Serological Correlate of Protection against Norovirus-Induced Gastroenteritis

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Background. Norovirus infection is the leading cause of acute nonbacterial gastroenteritis. Histoblood group antigens (HBGAs) are host susceptibility determinants for Norwalk virus (NV) infection. We hypothesized that antibodies that block NV-HBGA binding are associated with protection from clinical illness following NV exposure.

Methods. We developed an HBGA blocking assay to examine the ability of human serum to block the interaction of NV viruslike particles with H type 1 and H type 3 glycans. Serum samples from persons who were experimentally challenged with NV were evaluated.

Results. There was a high correlation between the H type 1 and H type 3 synthetic glycan assays ($r = 0.977$; $P < .001$); the H type 1 assay had higher quantitative sensitivity ($P < .001$). Among 18 infected secretor-positive individuals, blocking titers peaked by day 28 after challenge and were higher for individuals who did not develop gastroenteritis than for those who developed gastroenteritis on days 0, 14, 28, and 180 ($P < .05$ for each). In addition, 6 of 6 subjects without gastroenteritis had measurable prechallenge blocking titers ($>25$), compared with 2 of 12 subjects with gastroenteritis ($P = .002$).

Conclusions. Blocking antibodies correlate with protection against clinical NV gastroenteritis. This knowledge will help guide the evaluation of new vaccine strategies and the elucidation of the nature of immunity to the virus.

Trial registration. ClinicalTrials.gov identifier: NCT00138476.

Human noroviruses are the leading cause of epidemic nonbacterial gastroenteritis worldwide [1] and account for an estimated 23 million cases in the United States annually [2]. No small animal model exists, and human noroviruses have not successfully been grown in cell culture. Instead, researchers have relied on data from natural outbreaks and experimental human challenge studies to examine host immune responses to infection.

Human susceptibility to infection by the prototype genogroup I.1 virus, Norwalk virus (NV), has been linked in recent studies to the genetically determined expression of histoblood group antigens (HBGAs). Approximately 20% of Northern Europeans and Americans of Northern European ancestry lack a functional fucosyltransferase 2 (FUT2) enzyme, which is encoded by the FUT2 gene, and do not express H type 1 or Lewis $b$ (Le$b$) antigens on their mucosae or in secretions; this phenotype is termed nonsecretor or secretor-negative [3–5]. Individuals with blood type B or AB also are less susceptible to NV infection [4]. The development of recombinant expression systems, in which recombinant expression of viral capsid proteins leads to spontaneous self-assembly into viruslike particles (VLPs) [6, 7], has provided reagents to enable the study of virus-cell interactions and host immune responses to infection [8, 9]. These studies have shown that norovirus VLPs interact with a variety of HBGAs, including A, B, H type 1, H type 2, H type 3, Lewis $y$, Lewis $x$, and $Le^a$ [10–14].

Previous volunteer studies have yielded conflicting
evidence of a protective immune response to NV infection. Although studies showed short-term protection to experimental infection [15, 16], long-term immunity has been difficult to elucidate. When a group of volunteers were experimentally rechallenged with NV 27–42 months after the initial challenge, they were not protected against illness despite having developed serum antibodies against NV [17]. Another report showed that high levels of preexisting serum NV antibodies did not protect against infection [18]. These early studies were limited by the use of enzyme-linked immunosorbent assay (ELISA) to measure NV-specific binding antibody levels, because methods were not available to measure NV-neutralizing antibodies. More recent studies have used blocking assays as a surrogate for NV virus-serum neutralization [19–22], in which serum antibodies block the binding of NV VLPs to HBGAs. These studies reported that although all of the subjects tested had preexisting anti-NV antibodies as detected by ELISA, only 20%–30% had preexisting blocking antibody titers. Following NV challenge, 90%–100% of the subjects developed blocking titers [19, 20]. Although these studies evaluated HBG A blocking titers in samples from individuals with experimental and natural infections, none of the studies tested for a correlation between blocking titers and clinical outcomes. The purposes of this study were to optimize an assay to measure antibodies that block the binding of VLPs to HBGAs and to determine whether the presence of antibodies correlates with protection against disease.

