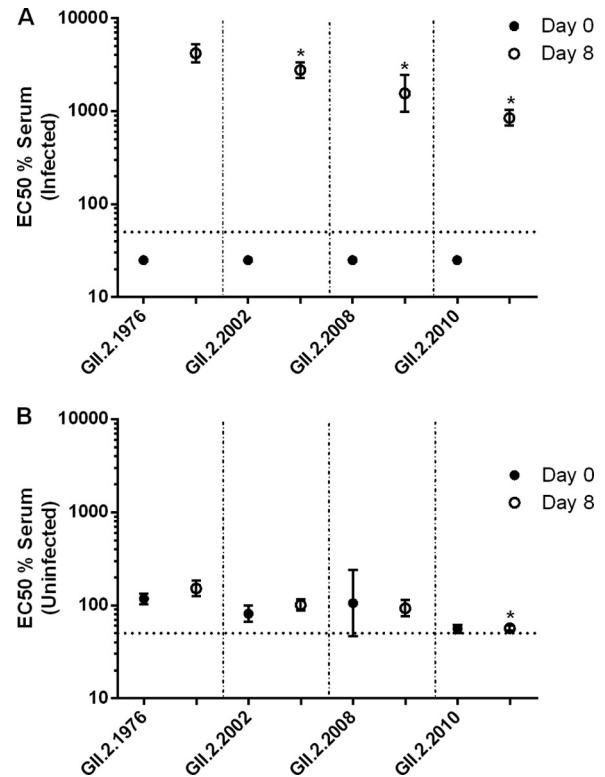


FIG 3 Serum blockade response modestly differentiates between GII.2 VLPs. Day 0 and day 8 sera were assayed for ability to block the time-ordered GII.2 VLP interaction with ligand in surrogate neutralization assays. Sigmoidal dose-response curves were fit to the mean percent control binding (percentage of VLP bound to ligand in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment), and the mean EC_{50} titers for blockade of GII.2.1976 SMV, GII.2.2002, GII.2.2008, and GII.2.2010 were calculated for infected (A) and uninfected (B) volunteers. Error bars represent 95% confidence intervals. Nonblockade sera are denoted by data markers on the graph below the lower limit of detection (dashed line) for visual comparison. *, day 8 titer significantly different from the GII.2.1976 SMV day 8 titer.

DISCUSSION

Human NoVs are the primary cause of severe acute gastroenteritis worldwide and in all age groups. Despite this significant disease burden, susceptibility to and immune protection from NoV infection are not well understood. The primary complicating factors in studying NoVs are the lack of a cell culture system for human NoV propagation and the high degree of genetic heterogeneity within the NoV family. This genetic heterogeneity translates to both differential cellular ligand binding properties and antigenic differences between the genogroups, genotypes, and even strains within the same genotype (18, 22, 26, 38, 44–46). These effects are most pronounced in the GII.4 genotype, where new strain emergence every 2 to 3 years correlates with loss of reactivity to antibodies that recognize blockade epitopes and altered affinity for different HBGAs (18, 19, 21, 34, 45). In contrast, in the GI.1 genotype antigenicity and HBGA binding have been relatively static over the past 40 years (28, 35). Little is known about the antigenicity of or susceptibility to strains within other genotypes or how previous exposure to different genotypes affects the immune response to a current virus challenge.

