Multiple Antigenic Sites are Involved in Blocking the Interaction of GII.4 Norovirus Capsid with ABH Histo-Blood Group Antigens

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Abstract

Noroviruses are major etiological agents of acute viral gastroenteritis. In 2002, a GII.4 variant (Farmington Hills cluster) spread so rapidly in the human population that it predominated worldwide and displaced previous GII.4 strains. We developed and characterized a panel of six monoclonal antibodies (MAbs) directed against the capsid protein of a Farmington Hills-like GII.4 norovirus strain that was associated with a large hospital outbreak in Maryland in 2004. The six MAbs reacted with high titer against the homologous virus-like particles (VLPs) by enzyme-linked immunoassay, but did not react with denatured capsid protein in immunoblots. The expression and self-assembly of newly-developed genogroup I/II chimeric VLPs showed that five MAbs bound to the GII.4 protruding (P) domain of the capsid protein, while one recognized the GII.4 shell (S) domain. Cross-competition assays and mutational analyses showed evidence for at least three distinct antigenic sites in the P domain and one in the S domain. MAbs that mapped to the P domain, but not the S domain, were able to block the interaction of VLPs with ABH histo-blood group antigens (HBGA), suggesting that multiple antigenic sites of the P domain are involved in HBGA blocking. Further analysis showed that two MAbs mapped to regions of the capsid that had been associated with the emergence of new GII.4 variants. Taken together, our data map antibody and HBGA carbohydrate binding to proximal regions of the norovirus capsid, showing that evolutionary pressures on the norovirus capsid protein may affect both antigenic and carbohydrate recognition phenotypes.
Introduction

Noroviruses are major etiological agents of acute viral gastroenteritis worldwide, and estimated to be responsible for approximately 200,000 deaths in children under 5 years of age each year, mainly in the developing world (48). Noroviruses are associated often with outbreaks in closed settings, such as schools, hospitals, hotels, cruise ships, nursing homes, or military facilities; and they are considered the most common cause of non-bacterial gastroenteritis outbreaks (18).

Noroviruses, belonging to the family *Caliciviridae*, contain a single-stranded, positive-sense, RNA genome that is organized into three open reading frames (ORFs 1-3). ORF1 encodes a polyprotein that is co-translationally cleaved into the nonstructural proteins by the viral protease; the ORF2 encodes the major capsid protein (VP1) and ORF3 encodes a small basic protein (VP2) that has been associated with stability of the capsid (6, 18). The expression of VP1 results in the formation of virus-like-particles (VLPs) that have been shown to be morphologically and antigenically similar to the native virion (28). X-ray crystallographic analysis of the capsid revealed a T=3 icosahedral symmetry with 180 VP1 molecules organized into 90 dimers. Each VP1 monomer is composed of two domains, the shell (S) and protruding (P) domains, linked by a flexible hinge. The S domain forms the internal icosahedral scaffold from which the P domain projects to form arch-like structures (49). The P domain can be further divided into P1 and P2 subdomains; with P2 being the most exposed and variable region of the VP1 (49). Recent X-ray analyses have shown that the P2 subdomain interacts with synthetic carbohydrates corresponding to various ABH histo-blood group antigens (HBGA) (9, 10).

Interestingly, the expression of the S domain alone results in smooth particles with a diameter of approximately 30 nm, while expression of the P domain alone yields particles with a diameter of approximately 20 nm (6, 56). The P particles (which can be produced rapidly in bacterial and yeast expression systems) are antigenically similar to the P domain of intact VLPs, and have been used in structure and function
studies to map specific amino acid residues in the P2 domain involved in carbohydrate interactions (9, 56-58).

Noroviruses have been classified into six genogroups (GI-GVI) and multiple genotypes (44, 68) based on VP1 sequences. Noroviruses from GI, GII and GIV have been shown to infect humans, with GII strains being the most prevalent worldwide (18). Since the mid-1990s, noroviruses from genotype GII.4 have been the most common cause of outbreaks of gastroenteritis in the United States and Europe (19, 53, 69). It has been reported that the chronological emergence of new variants (or phylogenetically-related clusters) of GII.4 noroviruses correlated with an increase in the occurrence of large epidemics throughout the world (19, 39, 52, 53, 61, 69). In 2002, a GII.4 variant designated as the Farmington Hills cluster, spread rapidly in the human population and displaced the previous GII.4 strains (53, 61). The mechanisms that have driven the fitness of this variant have not been completely established, but a number of mutations in the ORF1 and ORF2 have been detected and studied extensively. Interestingly, this strain presented a unique amino acid (aa) insertion (Gly394) in the P2 domain that was in close proximity to the HBGA binding site (9) and altered the binding of VLPs to synthetic carbohydrates (11, 39).

Studies of human noroviruses have been hampered by the lack of a cell culture system and small animal disease models, making it difficult to assess neutralization activity of antibodies. It has been reported that the measurement of antibodies that block the interaction between VLPs and HBGA carbohydrates might serve as a surrogate neutralization test (26, 40). Consistent with this, a correlation was found between the presence of serum HBGA-blocking antibodies and resistance to infection in adult volunteers challenged with Norwalk virus (50). Bok et al. (8) evaluated norovirus vaccine candidates in the chimpanzee model and found that chimpanzees vaccinated with Norwalk virus VLPs (Gl.1), but not with MD145-12 (GII.4 strain) VLPs, were protected against re-infection with Norwalk virus. The protection from infection in the chimpanzee model after vaccination correlated with the presence of serum antibodies that blocked the binding of Norwalk VLPs to HBGA
carbohydrates (8). Taken together, these data suggested that protective epitopes are
located at or near HBGA binding sites on the virion.

Because VLPs are antigenically similar to native virions (28), efforts have been made
to characterize the binding of monoclonal antibodies (MAbs) developed after
immunization of mice with VLPs from various norovirus genotypes (3, 25, 66, 67).
Several cross-reactive MAbs have been identified, and most of them have been
mapped to the S domain or the C-terminal region of the P1 domain (3, 4, 35, 45, 46,
51, 65, 66). It has been reported that certain MAbs block the interaction of VLPs
with cells or synthetic HBGA (38, 43), and two HBGA-blocking sites have been
mapped recently (12, 37).