**MATERIALS AND METHODS**

**Volunteer study.** NV challenge studies were conducted from September 2004 through March 2008 as described elsewhere [23]. The clinical protocol was reviewed and approved by the institutional review board at Baylor College of Medicine. After providing informed consent, healthy adults (age, 18–50 years) received an oral inoculation of either live NV at a range of doses (4.8–4800 reverse-transcription polymerase chain reaction [RT-PCR] units) or placebo. Serum samples were collected before inoculation (day 0) and on days 2, 7, 14, 28, and 180 after inoculation. All stool samples were collected for 21 d following challenge. Clinical signs and symptoms were evaluated every 4 h after inoculation for up to 96 h. NV infection was defined as excretion of virus in stool (detection of antigen or virus by ELISA or RT-PCR, respectively) or a ≥4-fold increase in serum antibody titer (from before inoculation to 28 d after inoculation) as detected by ELISA. Viral gastroenteritis was defined as either 1 episode of vomiting plus 1 other symptom (abdominal cramps or pain, nausea, bloating, loose feces, fever of ≥37.6°C, myalgia, or headache) or moderate diarrhea alone (watery feces of ≥200 g) for any continuous 24-h period.

The intent of the study was to enroll secretor-positive individuals, based on previous observations that nonsecretors were universally resistant to experimental infection with NV [3, 5]. The presence of HBGAs in saliva samples was determined by detection of A, B, Lewis α (Leα), or Leβ glycans in saliva samples by means of ELISA with the use of monoclonal antibodies against A (Immucor), B (Immucor), Leα (Immucor), and Leβ (Immucor). Persons who had A, B, or Leβ antigens in their saliva samples were identified as secretor-positive, whereas persons who had no detectable antigens or only Leα antigens were identified as secretor-negative or secretor status unknown (no reliable antibody against H type 1 was then available) and excluded from the study. After all subjects had been enrolled and all study procedures had been completed, the antibody against Leβ was found to cross-react with Leα, falsely identifying secretor-negative (expressing only Leα) persons as secretor-positive. A second anti-Leβ monoclonal antibody (Covance) that was Leβ-specific based on results from saliva samples from persons with known FUT2 genotypes, and Ulex europaeus agglutinin-1 (UEA-1) lectin (Sigma-Aldrich) was used to identify the presence of H type 1 glycans. These reagents were used to reanalyze saliva samples from enrolled subjects, and several subjects were found to be secretor-negative (only Leα antigens were present in saliva samples).

**HBGA blocking assays.** Blocking assays to measure the ability of serum antibodies to inhibit NV VLP binding to H type 1 or H type 3 synthetic carbohydrates were developed and optimized. All reagents and test samples were diluted in 0.1 mol/L sodium phosphate buffer (pH, 6.4) with 0.25% fatty-acid-free bovine serum albumin (Sigma-Aldrich), and all assay volumes were 100 μL. NV VLPs (0.32 μg/mL) for the blocking assays were produced using a baculovirus expression system, as described elsewhere [6], and were incubated with an equal volume of serum that had been serially 2-fold diluted from the starting dilution (1:25) for 1 h at 4°C in LoBind 1.5-mL tubes (Eppendorf). Neutravidin-coated, 96-well microtiter plates (Pierce Thermofisher Scientific) were washed and then coated with 2.5 μg/mL of either synthetic polyvalent Lewis d (H type 1)—polyacrylamide (PAA)—biotin or polyvalent (H type 3)—PAA-biotin (GlycoTech) for 1 h at 22°C. Plates were washed 6 times between each incubation step with 0.1 mol/L sodium phosphate buffer (pH, 6.4). The serum-VLP solutions were added and incubated at 4°C for 2 h. Plates were washed, and then NV-specific rabbit polyclonal serum (dilution, 1:5000) was incubated for 1 h at 4°C, washed, and followed by horseradish peroxidase-conjugated, goat antirabbit immunoglobulin G (dilution, 1:5000; Sigma) for 1 h at 4°C. The color was then developed by adding tetramethylbenzidine peroxidase liquid substrate (Kirkegaard and Perry Laboratory) and stopped after 10 min of incubation at 22°C by adding 1 mol/L phosphoric acid. Optical density (OD) was measured at 450 nm with the use of a SpectraMax M5 plate reader (Molecular Devices). Blank
wells were incubated with buffer instead of serum-VLP, and VLP binding to carbohydrates in the absence of a serum sample was used as a positive control. Results were rejected if OD values for the positive control were outside the range 0.7–1.3. The 50% blocking titer (BT$_{50}$), defined as the titer at which the OD reading (after subtraction of the blank) was 50% of the OD of the positive control, was determined for each sample. A value of 12.5 was assigned to samples with a BT$_{50}$ of <25. A blocking control serum sample was used as another control, with plates rejected if the BT$_{50}$ for the blocking control was >1 dilution above or below the known value of BT$_{50}$.

