Norovirus and Histo-Blood Group Antigens: Demonstration of a Wide Spectrum of Strain Specificities and Classification of Two Major Binding Groups among Multiple Binding Patterns

Pengwei Huang,1 Tibor Farkas,1,2 Weiming Zhong,1 Ming Tan,1 Scott Thornton,3 Ardythe L. Morrow,1,2 and Xi Jiang1,2*

Division of Infectious Diseases, Cincinnati Children’s Hospital Medical Center,1 and University of Cincinnati College of Medicine,2 Cincinnati, Ohio, and Navy Environmental and Preventive Medicine Unit 6, Pearl Harbor, Hawaii3

Received 24 September 2004/Accepted 15 January 2005

Noroviruses, an important cause of acute gastroenteritis, have been found to recognize human histo-blood group antigens (HBGAs) as receptors. Four strain-specific binding patterns to HBGAs have been described in our previous report. In this study, we have extended the binding patterns to seven based on 14 noroviruses examined. The oligosaccharide-based assays revealed additional epitopes that were not detected by the saliva-based assays. The seven patterns have been classified into two groups according to their interactions with three major epitopes (A/B, H, and Lewis) of human HBGAs: the A/B-binding group and the Lewis-binding group. Strains in the A/B binding group recognize the A and/or B and H antigens, but not the Lewis antigens, while strains in the Lewis-binding group react only to the Lewis and/or H antigens. This classification also resulted in a model of the norovirus/HGGA interaction. Phylogenetic analyses showed that strains with identical or closely related binding patterns tend to be clustered, but strains in both binding group can be found in both genogroups I and II. Our results suggest that noroviruses have a wide spectrum of host range and that human HBGAs play an important role in norovirus evolution. The high polymorphism of the human HBG system, the involvement of multiple epitopes, and the typical protein/carbohydrate interaction between norovirus VLPs and HBGAs provide an explanation for the virus-ligand binding diversities.

Norovirus, previously called “Norwalk-like viruses,” is one of the four genera of Caliciviridae that includes Norovirus, Sapovirus, Lagovirus, and Vesivirus (3). Noroviruses cause mainly acute gastroenteritis in humans. Human noroviruses are difficult to study because they remain refractory to be propagated in cell culture and to infect an animal model, although in vitro propagation of the murine norovirus recently has been reported (23). The recent advances in the study of the norovirus/host interaction and viral receptors have opened a new approach to the study of virus-specific host range and pathogenesis. The first study was performed on the prototype Norwalk virus and demonstrated that Norwalk virus recognizes human histo-blood group antigens (HBGAs) in the intestinal tissues and saliva of secretors (expressing H antigen) but not in those of nonsecretors (18).

Following the initial description of the binding pattern of Norwalk virus, we performed extended studies to characterize other noroviruses and demonstrated that at least four strain-specific binding patterns of noroviruses exist based on the ABO, secretor, and Lewis blood types of the saliva donors (8). The prototype Norwalk virus recognizes one of the four binding patterns and recognizes the types A and O secretors, but not type B secretors and nonsecretors. The other three binding patterns are binders of A, B, and O secretors (VA387), A and B secretors (MOH), and Lewis positive secretors and nonsecretors (VA207). According to the biosynthesis pathways of human HBGAs, the binding targets of each of the four binding patterns have been deduced (8). Using hemagglutination assays and oligosaccharides containing HGGA epitopes, Hutson et al. also showed that the recombinant Norwalk virus-like particles (VLPs) specifically interact with human HBGAs (10). The binding specificity of noroviruses to HBGAs also has been suggested by a study using the recombinant capsid proteins expressed in Venezuelan equine encephalitis virus replicons, although fewer binding patterns with fewer strains of noroviruses were studied (5). Strain-specific binding of noroviruses to HBGAs has also been demonstrated using authentic virions in stool specimens of patients infected with noroviruses, and factors in human stools were found to be able to promote VLP binding of some strains to HBGAs (6).

