MAJOR ARTICLE

Noroviruses Bind to Human ABO, Lewis, and Secretor Histo–Blood Group Antigens: Identification of 4 Distinct Strain-Specific Patterns

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We characterized the binding of 8 Noroviruses (NORs) to histo–blood group antigens (HBGAs) in human saliva using recombinant NOR (rNOR) capsid proteins. Among the 8 rNORs tested, 6 formed viruslike particles (VLPs) when the capsid proteins were expressed in insect cells, all of which revealed variable binding activities with saliva; the remaining 2 rNORs did not form VLPs, and the proteins did not bind, or bound weakly, to saliva. Four distinct binding patterns were associated with different histo–blood types, defined by Lewis, secretor, and ABO types. Three patterns (VA387, NV, and MOH) recognized secretors, and 1 pattern (VA207) recognized Lewis-positive nonsecretors. The 3 secretor-recognizing patterns were defined as A/B (MOH), A/O (NV), and A/B/O (VA387) binders. Oligosaccharides containing the Lewis and ABH antigenic epitopes were involved in binding. Our findings suggest that different strains of NORs may recognize different human HBGAs on intestinal epithelial cells as receptors for infection.

Noroviruses (NORs), previously known as Norwalk-like viruses, are a group of morphologically similar but genetically and antigenically diverse viruses that mainly cause acute gastroenteritis in humans. Genetically, NORs belong to 1 of 2 genera, the NORs and Sapoviruses (previously known as Sapporo-like viruses), of human caliciviruses. The NOR genus can be divided into 3 genogroups. Each genogroup can be further divided into genetic clusters; at least 15 genetic clusters of NORs have been identified [1]. Since the molecular cloning of Norwalk virus (NV) and several NORs and the subsequent development of new diagnostic assays, NORs have been recognized as the most frequent cause of nonbacterial epidemics of acute gastroenteritis in both developed and developing countries, and they affect individuals of all ages.

The prototype NV was discovered during the early 1970s, but, because of a lack of cell culture and animal models to study the virus, many aspects of NORs, such as host specificity, immunology, pathogenesis, and viral replication, remain unknown. Although limited studies on human volunteers have shown that the antibody response to NV challenge is protective against subsequent infection [2], other studies have found that preexisting antibodies against NV are not protective [3, 4]. Even more puzzling was that some individuals with high levels of antibody against NV were more susceptible to the virus than were individuals who did not have the antibody [3, 5] and that some individuals who did not have antibody against NV could not be infected by challenge with NV [6]. Furthermore, familial clustering of susceptibility to NV illness was observed in individuals exposed to contaminated water [7]. These observations suggested that, in addition to acquired host immunity, there might be a genetic predisposition toward infection with NOR [3].

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The search for host genetic factors associated with NOR infection was not possible until the cloning of NV during the 1990s [8, 9]. NORs encode a single capsid protein that self-assembles into viruslike particles (VLPs) when the recombinant capsid protein is expressed in baculovirus-infected insect cells [10, 11]. These VLPs are morphologically and immunologically indistinguishable from the authentic viruses found in human feces. The suggestion that NORs may recognize a receptor in intestinal cells was based on the ability of recombinant VLPs of NV to bind to and subsequently be internalized into CaCo2 cells, a human colon carcinoma cell line [12, 13].

A genetic basis of host specificity for NORs was suggested recently by our studies on the role of histo–blood group antigens in calicivirus infection. The rabbit hemorrhagic disease virus, an animal calicivirus of the *Lagovirus* genus, specifically binds to the H type 2 antigens of rabbit epithelial cells [14]. Furthermore, NV binds to human histo–blood group antigens present on intestinal epithelial cells and in the saliva of secretors but not of nonsecretors [15]. A recent study of human volunteers showed that susceptibility to NV infection is associated with the ABO blood type [16]. The results of these studies suggest that NV might recognize histo–blood group antigens as receptors for infection.

NV belongs to 1 of 15 known genetic clusters of NORs. To determine whether other clusters also have the same host specificity, we characterized the recombinant capsid proteins of 8 strains representing 7 genetic clusters [10, 11, 17, 18] for their binding to histo–blood group antigens in human saliva. The results expand our previous conclusion that, in addition to the secretor epitopes, the AB and Lewis histo-blood epitopes also are involved in NOR binding. Four binding patterns are described, and NV represents only one of them. These results indicate that the genetic polymorphism of human histo–blood group antigens may be associated with differential susceptibility to infection of different NOR strains. The significance and impact of this finding are discussed.

MATERIALS AND METHODS

Collection of saliva samples. A total of 81 saliva samples were collected from 51 European American, 14 African American, and 16 Asian American healthy adults. A total of 5–10 mL of saliva was collected by allowing each individual to spit into a sterile 50-mL centrifuge tube. The sample was processed immediately after collection. Except for sex and race, no other personal information was collected. The study was approved by the Institutional Review Board of Cincinnati Children's Hospital Medical Center.

The results of the European Americans are presented, because the genetic characteristics of this population are well established and because a comparable distribution of histo–blood types was found between our study and the published literature. The results of the other 2 populations as a whole are not presented because of the small number of subjects in each group and the need to confirm histo–blood types for some individuals. For example, we observed high rates (43%) of Le^{a+b+} in Asian Americans and of Le^{a-b-} (56%) in African Americans, compared with 11% for both categories in European Americans. However, as described in Results, the saliva samples of 3 Asian and African Americans of blood group type B were included in selected analyses, to have a sample size sufficient for ABO comparisons.

Measurement of recombinant NOR (rNOR) capsid antigen binding to saliva samples. Baculovirus-expressed recombinant capsid proteins of 8 NORs were studied: 3 of genogroup I NORs (NV, C59, and VA115) and 5 of genogroup II NORs (VA207, VA387, GrV, MxV, and MOH) [10, 11, 17–19]. Saliva samples were boiled at 100°C and centrifuged at 10,000 *g* for 5 min, and the supernatant was stored frozen until use. For testing rNOR binding to saliva, microtiter plates (Dynex Immulon; Dynatech) were coated with saliva samples at a dilution of 1:5000 in PBS (pH 7.4). After blocking with 5% dried milk (Blotto), rNOR capsid proteins at 0.4–1.0 μg/mL in PBS were added. The bound rNOR capsid proteins were detected using a pooled guinea pig anti-NOR antiserum (dilution, 1:3333), followed by the addition of horseradish peroxidase (HRP)–conjugated goat anti–guinea pig IgG (ICN) at a dilution of 1:5000. For each step, the plates were incubated for 1 h at 37°C and washed 5 times with PBS. The enzyme signals were detected by the TMB kit (Kirkegard & Perry Laboratories) and then read at a wavelength of 450 nm using an EIA spectrum reader (Tecan), as recommended by the manufacturer.

Determination of Lewis, secretor, and ABO histo–blood types. Saliva samples were diluted at 1:1000 in PBS and then coated onto microtiter plates (Dynex Immulon) overnight at 4°C. After blocking with 5% Blotto, monoclonal antibodies (MAbs) specific to Lewis and ABH antigens were added. MAbs anti-H1, -H2, -Le^x, -Le^y, –type A, and –type B were used at a dilution of 1:100; anti-Le^a was used at 1:300; and anti-Le^b was used at 1:200. After incubation for 1 h at 37°C, HRP-conjugated goat anti–mouse IgG or IgM antibodies were added. After each step, the plates were washed 5 times with PBS. The color reaction was developed and recorded as described above.