In this study, we developed MAbs against the capsid protein of a GII.4 strain
(Farmington Hills cluster) to explore the antigenic properties of this genetic cluster.
Using competition assays and newly-developed genogroup I/II chimeric VLPs, we
were able to show that multiple epitopes on the norovirus capsid can be involved in
the blocking of VLP:HBGA interactions. In addition, further analyses identified
HBGA-blocking MAbs that mapped to a region of the capsid that has been associated
with the emergence of new GII.4 variants.
Materials and Methods

Expression and purification of VLPs

To express VLPs, the ORF2 and ORF3 genes of norovirus strain Hu/NoV/GII.4/MD2004-3/2004/US (29) were amplified by PCR and cloned into a pENTR plasmid (Invitrogen, Carlsbad, CA) to yield pENTRMD2004-3. Recombination of plasmid DNA with baculovirus DNA was performed using the Baculodirect kit (Invitrogen), and the baculovirus stock was obtained following transfection of the recombination product into Sf-9 cells as recommended by the manufacturer (serum free adapted SF9 cells, Invitrogen). The baculovirus stock was used to infect SF9 suspension cultures for VLP production. Culture medium from baculovirus-infected cells was layered onto a 25% w/v sucrose cushion and subjected to centrifugation in a SW28 rotor at 76,200 x g for 4 h at 4°C (7, 20). The resulting pellets were dissolved in PBS pH 7.4 and further purified through a cesium chloride (CsCl) gradient by centrifugation in a SW55 rotor at 218,400 x g for 18 h at 15°C. The collected fractions (~1.3 g/ml density) were dialyzed against PBS, and the protein concentration was determined with a commercial Bradford assay kit (Pierce, Rockford, IL). The presence of VLPs was confirmed by electron microscopy.


Production of MAbs

BALB/c mice were immunized subcutaneously with 100 μg of MD2004-3 VLPs four times in 2-week intervals. After testing the serum titers, mice were boosted again
intravenously with 100 μg of MD2004-3 VLPs. Three days later, mice were
sacrificed, and spleen cells were isolated and fused with myeloma cells as described
previously (31). The reactivity of the single clone hybridoma supernatants was
tested against MD2004-3 VLPs and positive-cells were collected for further
characterization. Animal experiments and MAb production were carried out at
Creative Biolabs (Shirley, NY), and conducted under approved protocols at Stony
Brook University (IACUC Permit Number 2010-1632). All efforts were made to
minimize suffering. The isotype of each antibody was determined with the IOS-2
Mouse Antibody Isotyping Kit (Sigma, St. Louis, MO) following the manufacturer’s
recommendations.

Enzyme-linked immunosorbent assay (ELISA)

The reactivity of each monoclonal antibody (MAb) against norovirus VLPs was
examined by ELISA. Briefly, 96-well polystyrene microtiter plates (Thermo, Milford,
MA) were coated with 100 μL of purified VLP at a concentration of 0.5 μg/mL in
phosphate-buffered saline (PBS), pH 7.4 and incubated overnight at 4°C. Wells
incubated with PBS alone were used as a negative control for MAb binding. Wells
were washed with PBS containing 0.1% Tween 20 (PBS-T) and blocked with PBS
5% fat free milk for 1 h at room temperature (RT). Each MAb was used at 5 μg/mL
and adsorbed for 2 h at RT. The binding of antibodies to the VLP antigen was
detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse
immunoglobulin G (1:2,000 dilution; KPL, Gaithersburg, MD) and 2,2’-azino-bis(3-
ethylbenzthiazoline-6-sulphonic acid) (ABTS) (KPL). The binding of VLPs to the
plate was confirmed with guinea pig hyperimmune sera (1:500 dilution) raised
against each of the homologous VLPs; except for GII.2 VLPs where GII.1
hyperimmune serum was used.

Western blot analyses
The reactivity of each MAb with the MD2004-3 VLPs was analyzed by Western blot. Briefly, 2.5 μg of VLPs were mixed with Novex® 2X Tris-Glycine SDS loading buffer (Invitrogen), boiled for 5 min at 95°C, and separated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electroblotted onto a nitrocellulose membrane using the iBlot® Dry Blotting System (Invitrogen). The membranes were blocked with PBS 5% fat free milk for 1 h at RT. Each MAb (1:1,000) was adsorbed for 2 h at RT and the binding was detected with HRP-conjugated goat anti-mouse immunoglobulin G (1:2,000) and SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

**Detection of MD145-12 native proteins in stool samples**

Biotinylated MAbs were incubated overnight in NeutriAvidin-coated plates (Pierce, Rockford, IL), and MAb excess was washed with PBS-T BSA 1%. A 10% stool suspension of MD145-12 was incubated for 2 h and the binding of captured native proteins was determined by incubation with anti-MD145-12 VLPs guinea pig hyperimmune serum (1:2,000 dilution), followed by incubation with a peroxidase-conjugated goat anti-guinea pig immunoglobulin G (1:2,000 dilution; KPL), and peroxidase substrate ABTS (KPL). A suspension of MD145-12 VLPs (1 μg/mL) and a norovirus-negative stool were used as controls.

**Blocking of binding of VLPs to synthetic HBGA by MAbs**

The MD2004-3 VLPs were screened for binding to a panel of HBGA-associated oligosaccharides as described elsewhere (8). The oligosaccharides examined in this study were biotin conjugates type A, type B, Leα, Leβ, Leα, Leβ, H type 1 (H1), H2, and H3 (GlycoTech Corporation, Gaithersburg, MD). The ability of MAbs to block the binding of MD2004-3 to B carbohydrate was tested using serial two-fold dilutions of each MAb (starting concentration 15μg/mL). First, the antibodies were pre-incubated with 1.5 μg/ml of MD2004-3 VLPs for 2 h. The VLPs (in the presence or
absence of MAb) were added to carbohydrate coated-plates and incubated for 2 h. 
The binding of captured MD2004-3 VLPs was determined by incubation with guinea 
pig hyperimmune serum (1:2,000 dilution), followed by incubation with a HRP-
conjugated goat anti-guinea pig immunoglobulin G (1:2,000 dilution; KPL), and 
peroxidase substrate ABTS (KPL). The percent blocking was calculated using the 
value obtained from VLPs that were not incubated with MAbs or sera. A blocking 
value of \( \leq 50\% \) of binding was considered the cut-off value (26, 40).

Construction and expression of the chimeric VP1

The NV ORF2 sequence was PCR-amplified from plasmid NV FL101 (15) using 
specific primers NV-SalI\textsubscript{f} and NV-Not\textsubscript{r} (Table 1) that introduced the unique 
restriction sites SalI and NotI at the 5’ and 3’-ends. Briefly, the PCR fragment was 
gel-purified, digested with SalI and NotI and ligated into the corresponding sites of a 
cut pCI expression vector, generating a plasmid designated pCI-NV (Fig. 1A). The 
QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was employed 
to mutagenize pCI-NV using forward (NV-PspXI\textsubscript{f}) and reverse (NV-PspXI\textsubscript{r}) primers 
to introduce a PspXI restriction site at the hinge region (see Fig. 1B and Table 1). 
This clone, designated pCI-NV-PspXI, served as an intermediate vector for swapping 
capsid domains.

