MAJOR ARTICLE

Association of Histo–Blood Group Antigens and Susceptibility to Norovirus Infections

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Background. Noroviruses (NoVs) are the leading cause of viral gastroenteritis in humans of all ages. Challenge studies that used the NoV prototype strain Norwalk virus (NV) have shown that some individuals are not susceptible to infection, suggesting the absence of a receptor. Recent studies have identifie histo–blood group antigens (HBGAs) as possible receptors. Being a nonsecretor and presence of HBGA type B were associated with protection against infection with NV, a genogroup (GG) I NoV.

Methods. In the present retrospective study, we investigated the association between presence of HBGAs and the risk of infection with another NoV belonging to GGI (Hu/NV/I/Birmingham/93/UK). The study was done as part of an investigation of a waterborne outbreak in a group of schoolchildren and of a cohort of healthy adults. The ABH histo–blood group phenotype was determined by use of saliva or serum samples from these individuals.

Results. Presence of HBGA type B was significantl correlated with a lack of susceptibility to infection with GGI NoV and with an absence of antibodies. No correlation was found with GGII NoV. Although the infection rate in nonsecretors was lower, this difference was not statistically significant and several children lacking HBGAs in saliva were found to be infected.

Conclusions. Individuals with the HBGA type B may be protected against infection with GGI (but not GGII) NoVs. The association between susceptibility to NoV infection and being a secretor may be restricted to GGI NoV.

In recent years, Noroviruses (NoVs) have emerged as a leading cause of viral gastroenteritis in humans of all ages. They are transmitted by the fecal-oral route, either indirectly (by exposure to contaminated food or water) or directly (by person-to-person contact). The NoVs are genetically diverse, with >15 genotypes distributed over 3 genogroups (GGI, GGII, and GGIII) [1].

Several volunteer challenge studies of NoVs that used the prototype strain Norwalk virus (NV; 8FIIb) have shown that some individuals remain uninfected even when challenged with high or multiple doses [2–4]. Evidence of protective immunity to NoV infection is controversial; short-term immunity has been observed [3–5], but antibodies do not seem to confer protection against NoV infection [6].

Also, despite the high prevalence of antibodies in the population, NoV infection causes disease in people of all ages. The absence of antibodies to NV (GGI.1) in a group of infection-resistant volunteers suggested genetic nonsusceptibility to NV (e.g., through the absence of a receptor [3, 6, 7], which, so far, has remained elusive). However, recent studies have shown that recombinant NoV viruslike particles (VLPs) from different genogroups (GGI and GGII) display different binding patterns to histo–blood group antigens (HBGAs) present in saliva and expressed on red blood cells (RBCs) and gastroduodenal epithelial cells [8–12]. These antigens are widely distributed in human tissues [13, 14] and represent the terminal part of an oligosaccharide chain linked to proteins or lipids.

The HBGA determinants are carried on 4 main types of precursor saccharide structures. Two enzymes (α1,2-fucosyltransferases [FUT1 and FUT2]) are responsible for synthesis of H antigen from the precursor chain by addition of a fucose group. The H antigen then serves as the substrate for A and B glycosyltransferases, which

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add the A or B epitopes that determine the ABO histo–blood group phenotype. FUT1 preferentially acts on type 2 chains, whereas FUT2 preferentially acts on type 1, 2, and 3 precursor saccharides. Differential expression patterns have been observed for FUT1 and FUT2 in different tissues [14–16]. For example, on the surface epithelial cells of gastroduodenal tissues, the expression of H type 1 HBGA is under the control of the FUT2 gene. Several inactivating mutations have been identifie in the FUT2 gene, which result in the absence of HBGAs in secretions from ~20% of white individuals (termed "nonsecretors") [14, 17, 18]. The H type 1 HBGA was identifie as the main ligand for binding of recombinant NV (rNV) VLPs to gastroduodenal epithelial cells [12] and was, therefore, considered as a possible NoV receptor. Nonsecretors lack the H type 1 HBGA on gastroduodenal epithelial cells, which may explain their nonsusceptibility to NV infection [14].

In addition to the binding to H type 1 HBGA, an association between ABO histo–blood group phenotype and risk of NoV infection and disease has been demonstrated [19, 20]. Presence of HBGA type B was associated with protection against symptomatic NV infection [20]. Similarly, presence of HBGA type A on the subjacent H antigen diminished rNV VLP binding [12]. These observations suggest that presence of an HBGA interferes with the binding of NoV to the H antigen, possibly by partial blocking of the binding site.