An assay to confirm the specificity of the blocking was performed using the same protocol as that for the blocking assay, with the following exceptions: after the plates were coated with carbohydrates, serum samples were incubated directly on the plate without first preincubating with VLP. After washing, VLPs were then incubated on the plate and detected as for the blocking assay.

**ELISA to detect NV-specific antibodies and quantitative RT-PCR for NV quantitation.** NV-specific serum antibodies were detected by ELISA with the use of NV VLPs, as described elsewhere [24]. The concentration of the NV genome was determined for each stool sample by quantitative RT-PCR, as described elsewhere [23]. Total virus shedding was calculated by adding the product of the fecal virus concentration and the stool weight for each individual.

**Statistical analyses.** The Pearson correlation was used to compare the H type 1 and H type 3 assays. BT$_{50}$ values were converted to logarithmic values to calculate geometric mean titers, and the Wilcoxon signed rank test was used for the comparison of nonparametric data. The Mann-Whitney U test was used to compare BT$_{50}$ values between groups. The Fisher exact test was used to compare the presence of measurable blocking titers in each group. Fecal virus concentrations for subjects with and those without blocking titers were compared using the Student $t$ test. All reported $P$ values are 2-sided.

**RESULTS**

**Development of H type 1 blocking assay.** Noroviruses and recombinant NV VLPs bind to several HBGAs that are expressed on the surface of human cells, including in the epithelium of the gastrointestinal tract. Although the HBGA H type 3 is expressed within enterocyte mucosa, it is not expressed on the surface of enterocytes; in contrast, H type 1 is expressed on the surface of enterocytes [25]. In addition, H type 1 shows a stronger level of binding to NV VLPs than does H type 3 [22]. Due to the specific expression of H type 1 in the intestinal tract, the strong affinity of H type 1 for NV VLPs, and the observation that binding of the NV VLPs to the gastroduodenal junction correlates with the presence of H type 1 antigen and not with the presence of H type 3 [25, 26], we chose to focus on development of an H type 1 blocking assay.

The performance of the synthetic H type 1 assay was affected by several factors including pH, buffer ionic strength, incubation temperature, NV VLP protein concentration, and HBGA concentration. We found that the detection of VLP binding to H type 1 was optimal with the use of a 0.1 mol/L sodium phosphate buffer, at a pH of exactly 6.4, as the signal-to-noise ratio was lower for other pH levels. At a pH of $\leq 6.4$, the background binding was high, and at a pH of $>6.4$, the detection of binding was poor. The VLP-HBGA interaction was most stable in 0.25% bovine serum albumin in 0.1 mol/L sodium phosphate buffer, at a pH of exactly 6.4, as the signal-to-noise ratio was lower for other pH levels. At a pH of $<6.4$, the background binding was high, and at a pH of $>6.4$, the detection of binding was poor. The VLP-HBGA interaction was most stable in 0.25% bovine serum albumin in 0.1 mol/L sodium phosphate buffer and less stable in phosphate-buffered saline. Signals were higher when incubation steps were performed at 4°C compared with room temperature or 37°C.