To generate a NV S domain/MD2004-3 P domain chimera, the following was 
performed: with pENTRMD2004-3 as template, primers MD2004-3PspXI\textsubscript{f} and 
MD2004-3Not\textsubscript{r} were used to generate an amplicon of the MD2004-3 P domain 
with flanking PspXI and NotI restriction sites. The amplicon was digested with NotI 
and PspXI and ligated into the pCI-NV-PspXI backbone that had been digested with 
the same restriction enzymes, thereby replacing the NV P domain with the MD2004-
3 P domain. The chimeric ORF2 plasmid was designated as pCI-NV/MD2004-3 (Fig. 
1C). A similar approach was applied to obtain a MD2004-3 shell domain-NV P 
domain chimera. Using the same clone of the MD2004-3 VP1-VP2 sequence as a
template, we used primers MD2004-3-SalIF and MD2004-3-PspXIR to generate an amplicon of the MD2004-3 shell domain with flanking SalI and PspXI restriction sites. The amplicon was digested with SalI and PspXI and ligated into the pCI-NV-PspXI backbone that had been digested with the same restriction enzymes, thereby replacing the Norwalk shell domain with the MD2004-3 shell domain. The chimeric VP1 plasmid was designated as pCI-MD2004-3/NV (Fig. 1D). All constructions were confirmed by nucleotide sequencing. The expression of chimeric VLPs was achieved as described above.

Site-directed mutagenesis for epitope mapping

The ORF2 sequence of the MD2004-3 was amplified from pENTR MD2004-3 with primers MD2004-3-SalIF and MD2004-3-NotIR, digested with the corresponding enzymes and ligated into a pCI vector. Site-directed mutagenesis of pCI-MD2004-3 was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene), and complementary forward and reverse primers, which carried the nucleotide mutations (primer sequences available upon request). The restriction enzyme Dpn I (10 U/µl) was used to digest the parental DNA. Each of the mutated products was transformed into Epicurian Coli XL1-Blue supercompetent cells (Stratagene). Transformed cells were grown overnight in LB plates with carbenicillin (50 µg/ml), and individual colonies were used for plasmid amplification. The resulting plasmids were subjected to sequencing analysis to verify the entire VP1 coding region and confirm the presence of introduced mutations. MD2004-3 mutant VLPs were expressed and purified as described above.

Immunofluorescence microscopy

HeLa cells were plated in 96-well plates at 50,000 cells/well, and infected with modified vaccinia virus expressing bacteriophage T7 RNA polymerase (MVA-T7) at MOI=5 PFU/cell for 1 hour (62). After infection, cells were transfected with 400
ng/well of each DNA construct and Lipofectamine 2000 (Invitrogen) following the manufacturer’s recommendations. Cells were incubated for 24 hours and fixed with cold methanol for 20 min. The optimal dilution (1:200) of MAb was determined by serial dilutions. Goat anti-mouse immunoglobulin G (H+L) conjugated with Alexa Fluor 594 (Molecular Probes-Invitrogen, Carlsbad, CA) was used for detection. Guinea pig hyperimmune sera raised against MD2004-3 VLPs and goat anti-guinea pig immunoglobulin G (H+L) conjugated with Alexa Fluor 594 (Molecular Probes-Invitrogen) were used to confirm expression of the antigens.

Bioinformatic analyses

Nucleotide sequences from the prototype strains were downloaded from GenBank (Accession numbers are shown in Fig. 2), and aligned by using the translated aa sequence. The phylogenetic trees were constructed using Kimura 2-parameter as a nucleotide substitution model and Neighbor-Joining (NJ) algorithm as implemented in MEGA v4.0 (55).

The solved structure of the P domain of VA387 virus (GII.4) in complex with carbohydrate (Protein Data Bank [PDB] accession number 2OBT) was used to identify the residues involved in the binding with MAbs and visualized by using MacPyMol (DeLano Scientific LLC).

Competition assays

For competition assays, MAbs were biotinylated by using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Thermo Scientific, Rockford, IL) following the manufacturer’s recommendations. 96-well polyvinyl microtiter plates (Thermo) were coated with 100 μL (125 ng/mL) of MD2004-3 VLPs and incubated overnight at 4°C. Wells were washed with PBS-T and blocked with PBS-T with 1% bovine serum albumin (BSA) for 1 h at RT. Serial dilutions of each competitor MAb were made at a starting
concentration of 10 μg/mL and absorbed for 2 h at RT. The biotin-conjugated MAb was absorbed for 2 h at RT and the binding to the VLP antigen was detected with High Sensitivity streptavidin-horseradish peroxidase (1:10,000 dilution; Thermo Scientific) and ABTS (KPL). The concentration of MD2004-3 VLPs used for coating was optimized in homologous competitions. The optimal concentration of each biotin-conjugated MAb was determined to yield an OD450 of 0.8 to 1.5 by binding with MD2004-3 VLPs coated plates. The percent competition or enhanced binding was determined for all competitor MAb concentrations based on the value of the PBS control (i.e. the value of binding of biotin conjugated-MAb to MD2004-3 VLPs).

Blocking of MD2004-3 hyperimmune serum by MAb

The blocking activity of each MAb, or pools of MAbs, against the binding of polyclonal hyperimmune serum directed against MD2004-3 VLPs was examined by ELISA. Briefly, 96-well polyvinyl microtiter plates (Thermo) were coated with 250 ng/mL of MD2004-3 VLPs and incubated overnight at 4°C. Wells were washed with PBS-T and blocked with PBS-T 1% BSA for 1 h at RT. 10 μg/mL of each MAb, as single or pooled solutions, were absorbed for 2 h at RT. Wells were washed with PBS-T and 10-fold serial dilutions of a MD2004-3 hyperimmune serum were incubated for 1 h at RT. The binding of hyperimmune serum to the VLP antigen was detected with HRP-conjugated goat anti-guinea pig immunoglobulin G (1:2,000) and ABTS (KPL).
Expression and purification of MD2004-3 VLPs

The strain MD2004-3 was identified in 2004 as a causative agent of diarrhea in a Baltimore, Maryland hospital outbreak (29). This strain grouped within the Farmington Hills cluster of the GII.4 genotype (Fig. 2) that emerged in 2002, and became the global predominant GII.4 strain during 2002 and 2004 (53, 61). In order to develop VLPs for further study, the ORF2 (VP1) and ORF3 (VP2) genes of the MD2004-3 strain were engineered into a recombinant baculovirus and the norovirus capsid proteins were expressed in baculovirus-infected insect cells. Following concentration of the resulting VLPs by ultracentrifugation in CsCl gradients, visible bands were collected and dialyzed against PBS. Denaturing polyacrylamide gel electrophoresis of the proteins recovered from the CsCl gradient showed two bands that migrated between 64 and 50 kDa (Fig. 3A, Lane 2). The presence of VLPs was confirmed by electron microscopy (Fig. 3B).