Using a human challenge model with the GII.2.1976 SMV in-

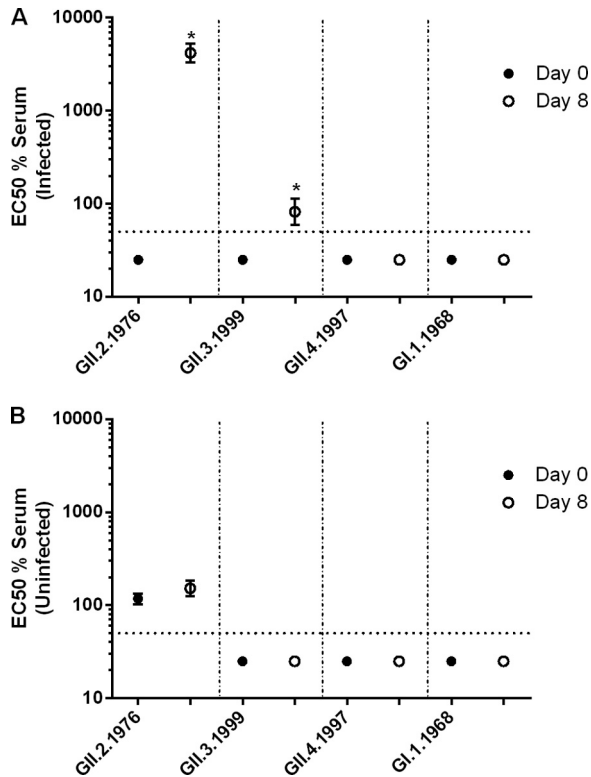


FIG 4 GII.2.1976 SMV infection induces a modest cross-genotype blockade response. Day 0 and day 8 sera were assayed for ability to block norovirus VLPs from additional genotypes in surrogate neutralization assays. Sigmoidal dose-response curves were fit to the mean percent control binding (percentage of VLP bound to ligand in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment) and the mean EC_{50} titer for blockade of GII.2.1976 SMV, GII.3.1999, GII.4.1997, and GI.1.1968 Norwalk calculated for infected (A) and uninfected (B) volunteers. Error bars represent 95% confidence intervals. Nonblockade sera are denoted by data markers on the graph below the lower limit of detection (dashed line) for visual comparison. *, day 8 titers at least 4-fold greater than and significantly different from day 0 titers.

oculum, we have tested the association between *in vitro* VLP-HBGA binding and virus susceptibility. In our earlier studies, SMV VLPs bound exclusively to type B saliva in single-dilution binding assays, but subjects of the O, A, and secretor-negative phenotypes became infected, identifying a discordance between these two observations that was also seen in similar studies with GI.1.1968 (28, 37). Here, using a dynamic range of VLP concentrations to test saliva binding, GII.2.1976 VLPs were found to bind preferentially to type B saliva but also to type A saliva, although at

TABLE 1 Characterization of anti-GII.2.1976 SMV VLP mouse MAbs

MAb	Isotype	Type of epitope ^a
SMV37	IgG1 κ	Conformational
SMV59	IgG1 κ	Linear
SMV114	IgG2b κ	Conformational
SMV129	IgG2a κ	Conformational
SMV130	IgG1 κ	Conformational
SMV187	IgG1 κ	Conformational
SMV276	IgG2b κ	Conformational

^a As determined by Western blotting.

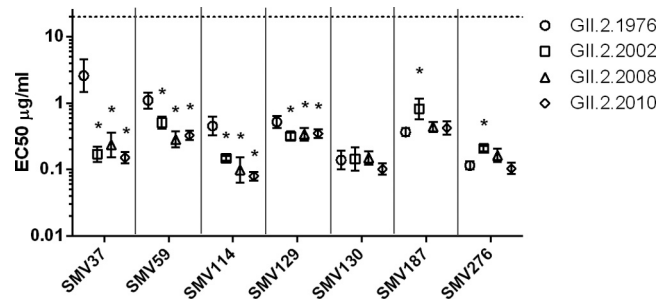


FIG 5 The epitopes recognized by anti-GII.2.1976 SMV mouse MAbs are conserved across the time-ordered panel of GII.2 VLPs. Antibody epitopes shared among GII.2 VLPs were measured by MAb binding to the time-ordered VLP panel by EIA, and the half-maximal binding was calculated in $\mu\text{g/ml}$. *, VLP with a significantly different EC_{50} from that of GII.2.1976. The horizontal dashed line indicates the upper limit of detection.

a lower affinity, agreeing with previously published reports (38). GII.2 VLPs did not bind to any tested type O or secretor-negative saliva samples in these assays. Even with the expanded HBGA binding repertoire identified here, the discordance between GII.2.1976 VLP binding to HBGA-phenotyped saliva and GII.2.1976 infectivity remains. The preferential binding of type B saliva over type A saliva is retained in GII.2.2002 VLPs, but both GII.2.2008 and 2010 VLPs bind type A and B saliva similarly. Although speculative, these data suggest that the more contemporary GII.2 strains may have altered affinities for HBGAs and thus target susceptible populations different from those targeted by the earlier GII.2 strains. Of note, residue 354 changed from glycine in GII.2.1976 and GII.2.2002 to alanine in GII.2.2008 and GII.2.2010. As previously shown (47), residue 354 of GII.2 strains is located within the HBGA binding pocket, suggesting that this change may account for the increased affinity of contemporary GII.2 strains for type A saliva. Only human challenge studies can determine if this change has any biological impact on the susceptible population, but cocrystal structures of different GII.2 particles bound to carbohydrates would provide insight into the effect of the G354A change on carbohydrate interaction and expand our understanding of the complex relationship between *in vitro* HBGA binding and infection.