The optimal concentration of NV VLPs was determined, and the concentration with the highest quantitative sensitivity for blocking that
yielded an OD signal of ∼1.0 was determined to be 0.16 μg/mL protein. The optimal concentration of H type 1 used for this amount of VLP was determined to be 2.5 μg/mL, with lower concentrations yielding unacceptable interwell variability. Conditions for the H type 3 assay were not as temperature- or pH-dependent as those for the H type 1 assay, but both assays were performed in the same conditions for ease of comparison.

Human serum can contain antiglycan antibodies. Huflejt et al. [27] found measurable levels of antibodies that recognized H type 1 and H type 3 carbohydrates in ∼50% of 103 serum samples from healthy persons. To determine whether the observed HBGA blocking activity was mediated by direct binding of serum antibodies to glycans, we developed a control assay to measure the blocking activity generated by preincubation of the capture glycan with serum prior to addition of NV VLPs. None of the samples tested showed antiglycan blocking activity. Thus, any measurable blocking effect was the result of antibodies binding to the VLP and preventing it from interacting with its carbohydrate ligand.

Comparison of the H type 1 and H type 3 blocking assays. Once the assays were optimized, we compared the performance of the H type 3 blocking assay with that of the H type 1 assay. There was a high correlation between the results from 52 serum samples tested by both assays under the same conditions (r = 0.977; P < .001) (Figure 1A). However, in most cases (50 [96%] of 52 samples) the H type 1 assay was more quantitatively sensitive, achieving higher blocking titers than those achieved by the H type 3 assay for matched serum samples (P < .001; Wilcoxon signed rank test) (Figure 1B). In 2 instances (4%), the H type 1 assay detected blocking activity whereas the H type 3 assay did not.

Validation of the H type 1 assay. To validate the assay, we tested prechallenge (day 0) and postchallenge (day 28) serum samples from human subjects and compared fold increases in blocking titers to total NV-specific antibody titers. All of the subjects who met the definition of infection and had a ≥4-fold increase in total anti-NV-specific ELISA results (18 of 18 subjects) also had a ≥4-fold increase in BT₀₅₀ (assay sensitivity, 100%). Conversely, for those who were not infected (including nonsecretors, individuals with blood type B or AB, or those challenged with placebo), 0 of 16 had a ≥4-fold increase in BT₀₅₀ (assay specificity, 100%). To further validate the assay, we established several internal controls. First, we imposed an acceptable range of OD values for our positive control (VLP in the absence of serum) of 0.7–1.3. As a positive blocking control, we included a pooled laboratory serum sample with a known BT₀₅₀ value on each plate and rejected plates in which the BT₀₅₀ value for the blocking control was >1 dilution above or below the known BT₀₅₀ value. None of plates were rejected on the basis of these criteria.

Evaluation of blocking titers in response to NV challenge. We tested samples from 34 subjects on days 0, 14, 28, and 180 after inoculation of the virus at a range of doses (4.8–4800 RT-PCR units). Of these subjects, 18 secretor-positive individuals became infected and also had a ≥4-fold increase in BT₀₅₀ by day 28 compared with that on day 0, and the majority (14 of 18 subjects) still had a ≥4-fold increase in BT₀₅₀ by day 180 (Table 1) (Figure 2). Blocking antibody titers peaked on day 14 for 6 subjects (33%) and on day 28 for 12 subjects (67%). All of the infected subjects still had detectable blocking titers on day 180. Twelve of the infected subjects had an illness that