The following MAbs specific to human histo–blood group antigen types were used for phenotyping. MAbs BG-4 anti–H type 1, BG-5 anti-Le^a, BG-6 anti-Le^b, BG-7 anti-Le^x, and BG-8 anti-Le^y were purchased from Signet Laboratories. MAbs BCR9031 anti–H type 2, BCR 9010 anti-A, and BCRM 11007 anti-B were purchased from Accurate Chemical and Scientific. MAbs 7-LE anti-Le^a, 2–25LE anti-Le^b, 19-0LE anti–H type 2, 12.4LE anti-Le^y, and 3–3A anti-A were obtained from Dr. J.

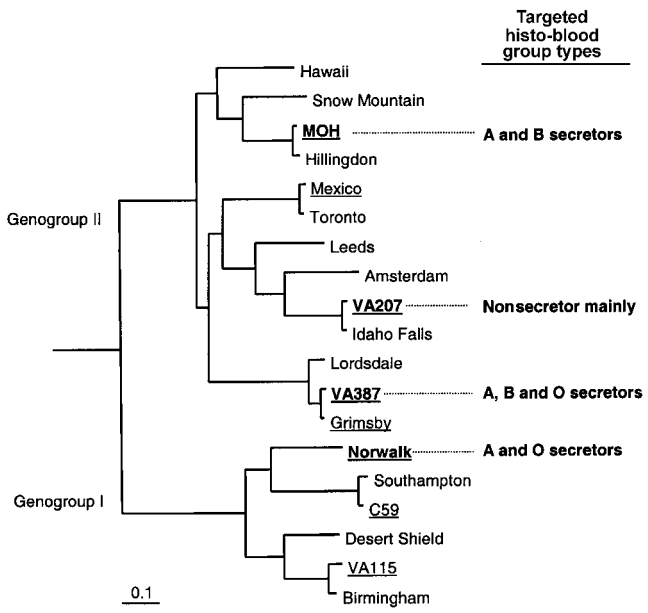

Figure 1. Maximum-likelihood phylogram (DNAML; Phylip version 3.52c) of Norovirus (NOR) capsid genes. The GenBank accession numbers of the 8 strains *(underlined)* tested for saliva binding are Mexico (U22498), MOH (AF397156), Norwalk US (M87661), C59 (AF435807), 115 (VA115, AY038598), 207 (VA207, AY038599), and 387 (VA387, AY038600). Other reference strains include Amsterdam (AF195848), Desert Shield (U04469), Grimsby (AJ004864), Hawaii (U07611), Hillingdon (AJ277607), Idaho Falls (AY054299), Leeds (AJ277608), Lordsdale (X86557), Snow Mountain (U75682), Southampton (L07418), and Toronto (U02030). The representative strains of the 4 binding patterns determined in the present study are in bold type. Strain VA207 mostly binds to nonsecretors but also binds to secretors.

Bara (INSERM U482, Villejuif, France). MAb ED3 anti-B was obtained from Dr. A. Martin (CRTS, Rennes, France).

Blocking of NORs binding to saliva samples by MAbs against human histo–blood group antigens. The same conditions of NOR/saliva EIAs were used, except that a step of incubation with MAbs was included. MAbs specific to the Le^a, Le^b, Le^x, and Le^y antigens described above were added onto the plate at a concentration of 1:5 or 1:10 with serial 2-fold dilutions. rNORs (rNV, rVA387, rVA207, and rMOH) were added at 0.4–0.6 μg/mL, and the captured rNOR capsid proteins were detected by the second antibodies and the HRP conjugates, as described above. A blocking of binding by a MAb was determined by a comparison of optical density values between wells with or without incubation with the MAb.

Binding of rNORs to synthetic oligosaccharides containing human histo–blood group antigen epitopes. Microtiter plates were coated with A-trisaccharide–bovine serum albumin (BSA; GalNAc α1–3 [Fuc α1–2] Gal β-O-space) n-BSA and B-trisaccharide-BSA (Gal α1–3 [Fuc α1–2] Gal β-O-space) n-BSA (Glycorex) at a concentration of 20 μg/mL at 4°C overnight. After blocking of the plates with 5% Blotto, rNOR capsid antigens (rNV, rVA387, rVA207, and rMOH) were added at 0.4–0.6 μg/mL. The captured recombinant capsid proteins were detected by the same procedures as those described above.

Binding of rNOR after a treatment of the synthetic oligosaccharides with glycosidases. The types A and B trisaccharide-BSA conjugates (8 μg) were digested with α-N-acetylgalactosaminidase or α-galactosidase (Glyko), respectively, at 37°C for 36 h. The same buffer, containing 100 mmol sodium citrate/phosphate, was used for both enzymes, but acetylgalactosaminidase was at pH 4.0 with an enzyme concentration of 0.13 U/mL, and galactosidase was at pH 6.0 with an enzyme concentration of 0.33 U/mL. After digestion, the enzyme-treated oligosaccharide conjugate was coated onto microtiter plates at a concentration of 20 μg/mL. The plates were incubated at 4°C overnight and then blocked with 5% Blotto. The remaining procedures of the binding and detection of rNORs (rNV, rVA387, rVA207, and rMOH) were done using the same conditions as those described above. The effect of the removal of the sugar residues by the glycosidases in rNOR binding was determined by comparison of samples that were and were not treated.

Detection of secretory IgA against NORs in saliva samples. The EIAs for the detection of antibodies against NORs

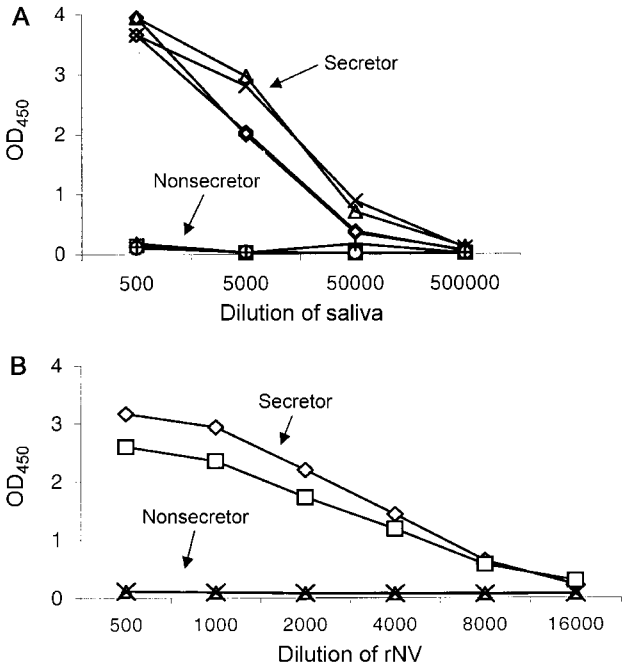

Figure 2. Dose responses of recombinant Norwalk virus (rNV) virus-like particle (VLPs) binding to saliva samples. Linear relationships of binding activities with saliva dilutions from 1:500 to 1:50,000 *(A)* and rNV VLP dilutions from 1:500 to 1:8000 *(B)* were observed using the conditions of EIA described in Materials and Methods. Saliva samples from 4 binders and 4 nonbinders were tested in the experiment shown in panel *A,* and saliva samples from 2 binders and 2 nonbinders were tested in the experiment shown in panel *B.*