HBGAs are complex carbohydrates linked to glycoproteins or glycolipids that are present on the red blood cells and mucosal epithelial cells or as free antigens in biological fluids such as blood, saliva, intestinal contents and milk. These antigens are synthesized by sequential additions of monosaccharides to the active portion of the antigen precursors by several glycosyltransferases that are controlled mainly by the ABO, Lewis, and secretor gene families.

The linkage of HBGGA recognition of noroviruses with clinical infection was first suggested by human volunteer studies. In one retrospective study, type O individuals revealed a significantly higher infection rate than those with other blood types in a group of volunteers challenged with the prototype Norwalk virus (9). The same situation was also observed in an outbreak possibly caused by a norovirus (7). Direct evidence

* Corresponding author. Mailing address: Division of Infectious Diseases, Cincinnati Children’s Hospital Medical Center, 3333 Burnet Avenue, Cincinnati, OH 45229-3039. Phone: (513) 636-0119. Fax: (513) 636-7655. E-mail: Jason.Jiang@cchmc.org.
that Norwalk virus recognizes HBGAs (specifically, secretor gene product) as receptors for infection was shown in a volunteer challenge study by a natural resistance of nonsecretors to Norwalk virus challenge and by a lack of binding of Norwalk VLPs to saliva samples collected from the nonsecretors (17). Saliva of type B individuals did not bind or bind weakly to Norwalk virus, and these volunteers had the lowest risk of infection following Norwalk virus challenge compared with individuals of other blood types (17).

In this study, we further characterized the norovirus strain specificity to human HBGAs on extended strains, including the four strains representing the four binding patterns described in our previous studies (8, 18). We performed binding and blocking experiments using saliva, synthetic oligosaccharides, and monoclonal antibodies (MAbs) to characterize norovirus binding specificities. To ensure the specificity of synthetic oligosaccharides used in the assays, we also performed validation experiments using known HBGAs-specific MAbs. Finally, to determine the potential interaction of different epitopes of HBGAs in norovirus binding, we also performed cross-blocking experiments using saliva and oligosaccharides as the blocking agents. Our results showed that noroviruses are highly diverse in recognizing human HBGAs, and up to seven binding patterns are described based on 14 strains studied. By further analyzing the binding patterns based on three major antigenic epitopes of HBGAs, the strains in the seven binding patterns have been grouped into two major binding groups and a model of the norovirus/HBGA interaction is proposed.

MATERIALS AND METHODS

VLPs of noroviruses. VLPs of 14 strains representing 13 genetic clusters of norovirus produced from baculovirus were used in this study, as follows: in genogroup I, Boxer (AF538679), C59 (AF435807) (2), Norwalk virus (M87661) (12, 13), Desert Shield virus (DSV; U044469), and VA115 (AY038598), and in genogroup II, BUDS (AY660568), Grimsky (GrV; AJ048664) (4), Hawaii (HV; J07611) (16), Mexico (MexV; U22498) (11), FAH (AF397156) (1), Parris Island (PIV; YH652979) (22), VA387 (AY038600) (14), VA207 (AY038659) (14), and Operation Iraqi Freedom 031998 (OIF, AY675545) (22). Among them, Norwalk, VA387, MOH, and VA207 represent the four previously published receptor binding patterns (8). The procedures of production of norovirus VLPs in insect cell culture have been published previously (11, 12). Briefly, a cDNA from the 3' end of the genome containing the viral capsid gene (open reading frame 2 [ORF2]) was cloned from the viral RNA extracted from stool specimens. The recombinant baculoviruses carrying the viral capsid genes were constructed from the cloned cDNAs using the Bac to Bac expression system according to the manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad, CA). Norovirus VLPs were produced in Sf9 or H5 insect cell cultures. VLPs were partially purified by sucrose gradient centrifugation and stored at –70°C. Protein concentrations were determined by measuring the optical density at 280 nm (OD280).