Isolation of MD2004-3 specific MAbs and reactivity with other VLPs

After two fusions, 16 hybridoma clones were screened as positive for reactivity with MD2004-3 VLPs. Isotyping identified ten IgM-secreting and six IgG-secreting hybridomas: this study focused on characterization of the IgG MAbs. The IgG MAbs were further isotyped as subclass IgG1 (A10, B11, and B12), IgG2b (A3 and B15), and IgG2a (A6) (Table 2). All six MAbs reacted with high titer (>10^4) against the MD2004-3 VLPs by ELISA, but none was reactive by Western Blot, suggesting that they each recognized conformational epitopes (Table 2). The reactivity of each MAb was further evaluated by ELISA using a panel of ten additional VLPs available in our laboratory (Fig. 4). Four distinct patterns of cross-reactivity could be seen with other GII VLPs (Fig. 4). The first pattern was represented by MAb B15, which showed cross-reactivity among all GII.4 VLPs in the panel and low cross-reactivity with a GII.3 strain. The second pattern exhibited by MAbs A10, B11, and B12
showed strong reactivity and specificity for the immunizing GII virus, MD2004-3, only. A third pattern represented by MAb A6 showed strong reactivity with MD2004-3, and weak reactivity with MD145-12. Finally, MAb A3 was strongly reactive with GII.4 strains detected in 1987 and 2004 (i.e. MD145-12, HS-191, and MD2004-3, respectively), and to a lesser extent, with GII.4 VLPs from 1974 and GII.1 and GII.2 viruses. To determine whether the MAbs described here could recognize native GII.4 norovirus virions, biotinylated MAbs were attached to NeutriAvidin-coated plates and tested against 10% stool-suspensions positive for MD145-12 virus. As expected, the three MAbs (A3, A6, B15) that reacted with MD145-12 VLPs were also able to detect viral native proteins present in stool samples (Fig. 5). None of the MAbs demonstrated reactivity with the VLPs representing GI (Norwalk and Desert Shield395) or GIV (Saint Cloud624).

HBGA binding and blocking analyses

In the absence of a cell culture system or a small animal model for the noroviruses, the blocking of VLPs binding to HBGA molecules has been used as a surrogate of neutralization (26, 40, 50). Thus, in order to identify the appropriate carbohydrate(s) recognized by VLPs and therefore suitable to perform blocking assays with the MAbs, we examined the HBGA binding profile of the MD2004-3 VLPs. As shown in Fig. 6A, the VLPs from the MD2004-3 virus bound strongly to carbohydrates B and H3.

To test the ability of MAbs to block the binding of VLPs to the H3 and B carbohydrates, each MAb was incubated with MD2004-3 VLPs as described in the Materials and Methods section. All MAbs (with the exception of MAb B15) blocked the interaction of the MD2004-3 VLPs with both the H3 (data not shown) and B (Fig. 6B) carbohydrates. MAb B15 did not block binding to H3, but it partially blocked the interaction of VLPs with B carbohydrate at the highest concentration tested (15µg/ml). Blocking of the VLPs-carbohydrate interaction occurred in a dose-dependent manner (Fig. 6B).
Identification of binding domain (S or P) specificity in the VP1

The capsid protein (VP1) of noroviruses is composed of two domains, S and P. In order to map the domain of the VP1 recognized by the GII.4 MAbs, a strategy was developed to generate chimeric VP1 proteins with S and P domain swaps between MD2004-3 (GII.4) and Norwalk virus (GI.1) in the pCI eukaryotic expression plasmid (Fig. 1). First, full-length cDNA clones of the ORF2 of MD2004-3 (pCI-MD2004-3) and Norwalk virus (pCI-NV) were constructed, and the expression of VP1 was verified with the corresponding hyperimmune serum by immunofluorescence (data not shown). A unique PspXI restriction endonuclease site was introduced between the S and P coding sequences in the Norwalk virus clone (yielding pCI-NV-PspXI) to allow the exchange of regions between Norwalk and MD2004-3. The resulting plasmids carrying chimeric VP1 genes as well as control plasmids, pCI-MD2004-3 and pCI-NV, were transfected into HeLa cells in the presence of MVA-T7 to increase expression efficiency (17, 62), and the binding of monoclonal antibodies was examined by immunofluorescence. All the MAbs reacted with cells transfected with pCI-MD2004-3, but not with pCI-NV or empty pCI vector (Fig. 7). MAbs A3, A6, A10, B11 and B12 reacted with the chimeric VP1 that carried the P domain from MD2004-3, while MAb B15 reacted with the chimeric VP1 that carried the S domain from MD2004-3 (Fig. 7).

To examine whether the conformational epitopes recognized by the MAbs in the eukaryotic expression studies would be present on intact VLPs, the chimeric VP1 genes were cloned and expressed using the baculovirus system as described in Materials and Methods. SDS-PAGE showed the presence of proteins that migrated between 50 and 64 kDa (Fig. 3A, Lanes 3 and 4). The presence of self-assembled VLPs was detected by electron microscopy (Fig. 3B), and their chimeric antigenic profile was confirmed by Western blot with GI- and GII-specific antisera (data not shown). The specificity of MAbs binding to the purified VLPs in an ELISA was...
identical to that observed with the pCI-based constructs in transfected cells (Fig. 3C).

