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Emergence of a Norovirus GII.4 Strain Correlates with Changes in Evolving Blockade Epitopes

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The major capsid protein of norovirus GII.4 strains is evolving rapidly, resulting in epidemic strains with altered antigenicity. GII.4.2006 Minerva strains circulated at pandemic levels in 2006 and persisted at lower levels until 2009. In 2009, a new GII.4 variant, GII.4.2009 New Orleans, emerged and since then has become the predominant strain circulating in human populations. To determine whether changes in evolving blockade epitopes correlate with the emergence of the GII.4.2009 New Orleans strains, we compared the antibody reactivity of a panel of mouse monoclonal antibodies (MAbs) against GII.4.2006 and GII.4.2009 virus-like particles (VLPs). Both anti-GII.4.2006 and GII.4.2009 MAbs effectively differentiated the two strains by VLP-carbohydrate ligand blockade assay. Most of the GII.4.2006 MAbs preferentially blocked GII.4.2006, while all of the GII.4.2009 MAbs preferentially blocked GII.4.2009, although 8 of 12 tested blockade MAbs blocked both VLPs. Using mutant VLPs designed to alter predicted antigenic epitopes, binding of seven of the blockade MAbs was impacted by alterations in epitope A, identifying residues 294, 296, 297, 298, 368, and 372 as important antigenic sites in these strains. Convalescent-phase serum collected from a GII.4.2009 outbreak confirmed the immunodominance of epitope A, since alterations of epitope A affected serum reactivity by 40%. These data indicate that the GII.4.2009 New Orleans variant has evolved a key blockade epitope, possibly allowing for at least partial escape from protective herd immunity and provide epidemiological support for the utility of monitoring changes in epitope A in emergent strain surveillance.

Noroviruses (NoVs) are the leading cause of severe viral gastroenteritis worldwide, causing ~50% of all acute gastroenteritis outbreaks in the United States and Europe (1). Disease severity is usually moderate, but morbidity and mortality rates due to NoV infection are increasingly becoming evident, with particularly high tolls taken on children and immunocompromised and aged populations (2–9), resulting in an estimated 200,000 deaths per year (10). In addition, postinfectious irritable bowel syndrome develops in a substantial portion of patients (11). These groups of people, in addition to military personnel, childcare and health care providers, and food handlers, would benefit from a NoV vaccine. Recent success has been demonstrated for a monovalent Norwalk virus VLP-based vaccine (12), but significant obstacles remain for the development of a broadly protective NoV vaccine, including the lack of a clear understanding of the complex antigenic relationships between the many NoV strains and the interaction between these strains and the host immune system over time.

Noroviruses contain positive-sense, single-stranded RNA genomes, and phylogenetic classification is based on the amino acid sequence of the major capsid protein encoded by ORF2 (13). Genogroup I (GI) and GII cause most human infections, and each genogroup is further subdivided into 9 and 21 different genotypes, respectively (1, 13). Exogenous expression of ORF2, the major capsid protein, results in virus-like particle (VLP) self-assembly. Each particle is composed of 90 copies of the major capsid protein dimer and a small number of copies of the ORF3-encoded minor capsid protein (14, 15). The capsid monomer is divided into two structural domains. The shell domain (S) forms the core of the particle and the protruding domain (P) extends away from the central core. The P domain is further divided into two subdomains: P1 consists of residues 226 to 278 and residues 406 to 520 and forms the stalks that support the extended, surface-exposed P2 subdomain (residues 279 to 405) (14). The P2 subdomain interacts with potential neutralizing/blockade antibodies and the NoV carbohydrate-binding ligands, the histo-blood group antigens (HBGAs) (16–22). Coinciding with these functions, changes in the P2 amino acid sequence of GII.4 strains occur frequently and correlate with the emergence of new epidemic strains with altered carbohydrate ligand binding and antigenicity profiles (16, 20, 23–29). The study of neutralization antibodies and epitopes is hindered by the lack of a cell culture or small animal model for human norovirus propagation. Therefore, we developed an *in vitro* surrogate neutralization assay that measures the ability of an antibody to block binding of a VLP to a carbohydrate ligand (16, 20, 30, 31). The blockade assay has been verified as a surrogate neutralization assay in infected chimpanzees (32) and Norwalk virus-infected humans (12, 33). Importantly, the blockade assay has been shown to differentiate between GII.4 strains antigenically too similar to be distinguished from each other by traditional EIAs (16, 20, 34). This enhanced sensitivity has been crucial in mapping GII.4 evolving blockade epitopes.

GII.4 strains have caused the majority of all norovirus outbreaks over the past 2 decades. Four GII.4 pandemics, each caused by a newly emergent GII.4 variant strain, have been characterized using molecular epidemiological methods. Strain US95/96 (GII.4.1997) caused the pandemic that occurred during the mid-1990s (35, 36). The second pandemic began in 2002 with the

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emergence of the Farmington Hills strain (GII.4.2002) (37), followed closely by the third pandemic in 2004 attributed to the emergence of the Hunter strain (GII.4.2004) (38–40). The most recent pandemic strain was Minerva 2006b (GII.4.2006), which emerged in 2006 and quickly replaced other circulating NoVs (5, 39, 41). Most recently, a new GII.4.2006 variant, GII.4.2009 New Orleans, has been the predominant outbreak strain, although GII.4.2006 Minerva continues to circulate at low levels (1, 42). GII.4.2009 New Orleans first emerged in October of 2009 and by November accounted for ~56% of the outbreaks documented by CaliciNet (42). In comparison, GII.4.2006 Minerva made up only 11% of the cases at that time, implying a circulating strain replacement. The emergence of GII.4.2009 New Orleans did not result in an increase in norovirus outbreaks, leading Yen et al. to suggest that GII.4.2009 New Orleans did not target an immunologically naive population (43).

Comparing the monoclonal antibody (MAb) reactivity of different GII.4 strains that have circulated over the past 2 decades has clearly demonstrated that new strain emergence is associated with changes in blockade antibody epitopes (20, 34). We and others have used bioinformatic tools to predict potential evolving epitopes. Residues 296 to 298 and residues 393 to 395 are consistently identified by different groups as putative epitopes that change between epidemic GII.4 strains. Surface residues at positions 333, 340, 356, 368, 372, 407, and 412 to 413 have also been predicted as antibody epitopes (16, 23, 24, 27, 38, 44–47). These amino acids cluster within the P2 subdomain, where they would be most available on the capsid surface for interactions with antibodies. Although bioinformatic predictions are useful, few studies have tested these predictions. Allen et al. (46) compared the VLP-binding reactivity of five MAbs against one pre- and one post-2002 pandemic GII.4 strain and found two conformational epitopes as differing between the two strains. Site A includes amino acids 294 to 296. Site B includes amino acids 393 to 395 and confirmed residue 395 as an important antigenic determinant in the GII.4.2002 Farmington Hills strain (16). Further, our group has used both human and mouse anti-NoV MAbs coupled with molecular biology approaches to exchange predicted epitopes between GII.4 strain backbones to identify evolving blockade epitopes between GII.4 strains that have circulated from 1987 until 2009. Using this novel approach we have identified three evolving blockade GII.4 antibody epitopes (20–22). Epitope A is comprised of amino acids 294, 296 to 298, 368, and 372 and is a highly variable blockade epitope that changes with new GII.4 strain emergence. Monoclonal human and mouse antibodies against epitope A have been described as highly strain selective, lacking reactivity to other GII.4 strains chronologically removed from the immunizing strain. GII.4.2006 and GII.4.2009 were found to differ in reactivity to a human MAb targeting epitope A, establishing a difference in human neutralizing epitopes between these two strains and supporting escape from herd immunity by antigenic variation at neutralizing epitopes as a mechanism for new NoV strain emergence. Epitope D (residues 393 to 395) has also been confirmed as an evolving blockade epitope using human anti-NoV MAbs. These findings are particularly interesting as epitope D has been shown to modulate HBGA binding of GII.4 strains, supporting the suggested correlation between epitope escape from herd immunity and altered HBGA binding (16). Mouse MAbs have confirmed epitope E (amino acids 407, 412, and 413) as a GII.4.2002 Farmington Hills-specific blockade epitope (21). Importantly, these empirical studies have validated our approach to predict evolving GII.4 blockade epitopes.