Binding of VLP to synthetic oligosaccharides containing human HBGA epitopes. Microtiter plates were coated with synthetic oligosaccharide-bovine serum albumin (BSA) conjugates containing human HBGA epitopes at a concentration of 20 μg/ml at 4°C overnight. Oligosaccharide-PAA-biotin conjugates (2 μg/ml) were coated to a microtiter plate by using streptavidin. After blocking with 5% BSA, norovirus VLPs were added at 0.4 to 1.0 μg/ml. The captured VLPs were detected by the same procedures described above. Oligosaccharides conjugated with two types of carriers were used in this study, including the polyclamylamine (PAA)-biotin conjugates H type 3, type A, and type B disaccharides; Leα, Leβ, H type 1, type 2, type A, and type B trisaccharides; and Leα, Leβ, Leγ, and Leθ tetrasaccharides (GlycoTech Corporation, Rockville, MD); and BSA conjugates A-trisaccharide-BSA ([Galα1-3(Fucα1-2) Galβ-O-spacer]n-BSA), B-trisaccharide-BSA ([Galα1-3(Fucα1-2) Galβ-O-spacer]n-BSA), and BSA conjugates containing a 5-atom spacer (Glycoexpress AB, Lund Sweden). Additional A-trisaccharide-BSA and B-trisaccharide-BSA with 20- and 3-atom spacer, respectively, were also used (V-Labs, Inc., Covington, LA).

Validation of synthetic oligosaccharide conjugates by MAbs. To determine the specificity of individual oligosaccharide products, we performed binding assays of the products with MAbs specific for HBGAs. Oligosaccharide-BSA conjugates (20 μg/ml) were coated directly, while oligosaccharide-PAA-biotin conjugates (2 μg/ml) were coated through streptavidin (10 μg/ml) as anchor. After removal of the uncoated conjugate and blocking with 5% BSA, MAbs were added at a dilution of 1:100 to 1:200. The captured MAbs were detected using HRP-conjugated goat anti-mouse IgG or IgM (ICN, Aurora, OH). MAbs used in this study included MAbs anti-B (BG-3), anti-H type 1 (BG-4), anti-Leα (BG-5), anti-Leβ (BG-6), anti-Leγ (BG-7), and anti-Leθ (BG-8) (Signet Laboratories, Inc., Dedham, MA) and MAbs anti-H type 2 (BCR9031), anti-A (BCR9010), and anti-B (BCRM 11007) (Accurate Chemical & Scientific Corporation, Westburg, NY).

Blocking of norovirus binding to saliva by MAbs. The same conditions of saliva binding assays described above were used. For blocking, saliva-coated plates were preincubated with MAbs at dilutions of 1:5 to 1:100 for 1 h at 37°C before adding norovirus VLPs to the plate. The levels (%) of blocking were calculated by comparing the OD490 values between wells with or without incubation with a MAb.

Cross-blocking assays with synthetic oligosaccharide conjugates. The same set of oligosaccharide binding assays described above were used. For blocking, norovirus VLPs were preincubated with 20 μg/ml of BSA-oligosaccharide conjugates or 2 μg/ml of PAA-biotin oligosaccharide conjugates for 1 h at 37°C before transferring to the oligosaccharide-coated plate. The levels (%) of blocking were calculated from the OD490 values between wells with or without preincubation with an oligosaccharide.

Cross-blocking assays with saliva samples. The same conditions of saliva binding assays with the principle of the oligosaccharide-blocking assays described above were used, except saliva samples were used for both coating and blocking. Saliva samples containing specific HBGAs were selected from our saliva bank, and the blood types of the saliva samples were determined by the MAB typing assays.

Sequencing and phylogenetic analysis. Sequencing was performed either directly with RT-PCR products or after cloning into the pGEM-T vector (Promega, Madison, WI). Sequencing reactions were read on an ABI PRISM 3700 DNA analyzer. Besides those previously mentioned, the following synthetic oligosaccharide sequences published in the GenBank were used in the phylogenetic analysis: Alphapton (AF195847), Amsterdam (AF195848), Arg320 (AF190817), Chiba (AB022679), Hesse (AF193779), Hillingdon (AJ277607), Jena (AJ011099), Leeds (AJ277608), Lorsdale (X65657), Melksham (X81879), Musgrove (AJ277614), Snow Mountain (AY134748), Southampton (LJ7418), Winchester (AJ277609), and Fayetteville (AY131106). Multiple alignments of deduced amino acid sequences of the capsid proteins were created by using Clustal v2.0 software (Oxford Molecular Ltd., Oxford, United Kingdom). Alignments were edited in GeneDoc v2.2 (19). Dendrograms were constructed by using the UPGMA clustering method of Molecular Evolutionary Genetics Analysis (MEGA version 2.1) with Poisson correction distance calculations and 125 bootstrap analyses (15).
RESULTS