**Fine mapping of putative epitopes**

An alignment of the aa sequences from the four VP1 proteins corresponding to the GII.4 VLPs used in this study showed the presence of 31 variable sites in the P domain that might be involved in epitope specificity (data not shown). To directly map residues involved in the formation of the various epitopes, we examined the predicted location of the aa residues on the mono- and dimeric models of the crystal structure of the VP1 protein, including exposure on the surface of the capsid and proximity to HBGA binding sites. We targeted 14 aa residues as possible determinants of antigenic specificity (Fig. 8), and the pCI-MD2004-3 DNA construct was subjected to site-directed mutagenesis (Fig. 8A). Even though residues 411 and 412 were not in close proximity with the HBGA binding sites, the mutagenesis was performed because these residues were identical in the MD2004-3, HS-191 and MD145-12 strains, and represented a potential site for the cross-reactive MAb A3 or A6. The resulting constructs were transfected into HeLa cells and binding of each of the MAbs was examined by immunofluorescence assay. One construct carrying the mutations AD294GI lost reactivity with MAbs A6 and A10 (Fig. 8A). To confirm this finding, we cloned the VP1 gene carrying the mutations AD294GI into a baculovirus vector. MD2004-3 mutant VLPs were used to confirm the loss of reactivity of MAbs A6 and A10 by ELISA (Fig 8B). Analysis of the X-ray structure of VA387 revealed that residues 294 and 295 (indicated in red) are located in a loop between the β-sheet (β) 2 and β3, at the top of the P domain at equal distance between the two HBGA binding sites (Fig. 8C)(9). Of note, all the other VLPs that tested negative by ELISA against MAbs A6 and A10 (Fig. 4), differed in their sequences at the aa residues 294 and 295 of the capsid protein (Table 3), providing further evidence of the importance of these aa residues in the binding of MAbs A6 and A10. The binding
Identification of antigenic sites

We next performed competition assays with biotinylated MAbs to define the number of antigenic sites in the capsid protein recognized by the MAbs in our panel. Of note is that unlabeled MAbs inhibited the binding of its biotinylated-MAb counterpart in a dose-dependent manner. Representative results from the A3 MAb (competed only against itself) and the A6 MAb (competed against several MAbs) are shown in Fig. 9A. Four different antigenic areas could be identified based on cross-competition assays (Fig. 9B). MAb A3 and B15 showed homologous competition only, suggesting that they bind to unique sites, which were designated as Antigenic Sites I and III, respectively (Table 4). In addition, competition analyses of MAbs A6, A10, B11 and B12 suggested that they map in two overlapping antigenic sites designated as Antigenic Sites Ila (A6 and A10) and Iib (B11 and B12, Table 4).

The ability to block the binding of GII.4-specific hyperimmune serum to MD2004-3 VLPs by each MAb, or combination of MAbs, was examined by ELISA. A pool of six MAbs was able to block hyperimmune serum binding in a dose-dependent manner; with the maximum blocking obtained between $10^{-3}$ and $10^{-4}$ dilutions of the hyperimmune serum (Fig. 10A). All the MAbs that mapped in the P domain of the capsid were able to block the binding of hyperimmune serum to MD2004-3 VLPs, whereas MAb B15 (which mapped in the S domain) was not (Fig. 10B). Interestingly, when pools of three MAbs that represent at least one MAb from each of the antigenic sites were used, the blocking titer was similar to the pool of six MAbs. Moreover, no statistical significance was found in the blocking titer of MAbs B11 or B12 when compared to that of the pool of MAbs, suggesting that B11 and B12 represent an immunodominant epitope.
Despite the role of noroviruses as major etiological agents of acute viral gastroenteritis, research on vaccines has been historically hampered by the lack of a permissive cell line or robust animal model (18). Currently, recombinant VLPs serve as a tool to study the antigenic structure of noroviruses (23, 25, 28, 40, 54) and are in development as vaccine candidates (13, 27, 47, 60). Yet, there are many unanswered questions regarding the effective design and development of norovirus vaccines, such as the identification of antigenic sites involved in virus neutralization and protection from disease.

Over the last decade, GII.4 noroviruses have been the most common cause of gastroenteritis outbreaks in the United States and Europe (19, 53, 69). In 2002, the Farmington Hills cluster of GII.4 displaced older variants of GII.4 globally, and became an important cluster from an evolutionary and epidemiological perspective (39, 61). In order to further elucidate the antigenic properties of this genetic cluster, we developed and characterized a panel of six IgG MAbs against the capsid protein of MD2004-3, a Farmington Hills-like strain.

In concordance with evidence that norovirus genogroups present major antigenic differences (20, 59, 63), none of the MAbs detected VLPs from Genogroups GI and GIV. Two (A3 and B15) of the six MAbs were cross-reactive with different GII VLPs and the remaining four MAbs reacted with one (A10, B11, B12) or two (A6) GII.4 VLPs. Interestingly, the epitopes of five of them mapped within the P domain and one (B15) within the S domain. Several cross-reactive MAbs have been described for noroviruses, and epitopes for most of them have been mapped within the most conserved regions of the norovirus capsid protein (C-terminal P or S domains). As suggested previously, cross-reactive MAbs could potentially be used in diagnostic assays (3, 24, 36, 46, 51); however, Oliver et al. (45) described a cross-reactive MAb that recognized a linear epitope in the S domain of the VLPs from bovine noroviruses that did not detect native virions in fecal samples from experimental
animals. They proposed that because the S domain forms the innermost domain of the capsid, the epitope was not accessible in native virions. In the 5-fold axis of the GII.4 capsid there is a small surface-exposed region of the S domain that is conserved among the GII strains (data not shown). Although it is possible that B15 might recognize this exposed region, it showed low reactivity with MD145-12 native virions in a 10% stool suspension. Together, these results suggest that certain S domain epitopes may not be optimal for detection of low quantities of viruses in stool, even though shown to be highly cross-reactive with purified VLPs.

The blocking of norovirus HBGA binding sites by sera from immunized animals or infected humans has been used as a surrogate of norovirus neutralization assay (26, 40). More importantly, it has recently been shown that the ability of the sera to block VLPs:HBGA interaction correlates with protection against infection in NV-vaccinated chimpanzees and illness in infected human volunteers (8, 50). Five of the six MAbs described here could block the interaction of MD2004-3 VLPs with two synthetic HBGA carbohydrates (H3 and B) and, as expected, their recognition epitopes mapped within the P domain. The S domain-specific MAb B15 did not block the interaction of MD2004-3 VLPs with carbohydrate H3, consistent with the likely internal location of its epitope in the capsid. Our unexpected finding that MAb B15 caused partial blocking of MD2004-3 VLPs with carbohydrate B (and not H3) suggests that binding of some antibodies in the S domain might also influence capsid interactions with certain carbohydrates.