Epidemiological studies of NoV outbreaks, including sequence analysis of outbreak strains, coupled with human anti-NoV MAbs that differentiate GII.4 Minerva and New Orleans strains, indicate that by 2009 the GII.4.2006 Minerva pandemic NoV strain was being replaced by the GII.4.2009 New Orleans strain. This strain replacement pattern is consistent with NoV strain epochal evolution. Based on documented changes in surrogate neutralization epitopes of previously emerged GII.4 strains, GII.4.2009 is likely a herd immunity escape variant of GII.4.2006. To test this hypothesis, we compared the antigenic features of GII.4.2006 and GII.4.2009 using MAbs developed to each VLP and polyclonal sera collected during a GII.4.2009 outbreak. MAbs coupled with epitope altered VLPs identified epitope A as a varying blockade epitope between GII.4.2006 and GII.4.2009. Confirming the importance of epitope A in the polyclonal immune response, ca. 40% of the antibody blockade reactivity in a set of GII.4.2009 outbreak sera was directed against epitope A. These data support the hypothesis that NoV GII.4 strain emergence correlates with changes in evolving blockade epitopes.

MATERIALS AND METHODS

VLPs. For all constructs except GII.4.2009, the synthetically derived, epitope-engineered or outbreak strain ORF2 genes were inserted directly into the VEE replicon vector for the production of virus replicon particles (VRPs) as previously described by our group (20, 22). Virus-like particles (VLPs) were expressed in VRP-infected BHK cells and purified by velocity sedimentation in sucrose, followed by simultaneous concentration and dialysis into phosphate-buffered saline (PBS) using a 100-kDa MWCO centrifugal filter units (Millipore). GII.4.2009 (21) VLPs were expressed in the baculovirus system, concentrated by centrifugation through 40% sucrose, and purified by cesium chloride gradient centrifugation. VLPs were resuspended in PBS. VLP protein concentrations were determined by a BCA protein assay (Pierce, Rockford, IL). The structural integrity of epitope engineered VLPs was confirmed by electron microscopy, enzyme-linked immunosorbent assay (ELISA), and carbohydrate binding as described previously (20).

EIAs. Mouse MAb reactivity was determined by enzyme immunoassay (EIA), as reported (20, 21). Briefly, plates were coated at 1 μg of VLP/ml in PBS before the addition of 1 μg of purified IgG/ml. Primary antibody incubation was followed by anti-mouse IgG-HRP (GE Healthcare) and color development with One-Step Ultra TMB ELISA HRP substrate solution (Thermo Fisher). Each step was followed by washing with PBS–0.05% Tween 20, and all antibodies were diluted in 5% dry milk in PBS–0.05% Tween 20. The data shown represent the average of at least three replicates and are representative of similar data from at least two independent trials. Establishment of EIAs using new MAbs included PBS-coated wells as negative controls and polyclonal anti-norovirus mouse sera as positive controls. Antibodies were considered positive for reactivity if the mean optical density at 450 nm after background subtraction for VLP-coated wells was >0.2 (48).

VLP-carbohydrate ligand-binding antibody blockade assays. Pig gastric mucin type III (PGM; Sigma Chemicals) has been validated as a substrate for NoV VLP antibody blockade assays (21, 22). PGM contains relatively high levels of H and A antigen and more moderate levels of Lewis Y antigen (21). The VLPs used in the blockade assays in the present study bind to both PGM and synthetic biotinylated-HBGA, and binding to PGM is consistent with synthetic Bi-HBGA binding profiles for α-1,2-fucose (H antigen) and α-1,4-fucose (Lewis antigen) containing molecules (16, 21, 49). For blockade assays, PGM was solvated in PBS at 5 mg/ml, coated onto EIA plates at 10 μg/ml in PBS for 4 h, and blocked overnight at 4°C in 5% dry milk in PBS–0.05% Tween

20. VLPs (0.5 μg/ml) were pretreated with decreasing concentrations of test MAb or outbreak sera for 1 h at room temperature before being added to the carbohydrate ligand-coated plates for 1 h. Bound VLP were detected by a rabbit anti-GII norovirus polyclonal sera made from hyperimmunization with either GII.4.2009 or a cocktail of GII.4.1997, GII.3.1999, GII.1.1976, and GII.2.1976 VLPs, followed by anti-rabbit IgG-HRP (GE Healthcare) and color developed with 1-Step Ultra TMB ELISA HRP substrate solution (Thermo Fisher). The percent control binding was defined as the binding level in the presence of antibody pretreatment compared to the binding level in the absence of antibody pretreatment multiplied by 100. All incubations were performed at room temperature. Each step was followed by washing with PBS–0.05% Tween 20, and all reagents were diluted in 5% dry milk in PBS–0.05% Tween 20. All antibodies were tested for blockade potential against GII.4 VLPs at 2-fold serial dilutions ranging from 0.08 to 2 μg of MAb/ml or 0.008 to 1% outbreak serum. Additional concentrations of purified antibodies were tested if needed to complete the sigmoid dose-response curve. The data shown represent the averages of at least two replicates and are representative of similar data from at least two independent trials. Blockade data were fit using sigmoidal dose-response analysis of nonlinear data in GraphPad Prism. The 50% effective concentration (EC_{50}) values were calculated for antibodies that demonstrated a dose-dependent (Hill slope ≤ -0.7) blockade of at least 50% at the dilution series tested. MAbs that did not block 50% of binding at the highest dilution tested were assigned an EC_{50} of 2× the assay upper limit of detection. Standard error was used to calculate the 95% confidence intervals (CI), and EC_{50} values between VLPs were compared using one-way analysis of variance with Dunnett's post test, when at least three values were compared or a Student *t* test when only two values were compared. A difference was considered significant if the *P* value was <0.05. Of note, VLP concentrations in blockade assays are in the low nanomolar range and therefore cannot discriminate between antibodies with subnanomolar affinities.

Mouse immunization, hybridoma production, and IgG purification. MAbs to GII.4.2009 were produced and purified as described earlier (21). Swiss-Webster mice were immunized on days 0, 20, 40, and 80 with 50 μg of VLP in PBS plus 50 μl of GERBU adjuvant (Fisher Scientific) for a total of 100 μl, and splenocytes were fused on day 84. The resulting hybridomas underwent two rounds of limited dilution subcloning, were isotyped (Roche), and were purified by protein G chromatography (GE Healthcare).