A broad spectrum of norovirus binding specificity to human HBGAs. In our previous studies, we described four major HBGA binding patterns represented by strains VA387, MOH, Norwalk, and VA207 based on a study of eight strains (VA387, GrV, Norwalk, C59, MxV, MOH, VA207, and VA115) representing seven genetic clusters of noroviruses (8). In this study, we characterized six additional strains representing six genetic clusters of noroviruses (PiV, BUDS, Boxer, OIF, DSV, and HV) (Fig. 1). When the binding results of all 14 strains with the 81 saliva samples were compared, three new binding patterns were revealed in addition to the four patterns previously described (Fig. 1).

Strain PiV revealed a binding pattern identical to that of MOH and MxV, which reacted with the type A and B secretors but not with the type O secretors and nonsecretors. Strain BUDS revealed a similar binding pattern but recognized only saliva of type A, not type B, secretors (Fig. 1). Boxer showed a binding pattern similar to that of VA207 (8), both reacting with nonsecretors, type O, and type A secretors, but not type B secretors; however, Boxer had an equal or even higher binding activity to type O secretors than to nonsecretors. OIF was another strain that bound the saliva of nonsecretors but with weaker binding activities to type O secretors than to nonsecretors (Fig. 1). In later studies using oligosaccharide- and MAb-based assays, significant differences in binding specificities to the Lewis and H epitopes were observed among these nonsecretor binding strains (VA207, Boxer, and OIF) (see Results).

Two strains (HV and DSV) did not react with any human HBGAs of the 81 saliva samples tested (Fig. 1), even at a high dilution of 1:1,000. The HBGA types of the individuals are shown at the bottoms of the panels (Non-sec, Non-secretors; A, type A secretors; B, type B secretors). Among the 14 strains compared, binding patterns of six strains (PiV, BUDS, HV, Boxer, OIF, and DSV) were characterized in this study. Four strains representing the four norovirus/HBGA binding patterns described previously are Norwalk, VA387, MOH, and VA207, respectively. Strain PiV was tested on a different set of saliva samples (52 in total) because of the consumption of the original set of the 81 saliva samples.
TABLE 1. Recognition of synthetic HBGA oligosaccharide conjugates by monoclonal antibodies specific to HBGAsa

<table>
<thead>
<tr>
<th>Oligosaccharide conjugate</th>
<th>Lea</th>
<th>Leb</th>
<th>Leb</th>
<th>H-1</th>
<th>H-2</th>
<th>Type A</th>
<th>Type B</th>
</tr>
</thead>
<tbody>
<tr>
<td>-PAA-biotin</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lea (tri-)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leb (tetra-)</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leb (tri-)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-1 (tri-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-2 (tri-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-3 (di-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type A (tri-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type B (tri-)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a Synthetic HBGA oligosaccharide conjugates were coated to plates directly (BSA conjugates) or through streptavidin molecules (PAA-biotin conjugates). Reactivity was scored from strong (+++) to completely negative (-). di, disaccharide; tri, trisaccharide; tetra, tetrasaccharide; ND, not done.