Recently, two binding sites for MAbs developed against VLPs from different GII.4 strains have been described. One site comprised a cluster of seven residues (i.e. 294-298, 368 and 372) (12), while the other one included three residues (i.e. 407, 412 and 413) (37). Importantly, these residues were shown to interact with HBGA blocking-MAbs, suggesting that they may be associated with protective immunity (50). We used the varying reactivity pattern of our panel of GII.4 MAbs against the VLPs to identify putative residues involved in determining their specificity, and designed MD2004-3 capsids carrying mutations in those residues. Allen et al. (2)
reported that after introduction of point mutations into recombinant GII.4 capsids, they failed to obtain recombinant VLPs in one of the four designed constructs, suggesting that the introduction of certain mutations can be structurally unfavorable for assembly. We showed that several mutant capsids engineered in this study reacted with the conformational MAbs and therefore acquired the correct folding. However, two of the MAbs (A6 and A10) in our study lost their reactivity when residues Ala 294 and Asp 295 were mutated to Gly and Ile, respectively, confirming that residues 294-295 are involved in the formation of an important antigenic site (1, 2, 12). Thus, these data are consistent with the involvement of this epitope in diversification of GII.4 strains and immune responses against norovirus infection.

The identification and characterization of viral B-cell epitopes has been important in establishing the role of different antigenic sites in protection (5, 16, 32, 64). Using overlapping fragments of the norovirus capsid protein, Yoda et al. (67) have shown the presence of three continuous antigenic sites for a GII.3 strain (NV36 virus), two in the S domain and one in the P domain. Because synthetic short peptides or overlapping fragments of the VP1 protein cannot generally be used to map discontinuous epitopes, competitive ELISAs have been widely employed in the topological mapping of MAbs that recognize conformational epitopes. Thus, using competitive ELISAs, Hale et al. (22) detected the presence of five antigenic sites in the capsid from NV (GI.1 strain); and the binding domain from one site was determined by mutagenesis analyses (46). Cross-competition assays using the panel of MAbs described here showed the presence of at least four antigenic sites in the capsid of GII.4 noroviruses, with two of these overlapping (Table 4). Using GI/GII chimeric VP1s, we identified the binding domain of the four antigenic sites. Thus, one unique antigenic site (Site I) and two overlapping sites (Site IIa and IIb) were detected in the P domain, while site III mapped in the S domain. MAbs A6 and A10 share the same antigenic site (IIa), which is consistent with their loss of reactivity when residues 294 and 295 from the capsid were subjected to mutagenesis. MAbs B11 and B12 recognize overlapping epitopes in the same antigenic site (IIb),
although competition assays and reactivity pattern with different VLPs suggests that these mAbs map to different residues.

The ability of the pool of six MAbs to efficiently block the binding of a high-titered hyperimmune serum raised against GII.4 VLPs suggests that the major antigenic sites of the capsid are represented in the MAb panel. Interestingly, when combinations of three MAbs representing each of the three antigenic sites described for the P domain were examined, the blocking effect was similar to that of the pool of six MAbs. Thus, these data suggest that the three antigenic sites described here are likely immunodominant sites of the GII.4 norovirus capsid. It will be important to examine whether serum antibodies from immune individuals block the binding of these MAbs. If so, epitope blocking assays employing these MAbs might serve as a surrogate for virus neutralization.

Three possible mechanisms can be involved in the blocking of the HBGA binding site: i. direct blocking; ii. steric interference; or iii. conformational changes that affect the structure. The fine mapping of MAbs A6 and A10 in conjunction with the fact that the other conformational MAbs, that map into different antigenic areas, did not significantly lose their reactivity after incubation with competitor MAbs (i.e. A3 and B15), provides evidence that steric interference is the mechanism by which these MAbs block the HBGA binding site of GII.4 noroviruses. Importantly, MAbs directed against the P2 domain have been shown to neutralize murine norovirus (MNV) infection (30, 42), and one MNV-MAb has been shown to neutralize by covering the outer surface of the P2 domain without causing any apparent conformational changes in the capsid protein (30). Thus, because our GII.4 MAbs bind to multiple epitopes of the P2 domain and block HBGA:VLPs interactions, they may prove useful in defining functional antigenic sites of human noroviruses in structural studies.

Norovirus vaccines based on VLP formulations have gained special interest because they have been shown to induce both systemic and mucosal immune responses.
when administered intranasally or orally (27). Recently, El-Kamary et al. have reported data from Phase I clinical trials in which NV VLPs were administered intranasally to humans with mono-phosphoryl lipid A (MPL) and chitosan as adjuvants (13). No vaccine-related serious adverse events were reported and high IgG and IgA titers developed when 100 μg of VLPs were administered. In addition, Bok et al. (8) have shown that chimpanzees vaccinated intramuscularly with GI VLPs, but not GII VLPs, were protected against infection with GI Norwalk virus, confirming the idea that multivalent vaccines may be required for protection against currently circulating noroviruses. In this regard, the S/P (S domain from one genogroup and the P domain from another) chimeric norovirus VLPs might present the advantage of inducing broadly-reactive immune responses and, thus, constitute a new platform for norovirus VLP-based vaccines. It has been shown that the CD4+ epitopes are located in different domains of VLPs, depending on the norovirus strain (41). Thus, even though it is believed that most of the B cell neutralizing epitopes might be located in the P domain of the capsid, the presence of T cell epitopes in the S domain suggest that vaccination with S/P chimeric VLPs might enhance the immune response and cross-protection against different genogroups.

In summary, we report the generation and characterization of a new panel of MAbs directed toward epitopes on the capsid of GII.4 norovirus, the predominant norovirus genotype. The VP1 domain specificity of each MAb was established with a panel of VLPs and GI/GII chimeric VLPs, and the ability to block HBGA binding corresponded to specificity for the P domain. Modeling and mutagenesis studies established that evolution in the norovirus capsid protein may affect both antigenic and carbohydrate recognition phenotypes, consistent with a link between HBGA blocking and the potential neutralization activity of antibodies. Further characterization of the reagents developed in this study may facilitate the establishment of surrogates of protection to norovirus illness as well as provide new approaches to candidate vaccines.
Acknowledgements

This research was supported by the Intramural Research Program of the NIH, NIAID.

References


Figure 1. Construction of chimeric VP1 proteins. (A) Schematic diagram showing wt NV VP1. Amino acid numbers are in bold, and nucleotide numbers are italicized. The amino acid and nucleotide numbers corresponding to the borders of the shell (S) and protruding (P) domain are shown. The amino acid and nucleotide sequence of the hinge region is shown below. (B) A recombinant NV VP1 was engineered in which a valine at position 224 was substituted for a leucine, generating a hinge region identical to the Hu/NoV/GII.3/Saga/5424/2003/JP strain (Genbank accession no. AB242256). The V224L substitution introduced PspXI as a unique restriction site (the nucleotide sequence of the PspXI restriction site is underlined). (C-D) Chimeric VP1 expression vectors were generated by cloning either the P or the S domain of the MD2004-3 strain into the pCI-NV-PspXI backbone vector.