Commercial MAbs. Commercial MAbs MAB223P, MAB224P, MAB225P, and MAB226P were developed against GII.4.2006 VLPs in collaboration with Maine Biotechnology (Portland, ME).

GII.4 outbreak serum samples. Deidentified convalescent-phase serum samples from eight subjects infected with GII.4.2009 during an outbreak that occurred in March 2010 were studied. Acute- and convalescent-phase serum samples were collected 3 to 4 days and 21 days after the onset of illness, respectively. Infection was defined as (i) IgA seroconversion as measured by EIA against GII.4.2009-VLPs and (ii) virus shedding in stool samples as detected by GI and GII NoV TaqMan real-time reverse transcription-PCR (RT-PCR) (42). Norovirus-positive samples were genotyped by sequencing the RT-PCR product from diagnostic regions C and D (50, 51). Sequence alignment of the outbreak strain and reference NoV genotypes was performed using the CLUSTAL W algorithm. Phylogenetic analysis was performed using TreeCon software (52), and phylogenetic trees were constructed using the neighbor-joining method. The P2 subdomain for each positive sample was amplified and sequenced (42). A P2 consensus sequence for the outbreak and P2 sequences of representative GII.4 variants (GII.4 Bristol [1993], GII.4 Farmington Hills [2002], GII.4 Hunter [2004], GII.4 Osaka, GII.4 Yerseke [2006a], GII.4 Minerva [2006b], and GII.4 New Orleans [2009]) were aligned, and phylogenetic analysis was performed as described above.

TABLE 1 VLP EIA reactivity of commercial anti-GII.4.2006 MAbs

MAb	VLP reactivity			
	MBS223P	MBS224P	MBS225P	MBS226P
VLP				
GI.1.1968	–	–	–	–
GII.1.1971	–	–	+	+
GII.2.1976	–	–	+	+
GII.3.1999	–	–	–	–
GII.4.1987	+	–	+	+
GII.4.1997	+	–	+	+
GII.4.2002	+	+	+	+
GII.4.2004	+	+	+	+
GII.4.2005	+	+	+	+
GII.4.2006	+	+	+	+
GII.4.2009	–	+	+	+

RESULTS

MAb antigenic differentiation between pandemic GII.4.2006 Minerva and epidemic GII.4.2009 New Orleans. Recent studies of GII.4 NoV antigenicity clearly indicate that new strain emergence is associated with changes in blockade epitopes (20–22). The emergence of GII.4.2009 New Orleans provides an opportunity to test this hypothesis in the context of a currently circulating strain. Previously, we characterized nine mouse MAbs that block GII.4 VLP interaction with carbohydrate ligand, i.e., MAbs that potentially recognize neutralization epitopes. To further characterize these MAbs and evaluate their utility in distinguishing between GII.4.2006 and GII.4.2009 VLPs as antigenic variants, we compared the EIA reactivity of GII.4.2006 and GII.4.2009 VLPs with each blockade mouse anti-GII.4 NoV MAb. MAbs GII.4.1987.G1, GII.4.1987.G4, and GII.4.1987.G5 (22, 34) and GII.4.2002.G6 (21) do not recognize GII.4.2006 or GII.4.2009 VLP by EIA, whereas MAbs GII.4.2006.G2, GII.4.2006.G3, GII.4.2006.G4, GII.4.2006.G6, GII.4.2006.G7, and GII.4.2002.G5 (21, 22, 34) demonstrated EIA reactivity with both GII.4.2006 and GII.4.2009 (data not shown). Thus, EIA reactivity to our mouse MAbs was insufficient to identify differential blockade epitopes between GII.4.2006 and GII.4.2009, since each MAb reacted with both VLPs similarly. Therefore, we expanded the pool of tested MAbs to include four new, commercially available mouse anti-GII.4.2006 MAbs (Table 1). First, we characterized these MAbs for reactivity to an assortment of NoV VLPs, including strains from both genogroups and a set of time-ordered GII.4 VLPs (20). All four commercial MAbs demonstrated broad reactivity with GII.4 time-ordered VLPs. MBS224P detected GII.4.2002 through GII.4.2009 VLPs. MBS225P and MBS226P detected the GII.4 panel and additional GII VLPs but neither GI.1.1968 nor GII.3.1999 (Table 1). These reactivity patterns have been described for anti-GII.4.2006 MAbs previously (34). Importantly, MAb MBS223P recognized GII.4.1987 through GII.4.2006 VLPs but did not recognize GII.4.2009, providing evidence at the epitope level that GII.4.2006 and GII.4.2009 have some distinct antigenic profiles, as described for human anti-NoV MAbs (20).

Divergence of GII4.2009 New Orleans from GII.4.2006 Minerva is associated with evolving antibody blockade epitopes. EIA reactivity to MAbs demonstrates that GII.4.2006 and GII.4.2009 have distinct antigenic profiles but the high degree of similarity between the two strains for most of the MAbs tested

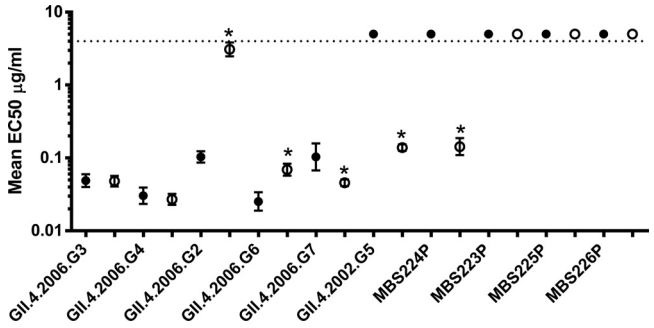

FIG 1 MAbs that recognize evolving GII.4 blockade epitopes distinguish GII.4.2006 from GII.4.2009. MAbs were assayed for ability to block GII.4.2006 and GII.4.2009 VLP interaction with carbohydrate ligand. Sigmoidal curves were fit to the mean percent control binding (percent of VLP bound to PGM in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment), and the mean EC_{50} (μg/ml) titer for blockade of GII.4.2006 Minerva (●) and GII.4.2009 New Orleans (○) was calculated. Error bars represent the 95% CI. *, The EC_{50} blockade titer for GII.4.2009 is significantly different from the EC_{50} for GII.4.2006. Nonblockade MAbs were assigned an EC_{50} of 5 μg/ml for statistical analysis and are denoted by data markers on the graph above the upper limit of detection (dashed line) for visual comparison.

suggests that a more sensitive measure of antigenic variation is required to identify functional antigenic changes. The antibody-mediated VLP-carbohydrate blockade assay measures the potential neutralization activity of an antibody and has proven to be highly differential between similar GII.4 NoV strains (16, 20). Therefore, each of the above-described MAbs was tested for blockade activity against both GII.4.2006 and GII.4.2009, and the EC_{50} values for each VLP were calculated for comparison (Fig. 1). When potential neutralization was measured instead of EIA reactivity, five of the MAbs reacted differently with the two VLPs. MAbs GII.4.2006.G3 and GII.4.2006.G4 blocked both VLPs similarly. GII.4.2006.G2 and GII.4.2006.G6 blocked GII.4.2006 at significantly lower titers than GII.4.2009. Conversely, GII.4.2006.G7, GII.4.2002.G5, and MBS224P blocked GII.4.2009 at significantly lower titers than GII.4.2006. MBS223P, MBS225P, and MBS226P did not block the interaction of either VLP with carbohydrate ligand at 4 μg/ml, indicating that these MAbs do not recognize a neutralizing epitope in either VLP, although the differential EIA reactivity of MBS223P in the absence of blockade activity indicates that GII.4.2006 and GII.4.2009 vary in at least one nonblockade epitope. Of the seven blockade MAbs tested, five differentiate GII.4.2006 from GII.4.2009 at potentially neutralizing epitopes. These data support the hypothesis that the emergence of GII.4.2009 is associated with changes in antibody blockade epitopes.