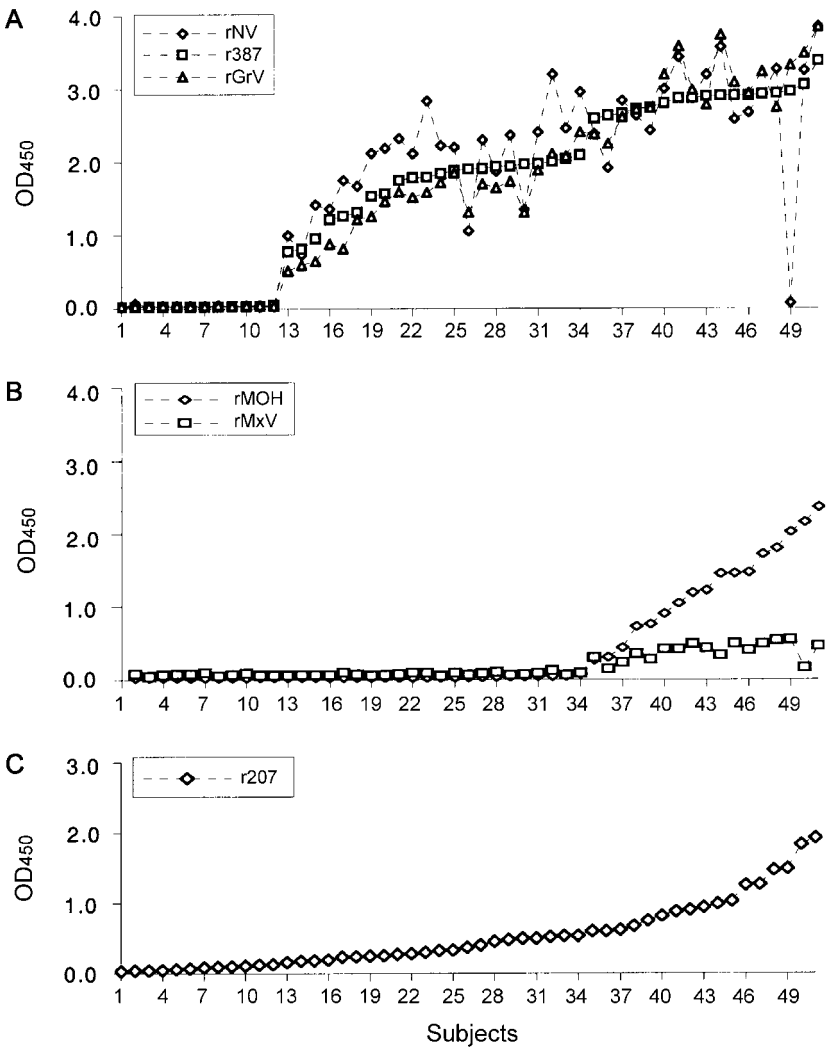

Figure 3. Binding of 6 recombinant Noroviruses (rNORs) to saliva samples from 51 European American volunteers. The optical density values of the 51 saliva samples were sorted from lowest to highest for the strains shown in each panel; therefore, the orders of subjects are different between panels. *A,* rNV (Norwalk virus), r387, and rGrV revealed a similar binding pattern, except for 1 individual (subject 49), whose samples reacted with r387 and rGrV but not with rNV. *B,* rMOH and rMxV revealed another type of binding curve. *C,* The binding curve of rVA207 was significantly associated with the Le^a concentration in saliva ($P < .001$, data not shown).

in serum samples were used to detect antibodies in saliva samples, according to protocols described elsewhere [10, 11, 18], except that saliva rather than serum samples were tested. Saliva samples used for these assays were not boiled. Recombinant capsid antigens derived from strains VA207, VA387, MOH, and NV were studied.

Statistical analysis. The χ^2 or Fisher's exact test was used to compare antibody titers between binders and nonbinders for individual rNORs. Pearson's correlation coefficients were calculated to examine the correlation between binding factors. To test differences in rVA387 and rVA207 binding of saliva between nonsecretors and secretors of O and A/B blood groups, we used analysis of variance followed by post hoc tests for pairwise comparisons.

RESULTS

NOR binding to saliva is a common phenomenon, but different strains reveal different patterns of binding. Eight rNOR capsid proteins representing 7 genetic clusters in genogroups I and II of NORs were studied (figure 1), 6 of which (NV, VA387, GrV, MOH, MxV, and VA207) revealed clear binding activities at variable levels among the 51 saliva samples tested from European Americans. These 6 rNORs formed VLPs when they were expressed in baculovirus-infected insect cell cultures. The other 2 rNORs did not form VLPs—C59 reacted weakly with some saliva samples, and VA115 did not react with any of the 51 saliva samples studied.