TABLE 2. Binding of synthetic HBGA oligosaccharide conjugates to norovirus VLPsa

<table>
<thead>
<tr>
<th>Oligosaccharide conjugate</th>
<th>Norovirus strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/B binding</td>
</tr>
<tr>
<td></td>
<td>VA387 Norwalk MxV PiV HV MOH BUDS</td>
</tr>
<tr>
<td>Lea (tri-)</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>Leb (tetra-)</td>
<td>++ + + + +</td>
</tr>
<tr>
<td>Leb (tri-)</td>
<td>- - - - - -</td>
</tr>
<tr>
<td>Leb (tetra-)</td>
<td>++ ++ + +</td>
</tr>
<tr>
<td>H-1 (tri-)</td>
<td>+ ++ + +</td>
</tr>
<tr>
<td>H-2 (tri-)</td>
<td>- - - -</td>
</tr>
<tr>
<td>H-3 (di-)</td>
<td>++ ++ + +</td>
</tr>
<tr>
<td>A (tri-)</td>
<td>+++ + ++ + +</td>
</tr>
<tr>
<td>B (tri-)</td>
<td>++ ++ ++ + +</td>
</tr>
</tbody>
</table>

a Synthetic HBGA oligosaccharide conjugates were coated to plates directly (BSA conjugates) or through streptavidin molecules (PAA-biotin conjugates). The binding of norovirus VLPs was detected by using a pooled guinea pig hyperimmune antibody. The same scoring system described in Table 1 was used. di-, disaccharide; tri-, trisaccharide; tetra-, tetrasaccharide.
type O saliva, but all three strains bound to synthetic Leb antigen (Fig. 1 and Table 2). It was noted that all these binding activities were dose related but with a low OD value (below 1.0). In fact, the binding of HV to synthetic Leb also has been reported by others (6). Thus, we believe that these reactions are strain specific, not an artifact. Possible reasons for the difference between the results obtained by the saliva- and oligosaccharide-based assays are considered in the Discussion.

The Lewis binding strains recognize the Lewis epitopes, but each may have a unique binding specificity. Since the MAb-based assays have already confirmed the involvement of the Lewis epitopes in the binding of VA207 (8), we performed additional blocking experiments on the three nonsecretor binding strains with MAbs against the four major Lewis epitope-containing antigens (Lea, Leb, Lea, and Leb). As expected, MAbs against Lea and Leb strongly blocked VA207 binding to saliva containing Lea and Leb (nonsecretor donors) (Table 3). However, they did not block Boxer and OIF binding to the same nonsecretor saliva samples. MAbs against Lea and Leb blocked VA207 and Boxer binding to saliva containing Lea and Leb (secretor donors) but did not block their binding to saliva containing Lea and Leb (nonsecretor donors) (Table 3). None of the four MAbs blocked OIF binding to the saliva of nonsecretors. Thus, we concluded that, although the MAb-based assays did not confirm all results by the saliva and oligosaccharide assays, the fact that all three strains bound to the saliva of the nonsecretors indicates that these strains are related by binding to the Lewis epitope (1,3/4 fucosyl residue). However, these strains are distinct, possibly by their variable affinity to the Lewis and H epitopes (see Discussion).

The A/B and H epitopes are independent but can act together in binding to A/B binding strains. Since most of the strains binding the A/B epitopes reacted with more than one epitope, we particularly studied the role of individual epitopes in association with other epitopes for binding VLPs. Specifically, the VA387-like strains (VA387, GrV, Norwalk, and C59) can react with almost all secretor-related antigens containing the A/B and H epitopes (Table 2). The A/B and H epitopes are linked to the same galactose molecule with α1-3 and α1-2 linkages, respectively, that are physically next to each other, suggesting that they are likely to act together. To test this hypothesis, we performed blocking experiments using oligosaccharides or saliva as the blocking agents.

Using oligosaccharides to block norovirus binding to oligosaccharides, we observed significant homologous blocking activities among oligosaccharide conjugates containing the A, B, and H epitopes (Tables 4 and 5). In addition, significant heterologous blocking activities among different oligosaccharide conjugates have also been observed in strains of the A/B binding group (Tables 4 and 5). For examples, the A and B antigens cross-blocked each other for their binding to VA387 (Table 4), MOH, and other MOH-like strains (BUDS, MxV, and PiV) (data not shown). The A and/or B trisaccharides also blocked the binding of VA387 and Norwalk virus to the H-containing antigens (H-1, H-3, Lea, and Leb), possibly at the common H recognition site of the capsids (Table 5). Similarly, the H-containing antigens also blocked the binding of VA387 and Norwalk virus to the A and B antigens (trisaccharides) with variable efficiencies. However, the Lewis epitopes (Lea and Leb) did not block the binding of VA387, Norwalk virus, MOH, or the MOH-like strains to the A/B and H epitopes, nor did the A and B trisaccharides block the binding of VA207, Boxer, or OIF to the nonsecretor saliva samples (data not shown).