NoV blockade antibodies primarily recognize epitopes within the P2 subdomain of the major capsid protein (20–22, 53). GII.4.2006 Minerva and GII.4.2009 New Orleans share 88% amino acid similarity within the P2 subdomain. Despite this high degree of similarity, the two strains vary at two previously identified blockade epitopes (Fig. 2) (20). Epitope E varies at V413I, a conserved amino acid change. Epitope A varies at A294P, S368A, and E372D, three of the six amino acids that comprise this immunodominant epitope (20, 22). To test whether variation in epitope A, D, or E accounts for the differences between GII.4.2006 and GII.4.2009 identified in blockade assays (Fig. 1), each blockade MAb was screened for EIA reactivity to GII.4.2006 VLPs containing alterations in blockade epitopes A, D, and E, previously confirmed as blockade antibody targets (20–22). In each case the GII.4.2006 epitope amino acids were replaced with the amino acids found in the GII.4.1987 epitope (Fig. 2). Binding of GII.4.2006.G4 and GII.4.2006.G7 was eliminated by exchange of epitope A amino acids found in of GII.4.2006 with the amino acids found in epitope A of 1987 (GII.4.2006/1987A), supporting previous findings indicating that GII.4.2006.G2, GII.4.2006.G3, GII.4.2006.G4, and GII.4.2006.G7 binding is impacted by changes in epitope A of GII.4.2006 (22; data not shown). Exchange of epitopes D and E within GII.4.2006 did not eliminate binding of any of the MAbs (data not shown).

Single dilution EIAs were insufficient to identify epitopes for any of the other blockade MAbs. Therefore, we analyzed the effect of epitope exchange on the blockade activity of each blockade MAb. In addition to the epitope exchange VLPs previously described, we constructed an additional epitope-exchanged VLP, GII.4.2006/2009A, comprised of GII.4.2006 backbone gene with the epitope A residue sequences replaced by the epitope A residue sequences of GII.4.2009 (Fig. 2). This chimeric VLP has three common epitope A amino acid residues (residues 296, 297, and 298) and three amino acid differences (residues 294, 368, and 372) between GII.4.2006 and GII.4.2009. This additional epitope A variant was designed to allow fine mapping of the residues essential for MAb blocking of epitope A. In contrast to the less-discriminating EIA, surrogate neutralization assays identified epitope A amino acids as required for blockade activity of all of the MAbs that blocked GII.4.2006. GII.4.2006.G2, GII.4.2006.G3, GII.4.2006.G4, and GII.4.2006.G6 all lost blockade activity when the GII.4.2006 epitope A sequence was replaced with the epitope A sequence found in GII.4.1987 (GII.4.2006/1987A, Fig. 3A and Table 2). Significant amounts of blockade activity were restored to these antibodies when only three amino acids were replaced (residues 294, 368, and 372 in GII.4.2006/2009A). These results indicate that GII.4.2006.G2, GII.4.2006.G3, GII.4.2006.G4, and GII.4.2006.G6 all recognize epitope A, and positions 296, 297, and 298 are essential for antibody blockade activity but insufficient to reconstitute GII.4.2006 blockade completely, since significantly more antibody was needed to block GII.4.2006/2009A than GII.4.2006. Further, blockade activity of GII.4.2006.G7 was ablated by exchange of amino acids 294, 297, 298, 368, and 372 in epitope A (GII.4.2006/1987A), but blockade activity was not significantly changed by exchange of amino acids 294, 368, and 372 (GII.4.2006/2009A). This demonstrates that GII.4.2006.G7 recognizes epitope A and that amino acids 296, 297, 298 are not only essential for blockade activity but also account for most of the activity, since GII.4.2006/2009A blockade was not significantly different from GII.4.2006 (Fig. 3B). Neither GII.4.2002.G5 nor MBS224P blocked GII.4.2006 (Fig. 1) and, subsequently, the exchange of epitope A, D, or E amino acids had no significant impact on blockade activity of these MAbs (Fig. 3C, Table 2, and data not shown). Further, although both of these MAbs block GII.4.2009, the exchange of GII.4.2009 epitope A into the GII.4.2006 backbone did not confer blockade of GII.4.2006, clearly indicating that GII.4.2002.G5 and MBS224P are not directed against epitope A but do target a blockade epitope that differentiates between GII.4.2006 and GII.4.2009.

Anti-GII.4.2009 New Orleans MAbs target blockade epitopes and differentiate GII.4.2006 from GII.4.2009. To broaden the

A

VLP	GenBank Accession Numbers	Epitope											
		A						D			E		
		294	296	297	298	368	372	393	394	395	407	412	413
GII.4.1987	AAK50355.1	V	S	H	D	T	N	D	-	H	N	T	G
GII.4.1987/2006A		A	S	R	N	S	E	D	-	H	N	T	G
GII.4.2006	JQ478409	A	S	R	N	S	E	S	T	T	S	N	V
GII.4.2006/1987A		V	S	H	D	T	N	S	T	T	S	N	V
GII.4.2006/1987D		A	S	R	N	S	E	D	-	H	S	N	V
GII.4.2006/1987E		A	S	R	N	S	E	S	T	T	N	T	G
GII.4.2006/2009A		P	S	R	N	A	D	S	T	T	S	N	V
GII.4.2009	ADD10375	P	S	R	N	A	D	S	T	T	S	N	I

B

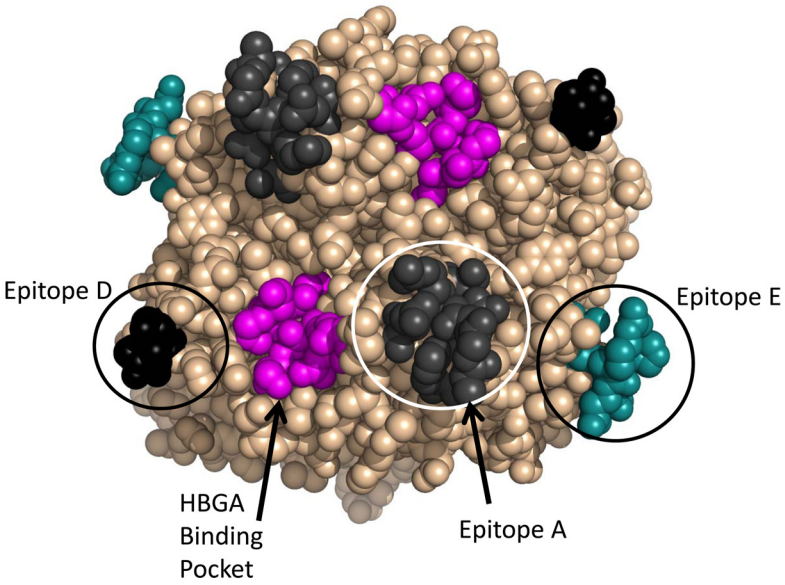

FIG 2 Amino acid sequence (A) and structural location (B) of confirmed GII.4 evolving blockade epitopes in GII.4 VLPs used in the present study.