This study is the first to measure potentially neutralizing

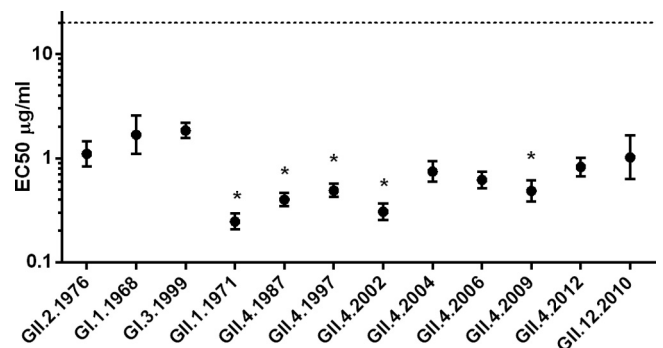


FIG 6 Anti-GII.2.1976 SMV mouse MAb SMV59 binds to an NoV conserved epitope by EIA. Half-maximal binding in $\mu\text{g/ml}$ of SMV59 to a panel of NoV VLPs was determined by EIA. Error bars represent 95% confidence intervals. *, VLPs with a significantly different EC_{50} titer from that of GII.2.1976. The horizontal dashed line indicates the upper limit of detection.

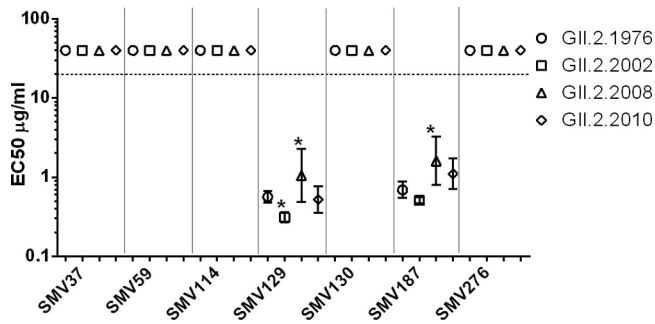


FIG 7 Anti-GII.2.1976 SMV mouse MAb blockade reactivity with the panel of GII.2 time-ordered VLPs suggests limited GII.2 antigenic divergence over 34 years. Blockade antibody epitopes shared among GII.2 VLPs were measured by surrogate neutralization assay. Sigmoidal dose-response curves were fit to the mean percent control binding (percentage of VLP bound to ligand in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment), and the EC_{50} was calculated in $\mu\text{g/ml}$. Error bars represent 95% confidence intervals. Nonblockade MAbs were assigned an EC_{50} of 40 $\mu\text{g/ml}$ for statistical analysis and are denoted by data markers on the graph above the upper limit of detection (dashed line) for visual comparison. *, VLP with significantly different blockade titer from that of GII.2.1976.

“blockade” antibody responses to GII.2 NoVs. As has been shown for GI.1 and GII.4 NoVs, GII.2.1976 SMV infection correlates with an increased blockade titer in all tested subjects by day 8 postchallenge, and titers remained elevated at day 21, the final sample collection day. Although all of the infected subjects had day 8 titers above 200 (the blockade titer associated with protection from infection in GI.1968-challenged subjects) (31), rechallenge studies are needed to evaluate the correlation between antibody blockade titer and protection from homotypic virus infection. Similarly, although five of six uninfected subjects had preexisting blockade titers to GII.2.1976, we are unable to determine the impact of antibody-mediated protection from infection in this study because five of the six uninfected subjects received the lowest dose of inoculum administered (37). This dosage difference prevents us from speculating on whether preexisting antibodies offer protection, but it is noteworthy that two out of six of the uninfected subjects mounted strong $CD4^+$ T cell responses to GII.2.1976 SMV postchallenge, suggesting that even at the lowest challenge dose, the inoculum was immunostimulatory in some individuals (37). Here, the same two subjects also had higher day 0 blockade titers and, together with a third uninfected volunteer, demonstrated more cross-strain blockade activity at day 0 (data not shown), further supporting previous claims (37) that some subjects may have been protected from GII.1976 infection by pre-existing acquired immunity.