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Figure 2. Geometric mean titers (GMTs) for the values of the 50% blocking titer (BT₀₅₀) determined by the H type 1 blocking assay. Among persons infected with Norwalk virus, BT₀₅₀ GMTs were significantly higher (*P < .05; Mann-Whitney U test) at each time point for those with no gastroenteritis (n = 6) compared with those with gastroenteritis (n = 12). Error bars represent 95% confidence intervals. GE, patients whose illness met the clinical definition of gastroenteritis; no GE, patients for whom the clinical definition of gastroenteritis was not met.
Figure 3. Effect of prechallenge serum 50% blocking titer ($BT_{50}$) level (determined by the H type 1 assay) on the development of gastroenteritis. Prechallenge serum samples from the 18 infected subjects were tested; 12 subjects had an illness that met the clinical definition of gastroenteritis (GE) (triangles), and 6 subjects did not have an illness that met the clinical definition of gastroenteritis (no GE) (squares). A, Values of $BT_{50}$. B, Total anti–Norwalk virus (NV) enzyme-linked immunosorbent assay (ELISA) titers. The 2 open triangles in panel A represent detectable values of $BT_{50}$ in the group of patients with gastroenteritis, and their respective anti-NV titers are shown in panel B.

met the definition of gastroenteritis; 6 did not. Blocking titers were higher at all time points for those without gastroenteritis than for those with gastroenteritis, including day 0 ($P = .002$; Mann-Whitney $U$ test), day 14 ($P = .008$), day 28 ($P = .017$), and day 180 ($P = .044$).

**Association of preexisting blocking titers with outcome.**
Analysis of the day 0 samples revealed that the absence of measurable blocking titers (<25) prior to inoculation was associated with the development of clinical gastroenteritis (Figure 3A). All infected secretor-positive subjects challenged with virus who did not develop gastroenteritis (6 of 6 subjects) had measurable blocking titers before inoculation, whereas blocking titers were detected in only 2 of 12 subjects who had gastroenteritis ($P = .002$; Fisher exact test). In contrast, there were no significant differences in ELISA antibody titers between subjects with and those without gastroenteritis (Figure 3B). These findings were confirmed with the H type 3 assay, with the same level of significance between the group of subjects with gastroenteritis and the group of subjects with no gastroenteritis.

Peak fecal viral shedding was higher in persons with no preexisting serum blocking antibodies compared with persons with preexisting blocking antibodies (11.2 vs 10.1 genomic copies/g; $P = .027$; Student $t$ test). Similarly, the total amount of fecal virus shedding after challenge was higher in persons with no preexisting serum blocking antibodies compared with persons with preexisting blocking antibodies (13.4 vs 12.3 genomic copies; $P = .01$; Student $t$ test).

**DISCUSSION**
This study reports for the first time (to our knowledge) a correlation between the presence of prechallenge HBGA blocking antibody titers and protection against clinical gastroenteritis induced by NV infection. Our findings support the hypothesis that HBGA blocking antibodies may be a surrogate method for measuring serum virus-neutralizing antibodies [19]. The blocking assay was used to screen serum samples from human volunteers who were experimentally challenged with NV. We also measured the ability of the serum antibodies to block recombinant NV VLP binding to either H type 1 or H type 3 synthetic glycans and observed a high correlation between the 2 assays. We found that blocking titers peaked by day 28 after challenge and were still elevated on day 180 relative to the prechallenge levels.

The lack of a reliable cell culture system for the prototype NV has hindered the study of specific immunological parameters such as the detection of serum virus neutralization antibodies that are proven correlates of protection for many other virus systems [28]. Previous volunteer studies have yielded conflicting observations of protective immune responses to experimental NV infection [15–18]. The ability of serum antibodies to block the binding of recombinant NV VLPs to their putative HBGA receptors in vitro has been hypothesized to be a surrogate measure for NV-neutralizing antibodies [19]. Although recent studies have evaluated HBGA blocking titers in serum samples from experimental and natural infections [19–22], none of those studies associated blocking titers with clinical outcomes. Our data indicate an association between the presence of blocking titers prior to inoculation and protection against disease (Figure 3). There is also an association between lower virus shedding (peak and total) and the presence of blocking activity. We reported the association between lower virus
shedding and the absence of symptoms of gastroenteritis elsewhere [23].