The binding of the 6 rNORs was highly specific: proteins

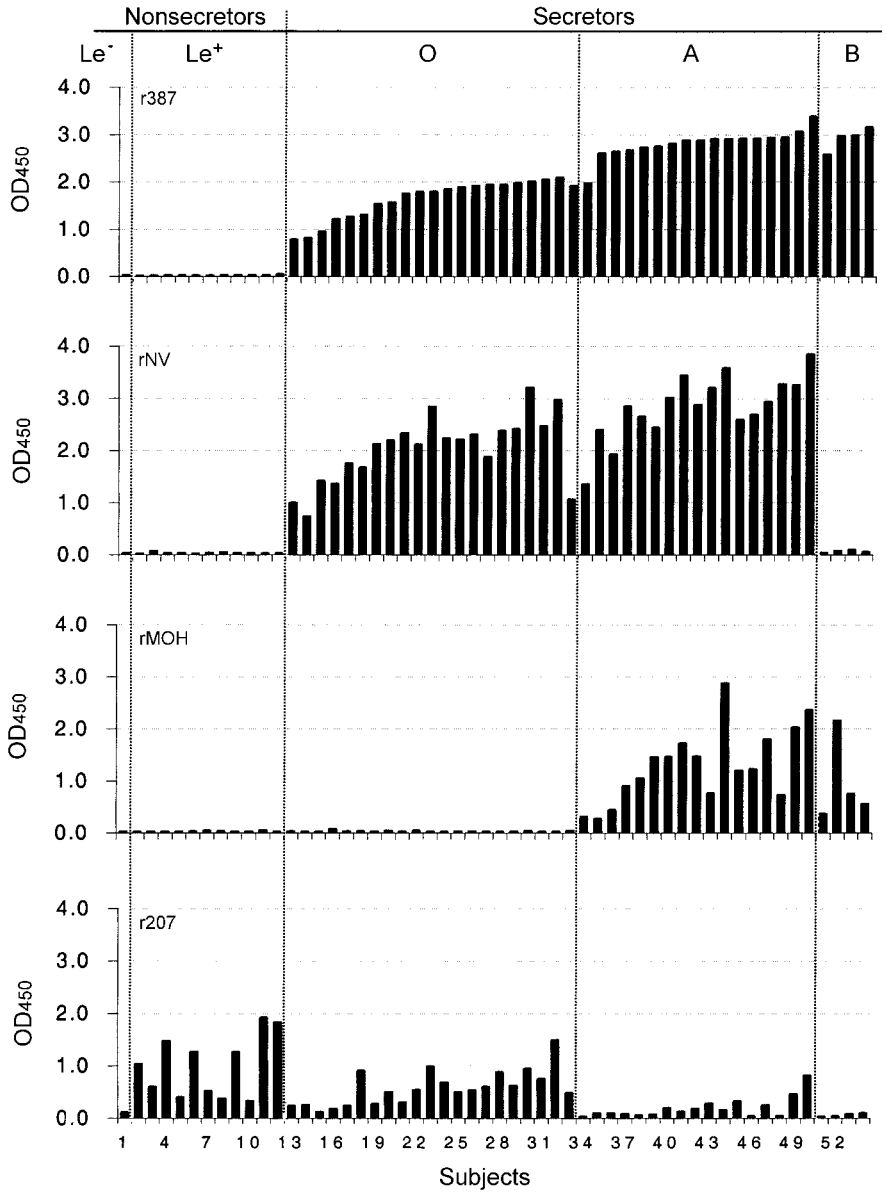

Figure 4. Binding of recombinant (r) Norovirus capsid antigens to saliva samples from 51 volunteers of European descent and 3 volunteers of non-European descent (type B). Saliva samples were tested at a dilution of 1:5000. *Top,* The histo–blood group types of the individuals are shown; *bottom,* subject numbers are shown. The 54 subject were grouped by their histo–blood types, and the magnitudes of saliva binding within each group were sorted by optical density readings from the lowest to the highest to strain VA387. NV, Norwalk virus.

from wild-type baculovirus–infected cells did not reveal any binding activity (data not shown). The observed binding activities were not caused by antibodies present in the saliva samples, because antibodies were inactivated by boiling the saliva samples before testing. When saliva samples were titrated for binding to rNORs by the EIAs, a clear division between binders versus nonbinders was observed at a wide range of saliva concentrations (1:500–50,000) tested (figure 2*A*). Similar dose responses of binding with the rNOR capsid proteins also were found (figure 2*B*). For optimal results, individual rNORs were quantified with a standardized procedure followed by titration that used saliva samples selected from binders and nonbinders for each strain. The final concentrations of the antigens used were in a range of 0.4–1.0 μg/mL.

When the 51 saliva samples were tested at a dilution of 1:5000, a clear separation between binders and nonbinders was observed for strains NV, VA387, GrV, MOH, and MxV (figure 3*A* and 3*B*). Strain VA207 did not reveal the clear division between binders and nonbinders, although a wide range of optical density values (0–2.0) was observed among the 51 saliva samples tested (figure 3*C*). Strains VA387, GrV, and NV revealed similar binding curves and bound to approximately two-thirds

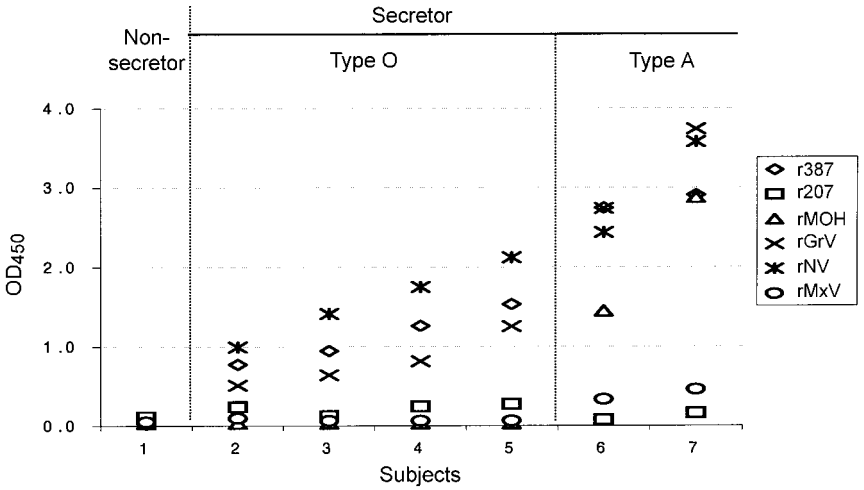

Figure 5. Binding of recombinant (r) Norovirus capsid antigens to saliva samples from Lewis (Le)–negative individuals. These individuals were assigned as Le-negative on the basis of their negative saliva reaction with Le^a- and Le^b-specific monoclonal antibodies (MAbs). Their secretor status was determined by an EIA that used an anti-H MAb. The histo–blood types of the individuals are shown at the top of the figure. NV, Norwalk virus.

of the 51 saliva samples (figure 3*A*), whereas strains MOH and MxV bound to only one-third of the 51 saliva samples (figure 3*B*). The binding patterns of the former 3 strains matched those of MAbs against H types 1 and 2, Le^b, and Le^y, whereas the latter 2 strains matched with MAbs against types A/B ($P <$ .001, data not shown). Strain VA207 did not show correlation with any NOR strain but did with the MAb against Le^a ($r =$ 0.48; $P <$ .001). Thus, the binding activities of all 6 strains revealed an association with certain histo–blood group antigens.

Distribution of Lewis, secretor, and ABO types of the 51 individuals. To further study the relationships of saliva binding with the histo–blood types of the saliva donors, the Lewis, secretor, and ABO types of the 51 individuals were determined. When tested by the MAb-based EIAs, 45 of 51 individuals had a clearly defined Lewis histo–blood type. Six individuals assigned as Le^{a+b+} had relatively high signals for both Le^a and Le^b; they were secretors, and, accordingly, they were grouped together with Le^{a-b+}. Therefore, a total of 11 Le^{a+b-} (22%), 33 Le^{a-b+} (65%; including 6 Le^{a+b+}), and 7 Le^{a-b-} (14%) were assigned, which is consistent with the frequency of 23% Le^{a+b-}, 72% Le^{a-b+}, and 6% Le^{a-b-} among Europeans living in the United States, as has been reported elsewhere [20]. Among the 7 Le^{a-b-} individuals, 6 were secretors and 1 was a nonsecretor, on the basis of their H antigen levels. Thus, among the 51 European American volunteers, there were 12 nonsecretors (24%) and 39 secretors (76%).

Among the 51 European American volunteers typed for ABO, 18 were type A, 1 was type B, 32 were type O, and 0 were type AB. Because the typing was done using saliva samples, the ABO types do not represent the true blood types among the 12 nonsecretors because of the lack of H type 1 or 2 antigens in their saliva as precursors for A or B antigens.

NOR binding to saliva determined by histo–blood types of the saliva donors. When the binding results were analyzed

Table 1. Predicted target products by Noroviruses (NORs) representing 4 major binding patterns, based on the biosynthetic pathways of the major histo–blood group antigen types.