To further confirm these results, we also performed saliva-saliva blocking experiments using a panel of saliva samples

<table>
<thead>
<tr>
<th>Norovirus strain</th>
<th>Type A trisaccharide conjugate</th>
<th>Type B trisaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lea</td>
<td>Leb</td>
<td>H-1</td>
</tr>
<tr>
<td>+++</td>
<td>+++</td>
<td>H-3</td>
</tr>
</tbody>
</table>

* Synthetic oligosaccharide BSA conjugates (type A or B) were coated to the plate directly. The binding of norovirus VLPs was detected by using a pooled guinea pig hyperimmune antibody. For blocking, oligosaccharide conjugates were preincubated with norovirus VLPs before adding to the plates. The same scoring system described in Table 3 was used. di, disaccharide; tri, trisaccharide; tetra, tetrasaccharide; NA, not applicable.
representing different HBGA types for the coating and the blocking steps, respectively. Figure 2 shows the typical results of the blocking assays for VA387. In summary of the results, the following conclusions have been made: (i) significant blocking was observed among homologous blood types, (ii) significant heterologous blocking activities were also observed among secretor saliva (types A, B, and O), (iii) saliva of nonsecretors did not block the binding to the A, B, and H antigens, and (iv) saliva samples with more complex antigens (e.g., type A and B secretors) were stronger blockers than saliva samples with less complex antigens (e.g., type O secretors).

In conclusion, both A/B and H are independent epitopes but they can act together depending on the structure of the binding interface of norovirus capsids. Strains MOH and BUDS apparently have one binding site to the A/B epitopes, while the remaining strains of the A/B binding group may contain two sites within the binding interface. The A and B epitopes may share the same binding site for the majority of the A/B binding group, except for Norwalk and C59, which recognize only the A but not B antigens. The H epitope is associated with the A/B epitopes in the binding, but it can act alone in the type O secretors. The fact that the A/B and Lewis epitopes do not cross-block each others indicates that strains in these two groups have distinct binding interfaces on the viral capsids.

Strains with similar binding patterns tend to be genetically clustered. The fact that the two groups mutually exclude the A/B or Lewis epitopes raised the possibility that the human HBGAs may play a role in norovirus evolution. However, we did not observe a clear segregation of the two binding groups with the genogroups of noroviruses when the entire capsid sequences were analyzed (Fig. 3). In our recent study, we demonstrated that the P domain is responsible and contains the essential elements for the binding of norovirus VLPs to HBGAs (20). Therefore, we performed a phylogenetic analysis focusing on the P domain, but still no genetic correlation was observed between the two binding groups (data not shown).

However, when the 14 strains were listed on the phylogenetic tree in relation to their binding patterns, clear relationships of genetic identities with binding patterns were observed among some strains (Fig. 3). For example, GrV and VA387 are the only two strains that bound types A, B, and O secretors and both belong to the same cluster (GI14) of genogroup II that shares a 98% overall amino acid sequence identity. Norwalk virus and C59 are two strains that bound to the A and O, but not B, secretors, and they are genetically close to each other. In addition, both BUDS and MOH bound only the A/B epitopes and they are more related to each other than to any other strains studied. Furthermore, HV, MxV, and PIV revealed similar binding patterns to the A/B and H antigens and they are related with the shortest phylogenetic distances to each other. Finally, strain VA115 and DSV are the only two strains that did not react with any HBGAs and they are genetically close to each other. Thus, we conclude that the strain specificities are genetically related, indicating that human HBGAs are involved in norovirus evolution. The scattering of strains of the two binding groups in both genogroups indicates that other factor(s) may also be involved in norovirus evolution.