Figure 2. Phylogenetic tree of noroviruses showing the different genogroups and GII.4 strains. Genogroups are indicated on the right of each phylogenetic cluster. MD2004-3 virus is marked by a filled square. Noroviruses used to express the VLPs used in this study are marked by a filled circle.

Figure 3. Expression and antigenic characterization of wild-type and chimeric VLPs (A) SDS-PAGE showing the mass of the expressed protein. VLPs were analyzed by Tris-Glycine SDS-PAGE in a 10% gel stained with Coomassie blue. (B) Electron micrographs of VLPs expressed in the baculovirus system and visualized by negative staining with 3% phosphotungstic acid, pH 7.2. (C) Reactivity of MAbs with chimeric VLPs. Binding of MAbs was measured by ELISA as described in the Materials and Methods. Black shaded squares represent positive binding and white squares no binding. The nomenclature of each VLP is described in Material and Methods.

Figure 4. Reactivity of monoclonal antibodies (MAbs) raised against MD2004-3 virus with a panel of VLPs. Binding of MAbs (labeled for each VLP experiment as indicated on bottom panels) was measured by ELISA as described in Materials and
Methods. Reactivity of VLPs with hyperimmune sera is represented by black bars. Student’s t test was used to compare reactivity differences of weak positives and negative control. Statistics were assessed using GraphPad Prism 5.0. Statistical significance is denoted by * p≤0.05; ** p≤0.001. Dashed line represents the cut-off value.

Figure 5. Detection of MD145-12 native proteins in stool samples. Biotinylated MAbs were incubated in NeutriAvidin-coated plates, and a 10% stool suspension of MD145-12 was incubated for 2h. Binding of captured native proteins was determined by incubation with anti-MD145-12 VLPs hyperimmune serum, followed by incubation with a peroxidase-conjugated goat anti-guinea pig immunoglobulin G and peroxidase substrate ABTS. A suspension of MD145-12 VLPs (1 μg/mL) and a norovirus-negative stool were used as controls. The experiment was performed twice in duplicate wells. Bars represent the mean; the error bars the S.E.M., and the dashed line the limit of detection.

Figure 6. MAbs block the binding of MD2004-3 VLPs with histo-blood group antigens (HBGA). (A) HBGA binding pattern of MD2004-3 VLPs. The ability of VLPs to bind to synthetic biotinylated-carbohydrates was measured by ELISA. The graph represents the results from two experiments performed in duplicate, with each dot representing the mean of duplicate wells. (B) Percent binding of MD2004-3 VLPs to B carbohydrate by MAbs (starting concentration 15μg/mL) was calculated as described in Materials and Methods. Error bars represent the S.E.M. Dashed line represents the 50% blocking cut-off value.

Figure 7. Expression of chimeric VP1 in eukaryotic cells. Immunofluorescence staining of HeLa cells transfected with different DNA constructs: pCI with VP1 from MD2004-3 (pCI-MD2004-3) and NV (pCI-NV) viruses, pCI with S domain from NV and P from MD2004-3 (pCI-NV/MD2004-3) and pCI with S domain from MD2004-3
and P from NV (pCI-MD2004-3/NV). MAbs binding was detected with goat anti-mouse IgG conjugated with Alexa Fluor 594.

**Figure 8. Fine mapping of epitopes from MD2004-3 MAbs.** (A) Immunofluorescence staining results from HeLa cells transfected with different DNA constructs. Mutations on the VP1 from MD2004-3 (pCI-MD2004-3; WT) were introduced by using specific primers and QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s recommendations. (B) Electron micrographs and MAb reactivity of VLPs carrying mutation AD294GI. Binding of MAbs was measured by ELISA as described in the *Materials and Methods*. (C) The localization of the mutations in the structure of the P domain are colored in light blue. Amino acids involved in the binding of MAbs A6 and A10 are shown in red. The HBGA binding sites are colored in pink and the carbohydrate in green.

**Figure 9. Competitive binding assays of MD2004-3 MAbs.** (A) Representative results from a MAb that competed only against itself (A3) and a MAb that competed against several MAbs (A6). The 100% and 50% (cut-off) of reactivity from biotinylated MAb is indicated by a dashed line. (B) Competition matrix showing the summary of the epitope mapping. Six MAbs were biotinylated and tested against the same panel of unlabeled MAbs. The reduction of binding from positive values obtained at the highest amount of competitor antibody is shown.

**Figure 10. MAbs block the binding of MD2004-3 hyperimmune serum to the homologous VLPs.** (A) Representative blockade of different dilutions of guinea pig hyperimmune serum by a pool of the six MAbs described in this work. (B) Reactivity of hyperimmune serum (dilution 10⁻⁴) against MD2004-3 VLPs in the presence of absence of MAbs. Reactivity of hyperimmune serum with VLPs was measured as described in the *Materials and Methods*. Each dot represents the mean of duplicate wells from a single experiment. Bars represent the OD average. Student’s *t* test was used to compare differences of blocking assays with unblocked control. Statistics
were assessed using GraphPad Prism 5.0. Statistical significance is denoted by *
\[ p \leq 0.05; \quad ** \ p \leq 0.001. \]
Table 1. Primers used for construction of GI.1/GII.4 chimeric capsid proteins

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ → 3’</th>
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<td>gtttttagtcccctctacgCtGagcagaaaaccaggc\textsuperscript{1}</td>
</tr>
<tr>
<td>NV-PspXIR</td>
<td>gcctggttttctgcgGcgtaggagggactaaaac</td>
</tr>
<tr>
<td>NV-Sallf</td>
<td>CGCGTGGTACCTCTAGAGTCGACatgatgtaggcgtctaagg</td>
</tr>
<tr>
<td>NV-NotIr</td>
<td>CATGTCTGTCGAAGCGGGCCGCttatcggcgacagacaagcc</td>
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</tr>
<tr>
<td>MD2004-3-NotIr</td>
<td>GTCTGTCGAAGCGGGCCGCttataatgcacgtctacgcctcccg</td>
</tr>
<tr>
<td>MD2004-3-PspXIr</td>
<td>gtttttagtcccctctacgCtGagcagaaaaccaggc\textsuperscript{1}</td>
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<tr>
<td>MD2004-3-Sallf</td>
<td>CACCGTGGTACCTCTAGAGTCGACatgagatgcccgtcg</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Mutations introduced are shown in \textbf{Bold} and upper case, NV sequences in \textit{Italics}, MD2004-3 sequences in \textbf{Bold} and lower case, and plasmid sequences in upper case.
Table 2. Characteristics of MD2004-3 monoclonal antibodies generated in this study