antibody pool and potentially identify additional evolving blockade epitopes specific for GII.4.2009, we hyperimmunized mice with GII.4.2009 VLPs and developed five anti-GII.4.2009 mouse IgG MAbs. This panel of MAbs had limited EIA reactivity with non-GII.4 VLPs (data not shown). None of the anti-GII.4.2009 MAbs reacted by EIA with NoV VLPs outside GII.4 or with any GII.4 VLP that circulated before 2004. NO37 reacted by EIA with GII.4.2005, GII.4.2006, and GII.4.2009. NO52 reacted with GII.4.2005 and GII.4.2009 but not with GII.4.2006. NO193 reacted with GII.4.2004, GII.4.2005, GII.4.2006, and GII.4.2009, while NO66 and NO224 exclusively recognized GII.4.2009. The tight GII.4.2009 MAb EIA reactivity profile was maintained in blockade assays when MAbs were tested against both GII.4.2006 and GII.4.2009 and the mean EC_{50} values for each VLP calculated for comparison (Fig. 4). All five MAbs blocked homotypic GII.4.2009 (mean EC_{50} range = 0.0641 to 0.2821 μg/ml). Only NO37 and NO52 blocked GII.4.2006, and both MAbs required significantly more antibody to block GII.4.2006 than GII.4.2009 ($P \leq 0.05$). NO193, NO66, and NO224 did not block GII.4.2006 at the MAb concentrations tested. Therefore, all five GII.4.2009 MAbs differentiated between GII.4.2006 Minerva and GII.4.2009 New Orleans VLPs in blockade assays. GII.4.2009 EIA and blockade data demonstrated that GII.4.2006 and GII.4.2009 may vary in at least three neutralization epitopes.

To potentially identify these differential blockade epitopes, the GII.4.2009 MAbs were tested for EIA reactivity to the GII.4.2006 epitope-exchange VLPs (data not shown). By EIA, NO37 reacted positively with each of the epitope-exchanged VLPs except GII.4.2006/1987A. Further, NO37 required significantly more MAb to block GII.4.2006 than homotypic GII.4.2009. Exchange of five epitope A amino acids (GII.4.2006/1987A) ablated the blockade activity of NO37 (Fig. 5A), identifying epitope A as a target for NO37. Exchange of only three amino acids from GII.4.2009 epitope A into GII.4.2006 (GII.4.2006/2009A) increased the blockade activity of NO37 to levels similar to the GII.4.2006 blockade, indicating that the amino acids in common between GII.4.2006 and GII.4.2006/2009A (i.e., amino acids 296, 297, and 298) are essential and sufficient for NO37 blockade activity of these VLPs. Supporting the importance of these sequential amino acids in antibody recognition, NO37 was the only MAb of 16 tested that detected VLPs by Western blot analysis (data not shown). By single dilution EIA, NO52 did not react with GII.4.2006 and consequently did not react with any of the epitope-exchanged VLPs (data not shown). However, NO52 did weakly block GII.4.2006 (Fig. 4 and 5B), confirming the increased sensitivity of the blockade assay compared to the single-dilution EIA. Exchange of five epitope A amino acids (GII.4.2006/1987A) ablated blockade activity of NO52, identifying epitope A as the an-