How the presence of preexisting antibodies from a lifetime of NoV exposures impacts the immune response to a current NoV challenge is unknown. The major capsid protein is a primary target of NoV antibodies (18, 41). Although this protein is highly heterogeneous between NoV strains, infection with one NoV can stimulate an antibody response that blocks other strain VLPs, suggesting that divergent NoV strains share common conserved blockade epitopes and/or antibody responses are influenced by original antigenic sin (35). The experiments reported here do not address which of these mechanisms provides the antibody cross-reactivity, but they do emphasize an important point for vaccine design. While challenge with GII.2.1976 SMV does not elicit a

broad cross-genotype blockade response universally, three of six infected subjects had at least a 4-fold increase in blockade titer to non-GII.2 VLPs. These data support other findings indicating that blockade antibodies are activated to not only the challenging NoV strain but also other NoV strains, a key benefit for a successful vaccine design against a diverse, evolving pathogen (35, 36, 48).

Further supporting the potential for NoV vaccine design, the data provided here suggest that a component protective against GII.2 NoV will not need to be reformulated frequently. Analysis of antibody blockade of VLPs representing GII.2 strains that have circulated from 1976 until 2010 suggests that limited evolution has occurred within the GII.2 genotype, as none of the polyclonal serum or MAbs lost reactivity to any of the time-ordered GII.2 VLPs. Interestingly, most of the amino acid substitutions that occurred over time resulted in changes that were exposed to the surface, with the majority of changes from 2008 and 2010 appearing to occur in or around the putative carbohydrate binding sites. These residue changes were not significant enough to ablate antibody binding, but they did moderately impact the blockade potential of antibodies. Sera from GII.2.1976-infected subjects blocked GII.2.1976 preferentially to GII.2.2008 and -2010, indicating some blockade epitope evolution between the GII.2 strains over 34 years. Limited epitope evolution is supported by MAb reactivity, as none of the VLPs lost reactivity to any of the MAb by EIA or blockade assay. At this time, it is not clear how much divergence in antibody epitopes is needed to escape from herd immunity. Studies with GII.4 human NoV, mouse NoV, and other RNA viruses have demonstrated significant changes in blockade or neutralization potential with only a single amino acid change (34, 49–51). It is possible that with an expanded panel of GII.2 blockade MAbs, we may have been able to more clearly antigenically differentiate the GII.2 VLPs. The lack of clear GII.2 antigenic phenotypes made it impossible for us to predict and test potential blockade epitopes as we have done in the past for GII.4 NoVs. Monoclonal antibodies did identify at least one blockade, conformation-dependent epitope and up to four nonblockade conformation-dependent epitopes. Another MAb, SMV59 recognizes a pan-NoV, nonblockade, linear epitope. This epitope likely resides within the shell domain of the major capsid protein (52). The high degree of cross-reactivity of this antibody makes it potentially useful as a diagnostic reagent. Although we were unable to map GII.2 epitopes, these antibody studies provide clinically relevant information about the timing, duration, and cross-reactivity of GII.2.1976 SMV-induced blockade antibody responses. The assays developed here and the data they provide will be fundamental for future GII.2.1976 SMV human challenge studies slated to begin in the first quarter of 2014 and provide further support for design of a successful NoV vaccine.

ACKNOWLEDGMENTS

We thank Victoria Madden and C. Robert Bagnell, Jr., of Microscopy Services Laboratory, Department of Pathology and Laboratory Medicine, University of North Carolina—Chapel Hill for expert technical support and Christine Moe for conducting the original GII.2.1976 SMV human challenge study.

This work was supported by a grant from the National Institutes of Health, Allergy and Infectious Diseases (AI056351) and a Gillings Innovation Laboratory Award from the UNC Gillings School of Global Public Health.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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