<table>
<thead>
<tr>
<th>Assay*</th>
<th>MAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A3</td>
</tr>
<tr>
<td>ELISA</td>
<td>+</td>
</tr>
<tr>
<td>Western Blot</td>
<td>-</td>
</tr>
<tr>
<td>Isotype</td>
<td>IgG2b</td>
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</table>

* ELISA and Western Blot used MD2004-3 VLPs as antigen.
Table 3. Comparison of aa residues involved in the binding of MD2004-3 MAbs among selected GII noroviruses

<table>
<thead>
<tr>
<th>Genotype/Strain/Year</th>
<th>AA position¹</th>
<th>MAbs Reactivity²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>294 295</td>
<td>A6 A10</td>
</tr>
<tr>
<td>GII.4/MD2004-3/2004</td>
<td>A D</td>
<td>++ ++</td>
</tr>
<tr>
<td>GII.4/HS-191/2001</td>
<td>A G</td>
<td>- -</td>
</tr>
<tr>
<td>GII.4/MD145-12/1987</td>
<td>V G</td>
<td>+ -</td>
</tr>
<tr>
<td>GII.4/CHDC4871/1977</td>
<td>G I</td>
<td>- -</td>
</tr>
<tr>
<td>GII.4/CHDC5191/1974</td>
<td>G I</td>
<td>- -</td>
</tr>
<tr>
<td>GII.3/Toronto24/1991</td>
<td>T S</td>
<td>- -</td>
</tr>
<tr>
<td>GII.2/SnowMountain/1976</td>
<td>L Q</td>
<td>- -</td>
</tr>
<tr>
<td>GII.1/Hawaii/1971</td>
<td>V P</td>
<td>- -</td>
</tr>
</tbody>
</table>

¹Residues present in wild-type viruses. For GenBank accession number of each strain refer to Figure 1A.
²Strong reactivity is represented by two plus signs; weak reactivity by one plus sign; no reactivity indicated by negative sign (see Fig. 4).
Table 4. Summary of antigenic sites defined by cross-competition analyses

<table>
<thead>
<tr>
<th>Antigenic Sites</th>
<th>Associated MAbs</th>
<th>Binding Domain</th>
<th>HBGA Blocking</th>
<th>Reactivity with GII.4 VLPs</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>A3</td>
<td>P</td>
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<td>MD2004-3, HS-191, MD145-12, CHDC4871, CHDC5191</td>
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<tr>
<td>IIa</td>
<td>A6, A10</td>
<td>P</td>
<td>+</td>
<td>MD2004-3, MD145-12</td>
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<tr>
<td>IIb</td>
<td>B11, B12</td>
<td>P</td>
<td>+</td>
<td>MD2004-3</td>
</tr>
<tr>
<td>III</td>
<td>B15</td>
<td>S</td>
<td>-</td>
<td>MD2004-3, HS-191, MD145-12, CHDC4871, CHDC5191</td>
</tr>
</tbody>
</table>
Figure 1.

A

pCI-NV

1

217 226

530 AA

Shell domain

P domain

Hinge

217 226

651 676

1590 nt

F L V P P T V E Q

ttt tta gtc cct cct acg gtg gag cag

B

pCI-NV-PsPXI

1

217 226

530 AA

Shell domain

P domain

Hinge

217 226

651 676

1590 nt

F L V P P T L E Q

ttt tta gtc cct cct acg ctc gag cag

C

pCI-NV/MD2004-3

1

217 226

544 AA

Shell domain

P domain

213 222

639 664

1632 nt

Norwalk (GI.1)

MD2004-3 (GII.4)

D

pCI-MD2004-3/NV

1

213 222

526 AA

Shell domain

P domain

213 222

639 664

1578nt

MD2004-3 (GII.4)

Norwalk (GI.1)
Figure 3.

**A**

**B**

**C**

<table>
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<tr>
<th>MAbs</th>
<th>VLPs</th>
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<tbody>
<tr>
<td>A3</td>
<td>MD2004-3</td>
</tr>
<tr>
<td>A8</td>
<td>MD2004-3/NV</td>
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<td>NV/MD2004-3</td>
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<tr>
<td>B11</td>
<td>MD2004-3</td>
</tr>
<tr>
<td>B12</td>
<td>MD2004-3/NV</td>
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<tr>
<td>B15</td>
<td>NV/MD2004-3</td>
</tr>
<tr>
<td>NV</td>
<td>NV</td>
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</table>

**Legend:**

- MAbs: Monoclonal Antibodies
- VLPs: Viral-like Particles
- MD2004-3: Reference strain
- MD2004-3/NV: Combination of MD2004-3 and NV
- NV/MD2004-3: Combination of NV and MD2004-3
- NV: Non-virulent strain
Figure 4.

- GI.1/Norwalk/1968
- GI.3/DesertShield395/1993
- GIV.1/SaintCloud624/1998
- GII.1/Hawaii/1971
- GII.2/SnowMountain/1976
- GII.3/Toronto24/1991
- GII.4/CHDC5191/1974
- GII.4/CHDC4871/1977
- GII.4/MD145-12/1987
- GII.4/HS-191/2001
Figure 6.

A

B
Figure 8.

A

<table>
<thead>
<tr>
<th>DNA Constructs</th>
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</table>

WTTHN296SHD MAb A3/A10

B

WTAD294GI

200 nm

C

MAbs A6/A10

HBGA Site III

Top View

Slide View
Figure 9.

A

mAb A3 Biotinylated

% binding

mAb A6 Biotinylated

ug/mL

B

<table>
<thead>
<tr>
<th>Competitor MAb</th>
<th>A3</th>
<th>A6</th>
<th>A10</th>
<th>B11</th>
<th>B12</th>
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</tbody>
</table>

Reduction from Positive Control

- ![Reduction](#) >75%
- ![Reduction](#) 50-75%
- ![Reduction](#) <50%
Figure 10.

A

OD 405 nm

Unblocked sera
Blocked with 6 MAb pool

Hyperimmune Sera Dilutions

B

OD 405 nm

Unblocked A3 A6 A10 B11 B12 B15
6 MAb Pool (A3, A6, B11)
3 MAb Pool (A3, A10, B12)

Blocking MAb