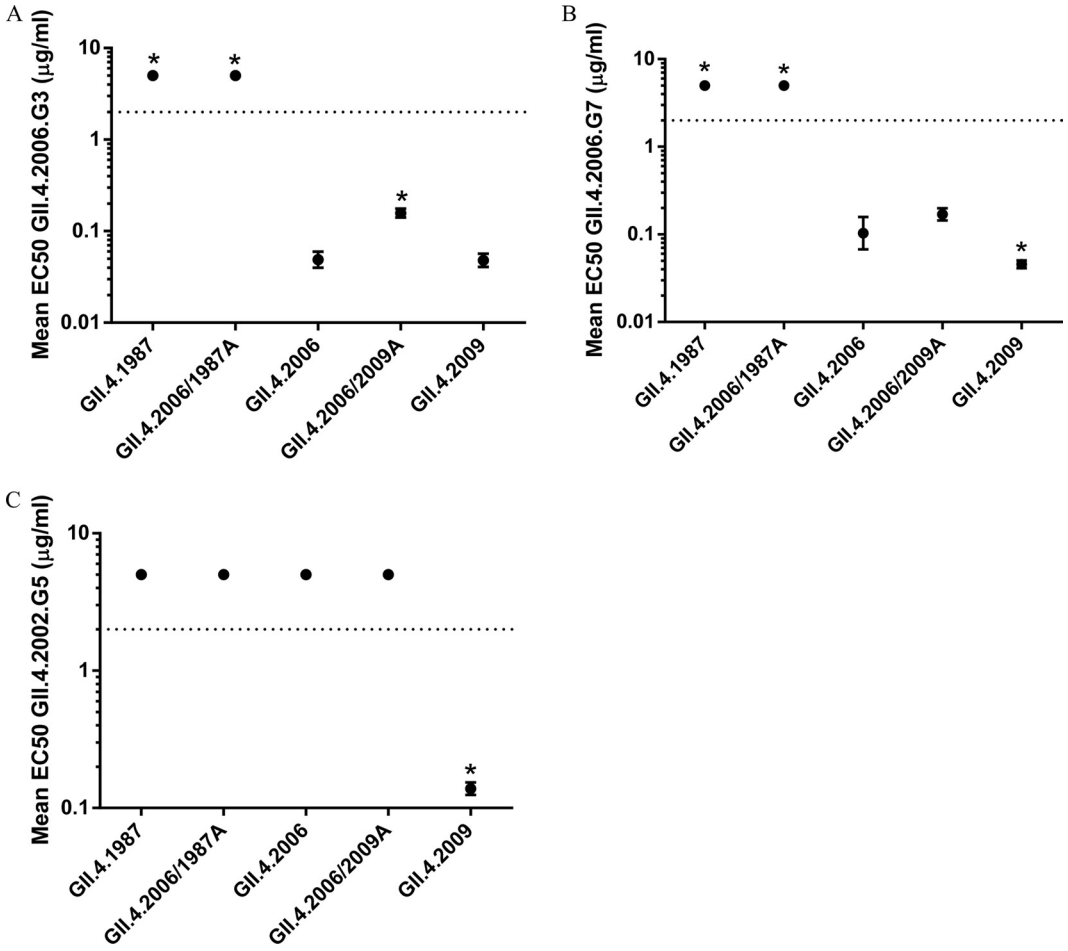

FIG 3 GII.4.2006 blockade MAbs recognition of epitope A. MAbs were assayed for ability to block VLP interaction with carbohydrate ligand. Sigmoidal curves were fit to the mean percent control binding (i.e., the percentage of VLP bound to PGM in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment) and the mean EC_{50} (μg/ml) titer for blockade of epitope-exchanged VLPs calculated. Error bars represent 95% CI. *, The mean EC_{50} blockade titer is significantly different from the mean EC_{50} for GII.4.2006. Nonblockade MAbs were assigned an EC_{50} of 5 μg/ml for statistical analysis and are denoted on the graph by data markers above the upper limit of detection (dashed line) for visual comparison. The mean EC_{50} for the blockade of epitope-exchanged VLPs identified three epitope A reactivity patterns. (A) The exchange of five amino acids (GII.4.2006/1987A) ablated the blockade activity and the exchange of three amino acids (GII.4.2006/2009A) lessened the blockade activity for GII.4.2006.G2, GII.4.2006.G3, GII.4.2006.G4, and GII.4.2006.G6 similarly to each other. (B) The exchange of five amino acids (GII.4.2006/1987A) ablated the blockade activity and the exchange of three amino acids (GII.4.2006/2009A) did not significantly change the blockade activity of GII.4.2006.G7. (C) The exchange of any epitope A amino acids had no impact on blockade activity on GII.4.2002.G5 or MBS224P.

tibody target. In contrast to the effect on NO37 binding, exchange of the three GII.4.2009 epitope A amino acids (294, 368, and 372) into GII.4.2006 (GII.4.2006/2009A) resulted in a significant gain in blockade activity of NO52 for GII.4.2006, approaching the GII.4.2009 mean EC_{50} value (Fig. 5B), identifying residues 294, 368, and 372 as essential for blockade activity of NO52. NO66, NO193, and NO224 blocked GII.4.2009 but did not block GII.4.2006. Exchange of the GII.4.2009 epitope A into GII.4.2006

TABLE 2 Mean blockade EC_{50} for each MAb tested

	Mean VLP EC_{50} (μg/ml)[a]				
MAb	GII.4.1987	GII.4.2006/1987A	GII.4.2006	GII.4.2006/2009A	GII.4.2009
GII.4.2006.G2	>2	>2	0.1032 (0.0863–0.1234)	0.4396 (0.5020–0.3849)	3.077 (2.472–3.8310)
GII.4.2006.G3	>2	>2	0.0487 (0.0398–0.0596)	0.1573 (0.1405–0.1762)	0.0480 (0.0407–0.0566)
GII.4.2006.G4	>2	>2	0.0303 (0.0238–0.0393)	0.0699 (0.0624–0.0782)	0.0270 (0.0227–0.0321)
GII.4.2006.G6	>2	>2	0.0253 (0.0189–0.0338)	0.2369 (0.2105–0.2666)	0.0689 (0.0569–0.0833)
GII.4.2006.G7	>2	>2	0.1034 (0.0675–0.1584)	0.1692 (0.1440–0.1987)	0.0456 (0.0411–0.0506)
GII.4.2002.G5	>2	>2	>2	>2	0.1383 (0.1248–0.1534)
MBS224P	>2	>2	>2	>2	0.1426 (0.1091–0.1863)

[a] The 95% CI is indicated in parentheses.

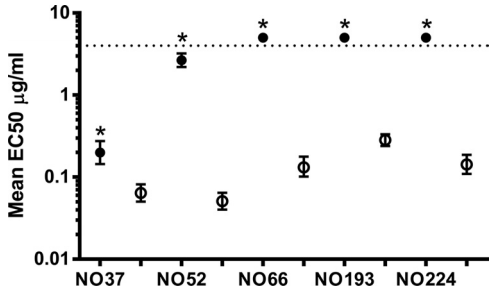

FIG 4 Anti-GII.4.2009 MAbs recognize blockade epitopes and distinguish GII.4.2009 from GII.4.2006. MAbs against GII.4.2009 were assayed for ability to block VLP interaction with carbohydrate ligand. Sigmoidal curves were fit to the mean percent control binding (the percentage of VLP bound to PGM in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment), and the mean EC_{50} (μg/ml) titer for blockade of GII.4.2006 Minerva (●) and GII.4.2009 New Orleans (○) was calculated. Error bars represent the 95% CI. *, The mean EC_{50} blockade titer for GII.4.2006 is significantly different from the mean EC_{50} for GII.4.2009. Nonblockade MAbs were assigned an EC_{50} of 5 μg/ml for statistical analysis and are noted on the graph by data markers above the upper limit of detection (dashed line) for visual comparison.

had no impact on blockade activity of NO66, NO193, and NO224, indicating that these MAbs do not target epitope A (Fig. 5C and Table 3).

Epitope A is a primary target for blockade antibodies in human polyclonal sera following GII.4.2009 New Orleans infection. Mouse MAbs to GII.4.2006 and 2009 identified blockade epitope A as an important distinguishing feature between these two GII.4 strains (Fig. 6). To determine the impact epitope A may have on the human polyclonal antibody response to NoV infection, we compared VLP blockade activity of convalescent-phase serum collected from individuals infected with GII.4.2009 (Fig. 7). GII.4.2009 convalescent-phase serum blocked GII.4.2006 (mean EC_{50} = 0.0205 μg/ml, 95% CI = 0.0154 to 0.0273 μg/ml), GII.4.2006/2009A (mean EC_{50} = 0.0163 μg/ml, 95% CI = 0.0121 to 0.0221 μg/ml), and GII.4.2009 (mean EC_{50} = 0.0160 μg/ml, 95% CI = 0.0131 to 0.0196 μg/ml) VLPs similarly. Significantly more serum was needed to block GII.4.1987 (mean EC_{50} = 0.0369 μg/ml, 95% CI = 0.0284 to 0.0479 μg/ml) ($P \leq 0.05$). Exchange of the 1987 epitope A amino acids into GII.4.2006 (GII.4.2006/1987A) significantly decreased the serum blockade activity by 38% compared to GII.4.2006 and reconstituted levels similar to GII.4.1987 blockade (mean EC_{50} = 0.0329 μg/ml, 95% CI =