DISCUSSION

Our study has significantly extended the understanding of the diversity of norovirus/HBGA interaction by the characterization of 14 strains representing 13 genetic clusters of noroviruses. Seven binding patterns have been identified based on
the saliva-, MAb-, and oligosaccharide-based assays according to the interaction with three major epitopes (A/B, H, and Lewis) of HBGAs. According to the blood types of the saliva donors, the seven binding patterns can be classified into two groups: the A/B binding group (VA387, GrV, Norwalk, C59, MOH, PiV, MxV, BUDS, and HV) and the Lewis (nonsecretor) binding group (VA207, Boxer, and OIF). All strains in the A/B binding groups bound to type A and/or B and O saliva of secretors but not to the saliva of nonsecretors, while all strains in the Lewis binding group bound to the saliva of nonsecretors and type O secretors but with weak binding or no binding to the type A and B secretors (Fig. 1).

Based on this grouping, a model of norovirus/HBGA interaction has been proposed (Fig. 4). Basically, each strain may have one binding interface that can accommodate a maximum of two of the three major epitopes of HBGAs (A/B, H, or Lewis). Binding activities with one or a combination of two of the three sugar side chains determine the binding patterns of the strains. The combinations of affinities to the A/B and/or H epitopes determine the binding patterns of the strains of the A/B binding group, while the combinations of the affinities to the Lewis and/or H epitopes determine the binding patterns of strains in the Lewis binding group. Thus, strains in each group can be further divided according to their combined binding activities to corresponding epitopes (Fig. 4).

According to this model, the complicated binding results observed in this study can be easily explained. For example, strains VA387 and GrV have a strong affinity to both A/B and H; therefore, they bound strongly to both type A/B and O saliva. Strains PiV, MxV, and HV have a strong affinity to A/B

FIG. 3. Phylogenetic tree and prediction of HBGA targets of noroviruses determined by binding and blocking experiments. The phylogenetic tree was constructed based on the amino acid sequences of the entire capsid genes using the UPGMA clustering method (MEGA v2.1) with Poisson correction distance calculations. Scale bar represents the phylogenetic distances expressed as units of expected amino acid substitutions per site. Bootstrap values are indicated as percentages of 125 replicates. Strains characterized in this study and strains representing the four previously described binding patterns are in bold. Strain SMV is characterized by Harrington et al. based on oligosaccharide binding assays (5). The potential HBGA targets for individual strains are shown on the right side of the panel. The binding results of each strain were assigned based on reactions in assays with saliva and/or oligosaccharide conjugates. "+" indicates a positive binding observed in any of the assays, "++", "+++", and "++++" indicate higher binding signals, and "-" indicates no binding.
but a relatively weak affinity to H, and they reacted strongly with the A/B antigens but weakly with the H epitope-containing antigen. Strains MOH and BUDS have only one site for the A/B epitopes, and they bound to the A/B secretors only. Norwalk virus and C59 may recognize A but not B, while SMV may recognize B but not A.

Similarly, in the Lewis binding group, Boxer may have a strong affinity to both Lewis and H epitopes and VA207 may have a strong affinity to the Lewis and a weak affinity to the H epitope, while OIF recognizes only the Lewis epitope. In other words, all three strains may recognize the Lewis epitope; the addition of an α1,2-linked fucose (H epitope) significantly increases the affinity of Boxer to the Lewis epitope, while this effect was less for VA207 and the least for OIF. Furthermore, the addition of the A and B epitopes may have a negative effect (epitope masking) on the binding to the Lewis and/or H epitopes for these strains. Therefore, VA207 revealed a typical three-step-interaction pattern that has the strongest binding activities with the saliva of the nonsecretors, intermediate binding activities with saliva of the types A and B secretors, and the lowest binding activities with saliva of the types A and B secretors (Fig. 1). The “masking effect” of the B epitope is probably stronger, as none of the three strains bound the saliva of type B secretors. The predicted affinity of Boxer and OIF also can be seen clearly in the saliva binding assays, in which Boxer had a stronger binding activity to the type O secretors than to the nonsecretors and OIF did not bind, or bound weakly, to the type O secretors.