		Secretor		
NOR (genogroup)	Nonsecretor	O	A	B
Blood antigen product	Precursor Le^a	Precursor Le^a; H type 1 Le^b	Precursor Le^a; H type 1; A type 1 A Le^b	Precursor Le^a; H type 1; B type 1 B Le^b
VA387, GrV (G II)	−	+++	++++	++++
NV (G I)	−	+++	+++	−
MOH, MxV (G II)	−	−	+++	+++
VA207 (G II)	+++	++	+/−	+/−

NOTE. Symbols −, +/−, ++, +++, and ++++ indicate the relative optical density values from lowest to highest for individual strains in the saliva–binding EIAs. These values are not comparable between strains. NV, Norwalk virus. For clarity, the Lewis-negative types are not represented on the table. In these types, the Le^a and Le^b antigens are absent. Strain 207 does not bind to saliva samples from such individuals (data not shown).

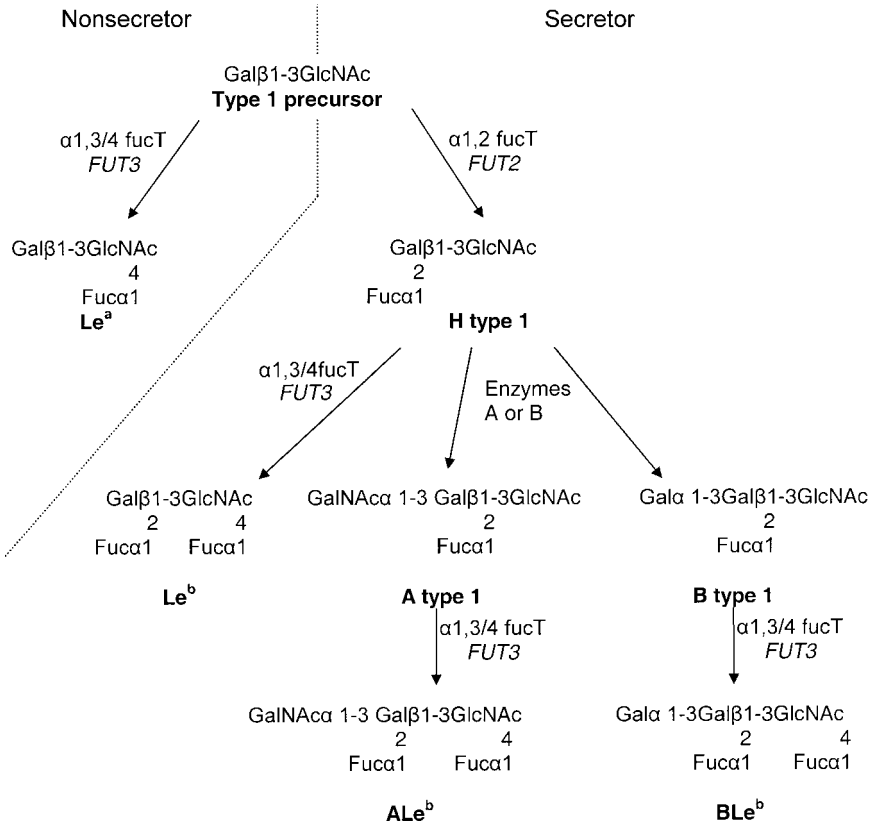

Figure 6. Biosynthetic pathways of human histo–blood group antigens based on H type 1 precursors

with the saliva donors' histo–blood types, a clear segregation of binding and blood types was observed. Figure 4 shows 4 representative binding patterns found in the 6 strains studied. None of the samples from 12 nonsecretors (1 Lewis negative and 11 Lewis positive) but samples from all 39 secretors (6 Lewis negative and 33 Lewis positive) reacted with VA387 (figure 4) and GrV (data not shown). The 6 strains had significantly higher binding activities to saliva samples from types A and B individuals than type O individuals (figure 4, VA387, $P < .001$; GrV, not shown). The binding pattern of NV was almost identical to that of VA387 and GrV, except that 1 secretor reacted with VA387 and GrV but did not react with NV (figure 3, subject 49; figure 4, subject 51). This individual was the only one among the 51 European Americans studied who had blood group type B. To confirm this result, the binding patterns of other 3 available type B secretor individuals (1 Asian American and 2 African Americans) were compared, and all 3 saliva samples from the type B secretors reacted with VA387 and GrV but not with NV (figure 4, subjects 52–54). All 19 MOH binders were type A or B secretors, and 0 of 20 type O secretors and all 12 nonsecretors bound MOH (figure 4). MxV had the same binding pattern as that of MOH (data not shown).

Although strain VA207 revealed a different binding pattern, an apparent 3-step curve was observed when the data were sorted against VA387 (figure 4). This curve was inversely associated with that of VA387 ($r = -0.48$; $P < .001$). Samples from individuals that did not react with VA387 reacted with VA207 strongly; samples from individuals that reacted intermediately with VA387 also reacted with VA207 intermediately; and samples individuals that reacted with VA387 strongly did not react or reacted weakly with VA207. The 3 groups were nonsecretors, type O secretors, and types A or B secretors, respectively.

Effect of the Lewis phenotype on the binding patterns of NORs to saliva. Of the 7 Lewis-negative individuals, 6 were assigned as secretors on the basis of EIA of the saliva samples with anti-H MAbs. Samples from 4 of 6 secretors were type O and bound with intermediate avidity to VA387, GrV, and NV but not MOH; the other 2 were type A and exhibited stronger binding with the 3 strains (figure 5). The 2 type A samples also reacted with MOH and, to a lesser extent, with MxV (figure 5). Therefore, these 6 Lewis-negative secretors had the same binding patterns as the corresponding Lewis-positive secretors. However, samples from 0 of 7 Lewis-negative individuals reacted with VA207. The saliva of the 1 individual that was negative for all 3 blood groups (ABO, Lewis, and secretor) did not bind with any of the 8 NORs studied (subject 1 in figures 4 and 5). This histo–blood type is present in <2% of the population.

In summary of the binding results of the 6 strains, 4 patterns

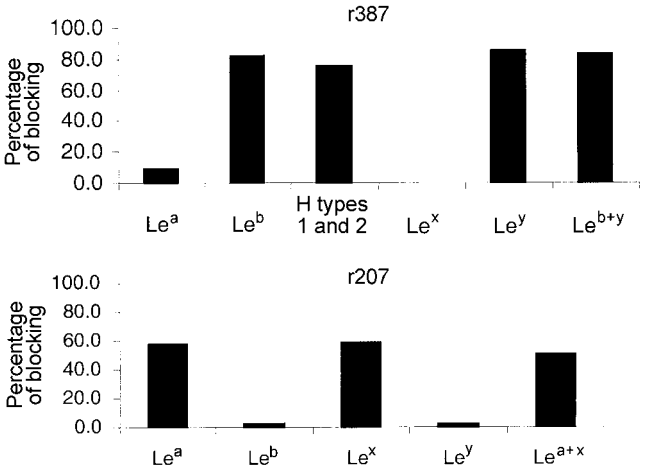

Figure 7. Inhibition by monoclonal antibodies (MAbs) specific for human histo–blood group antigens of recombinant Norovirus binding to saliva. Saliva samples from individuals with corresponding histo–blood types (a type O secretor saliva sample for VA387 and a nonsecretor saliva sample for VA207) were coated to plates, to capture VA387 or VA207 with or without preincubation with MAbs against H type 1 and 2, Le^a, Le^b, Le^x, and Le^y antigens. The MAb against H type 1 and 2 was specific to H type 2 but was also cross-reactive with H type 1 antigens, according to information from the manufacturer. The MAbs were used at a dilution of 1:10, with VA387 at 0.25 μg/mL or VA207 at 1.0 μg/mL. Saliva samples were used at dilution of 1:1000. Specific blocking was determined by the reduction (%) of the optical density values in wells with MAbs compared with wells without MAbs.

of binding defined by the Lewis, secretor, and ABO histo–blood types are proposed (figure 4; table 1): (1) strains VA387 and GrV recognize saliva of all secretors (types A, B, and O), (2) NV recognizes secretors with types A and O but not B, (3) MOH and MxV recognize secretors with types A and B but not O, and (4) VA207 binds to the saliva of nonsecretors as well as secretors, but with lower avidity to the latter, and does not bind to saliva of Lewis-negative individuals.