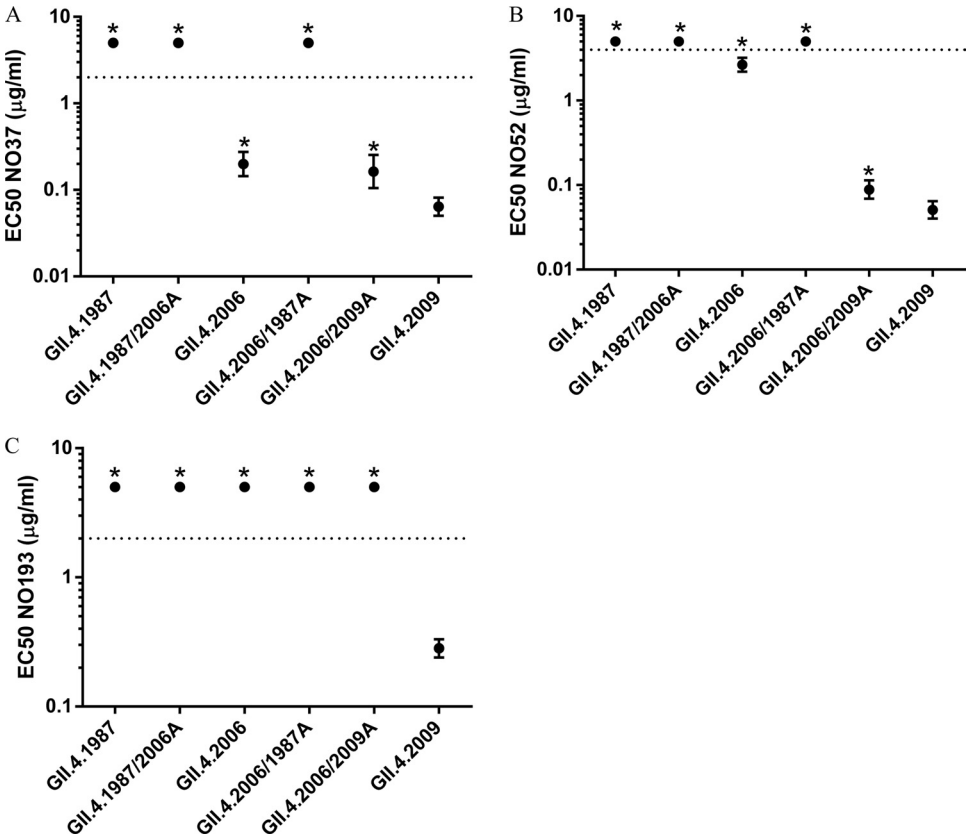

FIG 5 GII.4.2009 blockade MAbs recognition of epitope A. MAbs against GII.4.2009 were assayed for ability to block VLP interaction with carbohydrate ligand. Sigmoidal curves were fit to the mean percent control binding (the percentage of VLP bound to PGM in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment), and the mean EC_{50} titer was calculated. Error bars represent the 95% CI. *, The mean EC_{50} blockade titer significantly different from the mean EC_{50} for GII.4.2009. Nonblockade MAbs were assigned an EC_{50} of 5 μg/ml for statistical analysis and are noted by data markers above the upper limit of detection (dashed line) for visual comparison. The mean EC_{50} for blockade of epitope-exchanged VLPs identified three epitope A reactivity pattern. (A) The exchange of five amino acids ablated the blockade activity and the exchange of three amino acids lessened the blockade activity of NO37. (B) The exchange of five amino acids ablated the blockade activity and the exchange of three amino acids did not significantly change the blockade activity of NO52. (C) The exchange of epitope A amino acids had no impact on the blockade activity of NO66, NO193, and NO224.

TABLE 3 Mean EC_{50} for each GII.4.2009 New Orleans antibody tested

MAb	Mean VLP EC_{50} (μg/ml)[a]				
	GII.4.1987	GII.4.2006/1987A	GII.4.2006	GII.4.2006/2009A	GII.4.2009
NO37	>2	>2	0.1990 (0.1438–0.2754)	0.1628 (0.2527–0.1049)	0.0641 (0.0504–0.0816)
NO52	>2	>2	2.6560 (2.1970–3.2120)	0.0885 (0.0690–0.1137)	0.0508 (0.0401–0.0642)
NO66	>2	>2	>2	>2	0.1314 (0.1013–0.1774)
NO193	>2	>2	>2	>2	0.2821 (0.2395–0.3322)
NO224	>2	>2	>2	>2	0.1426 (0.1091–0.1863)

[a] The 95% CI is indicated in parentheses.

0.0277 to 0.0398 μg/ml). Conversely, exchange of the 2006 epitope A amino acids into GII.4.1987 (GII.4.1987/2006A) significantly increased the serum blockade activity by 37% compared to GII.4.1987 and reconstituted levels similar to GII.4.2006 blockade (mean EC_{50} = 0.0234 μg/ml, 95% CI = 0.0189 to 0.0290 μg/ml). These data indicate that ca. 40% of the polyclonal antibody response to GII.4.2009 infection is directed against epitope A.

DISCUSSION

GII.4 NoV strains have circulated worldwide for at least the past 2 decades, despite high population seroprevalence, leading some to conclude that NoVs induce only short-term or nonprotective immune responses that allow repeated strain infection. However, the detailed study of GII.4 strains that circulated from 1987 until 2012 at the bioinformatics level suggests that the GII.4 strains are complex and divergent from each other, as new strains emerge and replace ancestral strains. The viral evolution identified by bioinformatics has been confirmed by experimental data indicating that different GII.4 strains exhibit different RNA-dependent RNA polymerase rates (24), ligand-binding properties (16, 28), and antigenicity (16, 20, 34). Specifically, new GII.4 norovirus strain emergence is associated with changes in antibody blockade epitopes (20–22), supporting the hypothesis that GII.4 NoV persistence in the human population is driven by viral evolution that results in antigenic drift and escape from herd immunity. GII.4.2009 New Orleans is the first significant GII.4 strain to emerge since the pandemic GII.4.2006 Minerva strain (42) and the first since we developed a panel of MAbs that recognize GII.4 blockade epitopes, providing an opportunity to test this hypothesis in the context of a newly emerged, currently circulating strain.

GII.4.2006 and GII.4.2009 share 88% sequence similarity in the major capsid protein, explaining the high degree of similarity between the strains based on EIA reactivity to MAbs since only 4 of the 19 MAbs tested reacted with one but not the other VLP (Table 1 and data not shown). Reactivity to GII.4.2006 but not GII.4.2009 by MBS223P, a nonblockade antibody, suggests the strains differ antigenically in at least one nonblockade epitope that remains unidentified. Importantly, 10 of the 12 blockade MAbs tested differentiated GII.4.2006 from GII.4.2009, as defined by statistically significant differences in blockade titer (Fig. 1 and 4). These data support the hypothesis that the emergence of GII.4.2009 is associated with changes in blockade/neutralizing epitopes and subsequent loss of protective herd immunity, as previously described

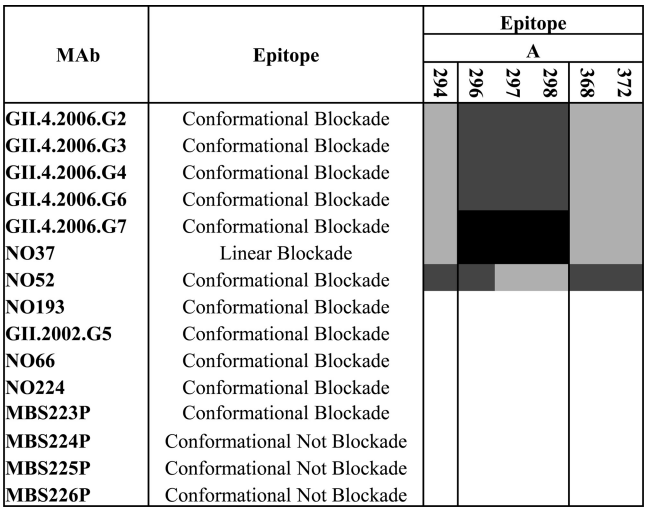

FIG 6 Summary of GII.4 MAb epitopes described in the present study. Antibody epitopes were determined to be conformational or linear based on Western blot reactivity. Binding of seven of the MAbs was dependent upon epitope A residues (light gray boxes). The impact of amino acids that comprise epitope A on each MAb were designated as either essential for blockade activity (dark gray boxes) or sufficient for complete blockade activity (black boxes) or had no effect on blockade activity (white boxes) as described in Fig. 3 and 5.