The inconsistent results obtained from the saliva- and the oligosaccharide-based binding assays may be due to some subtle differences between the synthetic products and the authentic antigens found in vivo. As observed in our validation experiments described earlier, many factors on the oligosaccharide determinants, the carriers, and the spacers of the conjugates can affect the affinity (data not shown). In addition, the layout or presentation of the HBGAs also may be different between the in vitro and in vivo conditions. HBGAs are likely to be presented on mucin or mucin-like molecules with a special array and density of the antigens, which may not be the same under the conditions of the synthetic products.

Because the norovirus/HBGA interaction is a typical protein/carbohydrate interaction, like the lectin/carbohydrate interaction that is highly diverse, a subtle change of the binding interface of the antigens could result in binding pattern change of the strains. Noroviruses are genetically highly diverse, and in our recent studies, we found that a single amino acid change of the P domain of the viral capsid protein could result in HBGA binding pattern changes (21). Thus, noroviruses could have a wide spectrum of binding specificities to HBGAs due to their wide genetic diversity. This is also true for MAbs, because MAb/HBGA interaction also is a typical protein/carbohydrate interaction. Therefore, it is not surprising when incongruous results are obtained between different assays with different reagents and among norovirus strains, because each of them could have subtle differences in HBGA recognition. The lack of interaction of the H-1 trisaccharide to the H-1 MAb observed in our study could be another example possibly due to different sources of these reagents.

Although variable results have been observed among different assays and different reagents, the cross-blocking experiments using the same types of reagents seemed highly consistent. One important finding of these studies is that different antigens containing shared epitopes can cross-block each other for binding to different noroviruses. This finding not only confirms the single binding pocket hypothesis (20, 21) but also provides a useful strategy to develop antiviral drugs against noroviruses. As demonstrated in the cross-blocking experiments, the trisaccharide A and B antigens were able to block
all A/B and H antigen-related binding. Thus, it may be possible to develop a single oligosaccharide molecule to block the binding of most norovirus strains in the A/B binding group and partially block some strains in the Lewis binding group to HBGAs. The finding that more complex HBGAs are more capable of blocking is particularly useful for designing such molecules as antiviral agents for norovirus. Theoretically, oligosaccharides such as the types A and B pentasaccharides (ALeα and BLeβ) that contain the A, B, H, and Lewis epitopes would be the ideal antiviral compounds for their potential blocking activities. Due to the possible masking effect of the A/B epitope, additional molecules containing the H and/or Lewis epitopes only may also be included.

This study has provided further understanding of the norovirus and human host interaction. However, there still are many questions to be addressed. For examples, the major epitopes of human HBG A that might be involved in norovirus recognition have been determined or deduced, but the precise targets, particularly for strains in the Lewis binding group, remain to be determined. Second, except for the seven found binding patterns, how many potential additional binding patterns exist? Third, what roles do HBGAs play during different norovirus infections? Although strong evidence has been obtained for Norwalk virus to support the correlation between HBG A binding and norovirus infection (17), a recent human volunteer challenge study on the Snow Mountain virus has challenged this conclusion (Christine Moe, personal communication). In addition, the strains DSV and VA115 did not recognize any human HBGAs tested in this study. What molecules on the host cell surface serve as receptors for these strains? On the other hand, it has been shown that a swine norovirus does not interact with either human and pig saliva (Tibor Farkas, unpublished results). Thus, these are the fundamental questions related to the origin, species specificity, and host range of noroviruses that need to be addressed in the future.

ACKNOWLEDGMENTS

The research described in this article was supported by the United States National Institutes of Health (National Institute of Allergy and Infectious Diseases [ROI AI37093-7] and National Institute of Child Health and Human Development [HD13021]) and by the Department of Defense (PR033018).

We thank Kim Green for providing the recombinant capsid protein of Hawaii virus and DSV, Richard Williams for providing the clinical specimens from the Parris Island outbreak investigation, and Yang Bai for his helpful scientific discussion of the contents of the manuscript.

REFERENCES