VA387 recognizes multiple epitopes of histo–blood group antigens in secretors. According to the biosynthetic pathways of human histo–blood group antigens (figure 6), the target molecules of the 4 binding patterns are predictable. VA387 may recognize the α1,2 fucose on the $Le^b$and H type 1 molecules because it reacted with type O and Lewis-negative secretors (table 1). VA387 also may recognize the N-acetylgalactosamine (A) and galactose (B) on the types A and B antigens because it had stronger binding signals in the types A and B individuals than type O individuals (figure 4; table 1). The mean optical density values were 2.8 (SE, 0.8) and 1.6 (SE, 0.9) for A/B and O types, respectively. The difference between the 2 groups was statistically significant ($P < .001$). Direct evidence of VA387 binding to these oligosaccharide-related epitopes was demonstrated by blocking the VA387 binding to a secretor saliva sample by MAbs against H types 1 and 2, Le^b, and Le^y (figure 7) and by binding rVA387 to synthetic oligosaccharides containing the A and B antigens (figure 8); the specificity of these bindings also was confirmed by a removal of the bindings by glycosidases specific to the A and B residues (figure 9). Therefore, the α1,2-fucosyl residue (added by the α1,2 fucosyltransferase encoded by secretor gene *FUT2*) on the H type 1, A type 1, and B type 1 antigens seemed to be the determinant responsible for VA387 binding, whereas the α1,4 fucosyl residue added by the α1,3/4 fucosyltransferase (encoded by Lewis gene *FUT3*) to convert them to Le^b, ALe^b, and BLe^b did not seem to be relevant for VA387 binding.

NV recognizes H and A but not B antigens. The similarity of binding patterns between NV and VA387 suggests that NV may also recognize the H (α1,2 fucosyl residue) and A (N-acetyl-galactosamine) epitopes on the histo–blood group antigens of secretors (table 1). The distinction is that NV recognizes type A but not type B antigens, which was confirmed by the binding of NV to synthetic A but not B antigens (figure 8) and by the loss of the ability to bind to A antigen after the epitope was treated with galactosaminidase (figure 9). The lack of NV binding to type B saliva suggested that the terminal galactose of type B epitope is not sufficient for NV binding. The removal of the B residue by galactosidase did not restore the binding by NV (figure 9), which suggests that the terminal galactose is not simply masking a binding site but that there is a specific requirement for acetylgalactosamine.

MOH recognizes A and B antigens. MOH may recognize the terminal N-acetyl-galactosamine (type A) and galactose (type B) on A type 1, B type 1, ALe^b, and BLe^b molecules (figure 6, table 1) but not the fucosyl residue (neither α1,2 nor α1,4 linkages), because MOH did not react with the type O secretors and Lewis-positive nonsecretors (figure 4). The fact that MOH bound equally well to Lewis-positive (A type 1, B type 1, ALe^b, and BLe^b) (figure 4) and Lewis-negative (A type 1 and B type 1 only) secretors (figure 5; table 1) also suggests that the Lewis

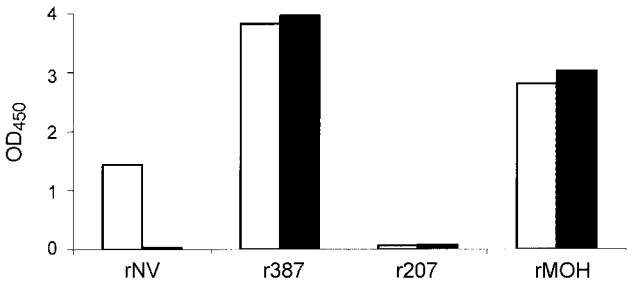

Figure 8. Binding of recombinant Noroviruses (rNORs) to synthetic oligosaccharides containing human blood types A *(white bars)* and B *(black bars)* epitopes. Oligosaccharides conjugated with bovine serum albumin were used to coat the plates. The 4 strains representing the 4 binding patterns of NORs were studied. The same conditions as those of EIAs using saliva samples were done, except that oligosaccharides were used to coat the plates. NV, Norwalk virus.

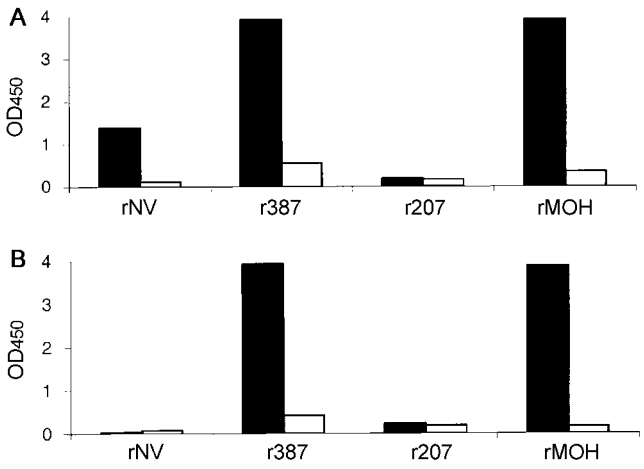

Figure 9. Binding of recombinant Noroviruses (rNORs) to synthetic A and B antigens treated with glycosidases. The conditions of saliva-binding EIAs were used to determine the binding of rNORs to synthetic A and B antigens that were pretreated *(white bars)* or not *(black bars)* with α-N-acetylgalactosaminidase *(A)* or α-galactosidase *(B)*. The decrease of binding due to specific removal of the sugar residues was determined by comparison of optical density values in wells with or without glycosidase treatment. NV, Norwalk virus.

gene (*FUT3,* which encodes α1,3/4 fucosyltransferase) is not involved in MOH binding. Direct evidence of MOH recognizing the N-acetyl-galactosamine (type A) and galactose (type B) residues is the MOH binding to synthetic oligosaccharides containing either A or B epitopes (figure 8); the binding specificity was confirmed by the loss of binding on the enzymatic removal of the N-acetylgalactosamine or galactose from A or B moieties (figure 9).