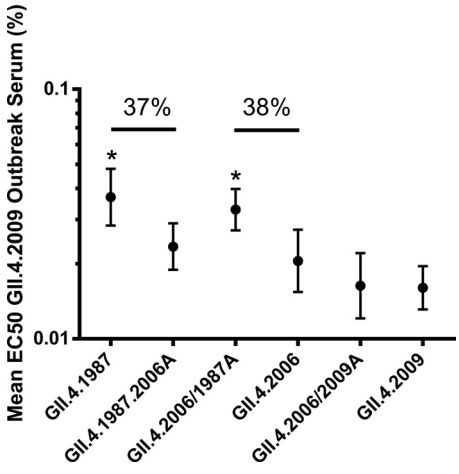

FIG 7 Epitope A constitutes ~40% of the polyclonal blockade antibody response in NoV outbreak human convalescent-phase serum. Convalescent serum samples collected from eight GII.4.2009-infected subjects were assayed for ability to block VLP interaction with the carbohydrate ligand. Sigmoidal curves were fit to the mean percent control binding (the percentage of VLP bound to PGM in the presence of antibody pretreatment compared to the amount of VLP bound in the absence of antibody pretreatment), and the mean EC_{50} (percent serum) titer for blockade of each VLP was calculated. Error bars represent 95% CI. *, The VLP mean EC_{50} blockade titer is significantly different from the mean EC_{50} for GII.4.2009. The exchange of epitope A between the GII.4.1987 and GII.4.2006 backbones resulted in significant changes (~40%) in the EC_{50} titer for both VLPs.

for other GII.4 strains (16, 20, 22, 34) and other RNA viruses such as influenza virus (54–56) and HIV and HCV (57).

Epitope A, a previously identified blockade epitope (20, 22), differs between GII.4.2006 and GII.4.2009. Predicted epitope A is conformation dependent and consists minimally of varying amino acid residues 294, 296, 297, 298, 368, and 372. Amino acid combinations including these residues have been predicted to form antibody epitopes by other groups as well (45, 46). Epitope A is targeted by a significant portion of isolated MAbs as well as polyclonal antibody responses elicited in both norovirus-immunized mice (22) and infected humans (Fig. 7) (20), indicating a primary function in the overall anti-GII.4 antibody response. Three of the six epitope A amino acids vary between GII.4.2006 and GII.4.2009: A294P, S368A, and E372D. The blockade activities of seven of the MAbs studied have been impacted by residue variation in epitope A. By comparing MAb blockade of GII.4.2006 with different versions of epitope A, we identified several epitope binding patterns. GII.4.2006.G2, GII.4.2006.G3, GII.4.2006.G4, GII.4.2006.G6, and GII.4.2006.G7 and NO37 and NO52 each completely lost GII.4.2006 blockade ability when the GII.4.2006 epitope A was exchanged with epitope A residues found in GII.4.1987. GII.4.2006.G2, GII.4.2006.G3, GII.4.2006.G4, and GII.4.2006.G6 each regained significantly more blockade activity when only amino acids 294, 368, and 372 were modified, compared to homotypic VLP blockade, suggesting that residues 296, 297, and 298 are essential for binding to epitope A for these MAbs. However, none of these MAbs detect VLP by Western blot analysis, despite the linear nature of this predicted binding region. Only NO37, which blocked GII.4.2006 and GII.4.2006/2009A similarly detected VLP by Western blotting, confirming that the linear sequence of residues 296, 297, and 298 is not only essential but sufficient for binding of this MAb. Similarly, GII.4.2006.G7 epitope A binding pattern is characterized by no change in blockade activity when 294, 368, and 372 were changed compared to homotypic VLP blockade activity. However, this MAb did not detect VLPs by Western blotting. NO52 provides an additional epitope A binding pattern characterized by almost all of the blockade activity conferred by the conformational amino acids 294, 368, and 372. These varied patterns of epitope A reactivity suggest that amino acids 294, 296 to 298, 368, and 372 actually constitute several distinct, overlapping blockade antibody epitopes, and our panel of MAbs consists of individual antibodies that bind to different combinations of amino acids that include components of epitope A, in particular residues 296 to 298. This suggestion is supported by the diverse EIA reactivity patterns documented for several of these MAbs now defined as epitope A dependent. Interestingly, these diverse strain EIA reactivity patterns coupled with highly selective blockade patterns are contrary to findings with human MAbs that recognize epitope A. Previous studies with human MAbs demonstrated that epitope A has a complex reactivity pattern between strains (20). Three of seven anti-GII.4 MAbs developed from a healthy individual of unknown NoV exposure history were targeted to epitope A. One MAb only recognized and blocked epitope A as it is presented in the early strains GII.4.1987 and GII.4.1997. Two other MAbs only recognized and blocked epitope A as it is presented in GII.4.2006 and GII.4.2009, although significantly more antibody was needed to block GII.4.2009 than was needed to block GII.4.2006. Similar but very limited cross-strain blockade activity was also shown for mouse MAbs that bind to epitope A (22, 34), but, surprisingly, mouse MAbs targeting epitope A display a wider EIA binding profile than human MAbs directed to epitope A (22, 34), possibly suggesting a difference in norovirus antibody specificity between antibodies that result from hyperimmunization of mice with VLP and antibodies generated as the result of a life-time of norovirus infection. Crystal structures of GII.4-MAb complexes are needed to fully understand these complex reactivity patterns.

Epitope A is continuing to evolve, likely in response to protective herd immunity, and the data presented here suggest that a surveillance system that monitored changes in epitope A may be an effective predictor of new norovirus strain emergence. Although, at this time, it is unclear exactly how many or which amino acids within epitope A would need to change to result in complete escape from anti-GII.4.2006 immunity, clearly changing five of the six amino acids (294, 297, 298, 368, and 372) results in complete loss of blockade activity of all of the MAbs that are impacted by epitope A sequence while changing only three amino acids (294, 368, and 372) had anywhere from no effect to almost complete restoration of the blockade activity, depending on the antibody tested. Comparison of a large panel of MAbs for blockade activity of both GII.4.2006 and GII.4.2009 indicated that GII.4.2009 is an intermediate escape variant, sensitive to blockade by some but not all anti-GII.4.2006 MAbs. These partial epitope changes may explain the documented transition from GII.4.2006 to GII.4.2009 as the primary cause of norovirus outbreaks in the absence of an increase in the total number of norovirus outbreaks (43).

The continued viral adaptation of blockade epitopes, including epitope A, will require development of proactive norovirus surveillance systems coupled with a formulation-adaptable vaccine to provide protection from norovirus infection, not unlike the successful approach used for global control of influenza virus. Norovirus surveillance systems have already been established in Europe, Japan, and the United States and shown to be able to rapidly detect newly emerging strains (42). Mapping of the crucial epitopes for virus neutralization will facilitate the predicative power of these surveillance systems and provide potential vaccine targets.

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