Many of the subjects tested in our study had measurable preexisting blocking titers, in contrast to results recently reported by Lindesmith et al [20], in which few of the preinoculation samples tested were able to block 50% binding at the minimum dilution tested (1:400). Most likely these different observations are due to technical differences that resulted in increased quantitative sensitivity in our assay, which enabled the detection of blocking titers starting at a lower serum dilution (1:25). Because there was no mention of either the selection criteria for inclusion of volunteers or the clinical data for those selected in the study by Lindesmith et al [20], it is not clear why they found no correlation; however, our results predict that the lack of detectable blocking titers would correlate with a lack of protection from illness. We found that assay pH and temperature were critical factors in order to obtain a sufficient OD value for VLP-HBGA binding. Inclusion of a laboratory standard blocking control to monitor assay variability was also necessary to accurately interpret and validate the results. In addition, it is known that some individuals possess anti-HBGA serum antibodies [27, 29], which would cause false positive results in our assay. This concern led us to develop an assay to test for anticarbohydrate antibodies and allowed us to confidently interpret the blocking titers because none of the volunteer samples tested had anti-HBGA antibodies.

There was a strong correlation for NV VLP–HBGA blocking activity of serum antibodies between H type 1 and H type 3 glycans as assessed by BT 50 levels (Figure 1A). This is expected because NV VLPs bind to terminal fucose and galactose moieties that are present on both H type 1 and H type 3 [30]. Previous studies have suggested that the H type 1 glycan has a greater affinity for NV VLPs than does the H type 3 glycan on the basis of greater in vitro VLP binding to the H type 1 glycan [12] and greater inhibition of VLP binding to gastroduodenal tissue sections compared with H type 3 [25]. Our results are consistent with these data and may explain why the H type 1 blocking assay was more quantitatively sensitive in detecting blocking titers than H type 3 (Figure 1B). H type 1 is also the most biologically relevant HBGA, given its specific expression on the surface of the gut mucosa [25]. Despite the advantages of the H type 1 glycan, H type 3 is currently more easily obtained; therefore, until H type 1 becomes readily commercially available, the use of H type 3 for blocking assays is a reasonable alternative.

The results of this study highlight a number of issues to be addressed in future studies. First, a larger number of test samples is needed to confirm an association between blocking titers and protection from illness. Second, what is the minimum threshold blocking titer that protects from illness? In this study, we evaluated the presence or absence of a blocking titer on the basis of a minimum serum dilution of 1:25. Third, what is the duration of immunity associated with blocking antibodies? We tested samples up to 180 d after challenge, and infection-induced blocking activity was still measurable 180 d after challenge for all infected subjects (Figure 2). Fourth, the interaction between the inoculum dose and the blocking activity needs to be assessed because the protective effect of even high blocking titers may be overcome if individuals are exposed to a sufficiently high dose of the virus. Fifth, do candidate vaccines against NV induce serum blocking activity, and does this activity correlate with protection from illness? Finally, are blocking antibodies cross-reactive with VLPs of other Norwalk-like virus genotypes?

The immune response to norovirus infection is complex, with some reports indicating that high levels of preexisting antibodies as measured by ELISA do not protect against infection, and long-term immunity is not consistently observed [11, 18, 31–33]. However, NV antibody titers measured by ELISA only detect antibodies that bind to VLPs and not functional antibodies; therefore, it is not surprising that ELISA antibodies do not predict protection against future infection and symptomatic illness. In contrast, we have been able to link NV blocking titers with the development of clinical symptoms following experimental challenge, and thus we have identified a potential correlate of protection. This knowledge will help guide the development and evaluation of new vaccine strategies and will help to elucidate the nature of host immunity to the virus.

Acknowledgment

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References