VA207 recognizes the Lewis epitope (α1,4-fucose). Strain VA207 apparently recognizes the α1,4-fucose residue containing antigen Le^a, because it is the only antigen found in saliva that is produced in Lewis-positive nonsecretors (table 1). Secretors also can produce Le^a, consistent with VA207 binding to saliva samples from secretors. The mean optical density values were 1.0 (SE, 0.2) and 0.6 (SE, 0.1) for Lewis-positive nonsecretors and secretors of O type, and the difference was statistically significant ($P = 0.046$). That VA207 binds with less avidity to saliva samples from secretors relative to nonsecretors (figure 4) may be a reflection of the smaller amounts of Le^a present in the saliva of secretors because of the presence of alternative competing pathways by the α1,2 fucosyltransferase expressed by the secretor gene *(FUT2)*. Variable expression of *FUT2* fucosyltransferase in secretors may account for the lack of a clear demarcation between VA207 binders and nonbinders. Consistent with this interpretation, strain VA207 did not bind to saliva samples from Lewis-negative individuals who lack *FUT3* and thus do not make Le^a (figure 5). This interpretation is supported by the correlation ($P < .001$) between the binding of rVA207 and the Le^a concentration in the 51 saliva samples (data not shown). Direct evidence of VA207 binding to α1,4 fucose was obtained by the ability of anti-Le^a MAb to block the binding by VA207 to nonsecretor saliva (figure 7).

Type 2 precursor–based molecules also are involved in NOR binding to saliva samples. The studies described above focused on the type 1 molecules of human histo–blood group antigens. When the type 2–based histo–blood types of the 51 individuals were analyzed, a clear correlation of H types 1 and 2 was observed, as shown by the parallel binding curves among MAbs against Le^b, Le^y, and H types 1 and 2 ($P < .001$, data not shown) and those against Le^a and Le^x ($P < .05$, data not shown). Direct evidence of type 2 epitope involvement in rNOR binding was obtained by the blocking of VA207 binding to a saliva sample from a Lewis-positive nonsecretor by the anti-Le^x MAb and of VA387 to a saliva sample from a secretor by the anti–H-2 and anti-Le^y MAbs (figure 7).

NOR-specific IgA in saliva samples from NOR binders and nonbinders. Salivary IgA against VA207, VA387, MOH, and NV was measured by type-specific recombinant NOR EIAs developed in our laboratory. Four of 10 VA387 binders had significantly higher levels of salivary IgA against VA387 than did the 10 nonbinders (figure 10), which indicates that binders are more likely to have been infected than nonbinders. The mean salivary IgA value at a dilution of 1:20 was 0.4 (SE, 0.1) for VA387 binders and 0.1 (SE, 0.1) for nonbinders ($P = .02$); the difference in IgA levels between binders and nonbinders also persisted at a higher dilution (1:160, $P = .02$). One of 10 MOH binders appeared to have a higher level of salivary IgA response against MOH than the 10 nonbinders (figure 10). Five of 11 VA207 binders but 0 of 4 nonbinders (Lewis negative) appeared to have higher levels of salivary IgA against VA207 (figure 10). No significant difference in salivary IgA levels for NV was observed between binders and nonbinders (figure 10). Strains VA387, MOH, and VA207 are currently circulating [21], whereas NV is the prototype of NORs that was isolated 30 years ago and is rarely detected today.

DISCUSSION

Four patterns of rNOR binding to human histo–blood group antigens were observed. Histo–blood group antigens are generated by multiple genes and gene products (enzymes) acting on the same antigen precursors, which results in the complex expression of histo–blood group antigens in each individual and a corresponding complexity in rNOR binding to saliva. However, when the binding was grouped by the histo–blood types of the saliva donors, individual binding patterns were clearly related to the specific blood types for all 54 subjects studied, allowing the targeting antigens for each of the 4 binding patterns to be deduced.

The saliva binding experiments were done before the histo–

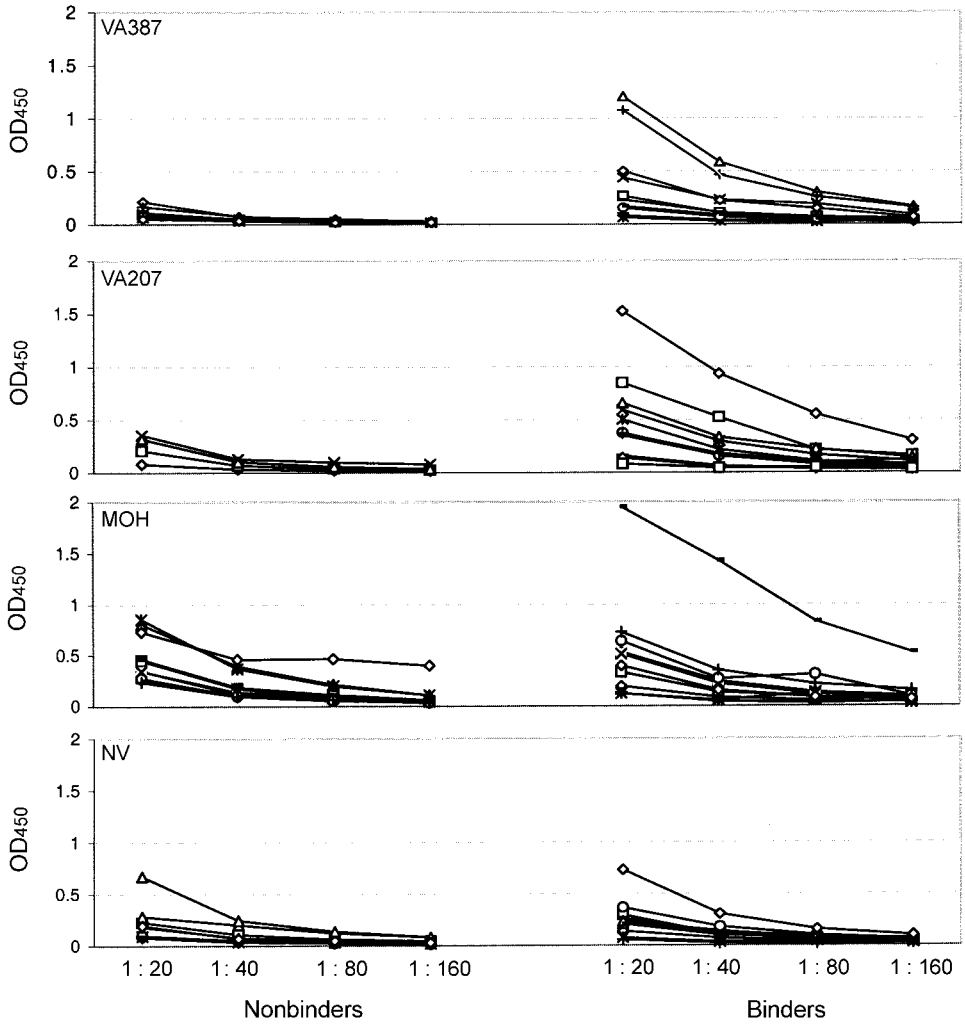

Figure 10. Specific levels of IgA against 4 Noroviruses, measured by direct antigen-coating EIAs in saliva samples from binders and nonbinders. Saliva samples from 10 binders and 10 nonbinders were compared for VA387, Norwalk virus (NV), and MOH IgA. Eleven binders and 4 nonbinders (Lewis negative) were studied for VA207.

blood types of the individuals were determined; thus, the data were generated blindly. The blood typing experiments also were done independently and blindly in 2 laboratories (Cincinnati Children's Hospital, Cincinnati, OH, and Institute de Biologie, Nantes, France), and complete concordance between the 2 sets of results was obtained. The binding signals of rMxV were relatively lower than other strains even when a higher concentration (1:500) of saliva samples was used. We found that the rMxV capsid antigen was easily degraded, compared with other rNOR capsid antigens; thus, a low concentration of VLPs could be the reason for low binding signals.

Deducing the binding targets for VA207 and MOH is relatively straightforward, because they seem to be binding to the products of a single gene family. VA207 apparently binds to Lewis epitopes containing α21,3/4 fucose in Lewis-positive individuals, regardless of their secretor status and ABO type. However, the avidity of binding to saliva samples from ABO secretors varied and generally was lower than that to saliva samples from nonsecretors, possibly because of the presence of the ABH genes in these individuals that express enzymes that divert substrates into epitopes that do not bind to this strain. Because the Lewis enzyme can act on both types 1 and 2 and possibly on type 3 [15], precursors, products of both type 1 and 2, are expected to be reactive to VA207. In contrast, MOH recognizes the A and B epitopes in both types 1 (A type 1, B type 1, ALe^b, and BLe^b) and 2 (A type 2, B type 2, ALe^y, and BLe^y) molecules whose synthesis is controlled by the A and B enzymes, irrespective of the presence or absence of other sugar epitopes of the Lewis and secretor gene families.

The basis of binding for VA387 and NV is more complicated, because the ligands likely are products of 2 gene families—for example, molecules containing H or A/B epitopes, or both, seem to be recognized by these 2 strains. In type O individuals, the binding likely only involves the secretor epitope (α1,2 fu-

cose residue or H antigen). In types A and B individuals, additional epitopes (types A and B) may also participate in binding, strengthening the avidity or affinity of binding. Thus, VA387 may have 3 binding sites for A, B, and H antigens, and NV may have 2 binding sites for A and H antigens.

The association of binding specificity with the primary sequences of NOR capsid genes remains to be determined. Genetically related strains appear to have similar binding patterns, such as that between VA387 and GrV, which belong to the same genetic cluster and share 98% amino acid sequence identity in the capsid genes. However, viruses within genogroups had different binding patterns, and viruses with similar binding patterns were found in different genogroups (figure 1). For example, NV and VA387 belong to distinct genetic clusters in separated genogroups, but they share common epitopes of H and A antigens. These results raise questions about the biological meaning of the current genetic and antigenic classification of human caliciviruses. Future studies to elucidate the molecular basis for such binding specificity also are necessary.

Several bacterial pathogens are thought to use histo–blood group antigens as receptors, and blood group type has been linked to an increased susceptibility to disease [22–33]. The linkage of histo-blood group antigens with NOR infection has been suggested by the antibody prevalence data described in the present study. Moreover, recent reports of volunteer studies have provided a direct evidence for the involvement of histo–blood group antigens in NV infection. In one study, among 50 volunteers challenged with NV, 0 of 15 nonsecretors became infected, and none of their saliva samples bound NV [34]. The 35 secretors, however, were significantly more likely to become infected after the challenge; and most of their saliva samples bound NV VLPs. This could explain the consistent finding that some of volunteers who do not have preexisting antibodies against NV do not become infected after challenge [4–6]. In another volunteer challenge study, individuals with blood group type B were reported to be less likely infected by NV than those with blood group types A and O [16]. These observations agree with our findings. Although NV does not recognize type B antigen, the fact that some individuals with blood group type B became infected may be due to the residual H antigen in those individuals. Thus, we predict that each NOR may have its own host specificity that depends on combined ABO, secretor, and Lewis phenotypes, in parallel to what we have observed with the saliva-binding assays, and saliva binding may serve as a biomarker for the risk of NOR infection.

The current epidemiological features of the other 3 NORs also support the association of histo–blood group antigens with infection. The VA387-like strains, which belong to the Lordsdale cluster, are globally dominant, causing 50%–90% of all NOR-associated outbreaks of acute gastroenteritis in the United States and several other countries [35–37]. Our data suggest that VA387 has the ability to infect any secretor, which includes ~80% of European-derived populations. The MOH-like strains, which our data indicate would have a narrower host range, are not as epidemiologically prevalent as the VA387-like strains. MOH was isolated in a family outbreak that involved almost all of the family members [17]. In summary, we predict that NORs may infect >98% of European-derived populations, but not all blood types should be susceptible to all NORs.

This prediction may explain why some individuals who have a high level of antibodies against NV are more susceptible to the virus than are individuals who do not have the antibody [3, 5]. These individuals could be types A and O secretors. Although NORs are antigenically diverse, cross-reacting antigenic epitopes exist among different strains of NORs [21, 38]. Cross-reacting antibodies may not be protective against subsequent infection with a different strain. Therefore, the high level of preexisting antibodies in these individuals could be a marker of previous exposure to antigenically related strains that share common receptors, but not common neutralization epitopes, with NV.

Defining the target histo–blood group antigens and binding specificity for each strain of NOR could potentially lead to the development of synthetic antiviral compounds. The saliva-binding assays described in the present study, which are highly specific and simple, could be used to search for naturally occurring antiviral compounds. The sequences of the capsid genes of the studied strains and the 3-dimensional structure of the prototype NV are known and could be used to model ligand-receptor interactions and facilitate antiviral drug design.

The relationship between the expression of specific histo–blood group antigens among individuals and differential host specificity for specific NORs implies that NORs could be cultivated in vitro if the host cells are matched for compatibility with the strains to be cultivated. Susceptible cell lines also could be generated by transfection with human histo–blood group genes. Similar transgenic techniques could also prove to be useful in the development of animal models.

Two of 4 calicivirus genera, NOR and *Lagovirus,* bind to specific histo–blood group antigens [14, 15]; the specificity of the other 2 genera *(Sapovirus* and *Vesivirus)* remains unknown. Some animal caliciviruses are genetically related to NORs [39–41], which suggests the possibility that NORs transmit across species. Some histo–blood group antigens are shared among some species; however, at present, there is no direct evidence of the cross-species transmission of NORs.

A growing number of infections by viral and bacterial pathogens have been linked to the presence of specific histo–blood group antigens in the host [22–33]. The present study provides linkage of 3 major histo–blood group antigens (ABO, secretor, and Lewis) with NOR binding. The data strongly suggest that glycoconjugates containing ABH and Lewis antigens are major

determinants for NOR binding. The diversity of these glycoconjugates appears to constitute a first line of defense by humans against rapidly evolving pathogens. It is controlled by multiple glycosyltransferase gene families, many of which contain multiple silent (recessive) alleles, resulting in a highly heterogeneous expression in humans. Meanwhile, the high mutation rate of the NORs results in multiple clusters and strains with diverse abilities to bind to host glycoconjugates. Thus, the study of human NOR infection provides an excellent example of the evolutionary relationships between pathogen and host. The elucidation of these interactions promises to lead to new strategies for the therapeutic control of emerging pathogens.

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