The association found with HBGA-ABH has been observed in vitro for several genotypes of NoV. In vivo data on the association between presence of HBGAs, being a secretor, and susceptibility to infection with NoV genotypes other than NV are limited [19].

In the present retrospective study, we investigated whether the association between presence of HBGAs, being a secretor, and susceptibility to NV infection is specifi to the NV genotype or whether it can be generalized to other NoV genotypes within the same or more distantly related genogroups. We studied the association between presence of HBGA-ABH and infection with another GGI virus (Hu/NV/I/Birmingham/93/UK) after natural infection in a common-source waterborne outbreak [21]. The Hu/NV/I/Birmingham/93/UK lineage has been described elsewhere [22] and has caused sporadic outbreaks since it was firs described. It clusters within GGI and exhibits 64.3% amino acid identity with NV in the capsid protein. Also, the association between presence of NoV-specifi antibodies, as a measure of infection history, and the ABO histo–blood group phenotype was studied in a cohort of healthy adults.

SUBJECTS, MATERIALS, AND METHODS

Study samples from persons involved in an outbreak. In June 2002, an outbreak of gastroenteritis occurred in a group of primary schoolchildren after a fiel trip to a recreation ground [21]. Stool samples from these children were obtained and used for detection and typing of virus. Information on clinical symptoms and exposure was obtained through questionnaires. Infection was define as the presence of virus in stool samples and/or the presence of clinical symptoms (diarrhea or vomiting). A recreational water fountain was found to be the source of the Hu/NV/I/Birmingham/93/UK, which was detected in both water samples and materials from patients. Saliva samples were obtained from children who had played in the fountain and were typed for presence of ABH antigens in relation to histo–blood group phenotype and secretor status.

Study samples from a cohort of healthy adults. Detailed information about the cohort will be given elsewhere [23]; it included 212 veterinarians who attended an annual practitioners' association meeting in The Netherlands. Ages ranged from 20 to >50 years (20–29 years, $n = 19$; 30–39 years, $n = 53$; 40–49 years, $n = 76$; and >50 years, $n = 62$), with 80% men and 20% women. Five-milliliter blood samples were collected from these individuals by venipuncture, refrigerated (4°C), and transported to the laboratory, where the serum was separated, heat-inactivated for 30 min at 56°C, and frozen at −20°C within 24 h of collection. Informed consent was obtained from all participants in the present study.

Secretor status phenotyping. The secretor status of the exposed children was determined by testing for presence of H antigen in saliva samples by use of a microplate hemagglutination (HA) inhibition (HI) assay [24]. A total of 50 μL of anti-H lectin was serially diluted (2-fold) in saliva (diluted 1:64 in PBS); 50 μL of a 0.5% RBC solution in saline (blood group type O^-; CLB) was added and incubated for 30 min at room temperature. If present in saliva (in secretors), the H antigen will inhibit HA of O^- RBCs by anti-H lectin. A saliva sample from a known secretor and saline were included as positive and negative controls, respectively. An HI assay result was considered to be positive when the HA titer was reduced by >2-fold, compared with the HA titer for the saline control. A positive HI assay result indicated that soluble H antigen was present in the saliva (secretor).

ABO histo–blood group phenotyping. The ABO histo–blood group phenotype of secretors was determined by use of a microplate HI assay with saliva samples. A total of 50 μL of anti-A and anti-B monoclonal antibodies (CLB) was serially diluted (2-fold) in saliva (diluted 1:64 in PBS); 50 μL of a 0.5% RBC solution in saline (blood group type A_1 or B; CLB) was added and incubated for 30 min at room temperature. Saliva samples with known A, B, or O phenotype and saline were tested in parallel as positive and negative controls, respectively. An HI assay result was considered to be positive, indicating presence of type A and B HBGAs in the saliva, when the HA titer was reduced by >2-fold, compared with the HA titer for the saline control. The HI assay was validated by determining the histo–blood group phenotype of saliva samples from 10

Table 1. Association between secretor status and outcome of exposure to Hu/NV/I/Birmingham/93/UK.

	Outcome of exposure		
Secretor status	Infected	Not infected	Overall
Secretor	20 (83)	2 (40)	22 (76)
Nonsecretor	4 (17)	3 (60)[a]	7 (24)

NOTE. Data are no. (%) of individuals.

[a] Odds ratio, 0.133 (95% confidence interval, 0.019–0.938). $P = .075$, Fisher's exact test.

individuals with known histo–blood group phenotypes, as determined by serum phenotyping, which is described below. No discrepancies were observed. ABO histo–blood group phenotyping of serum from the healthy adult cohort was done by adding 50 μL of serum to an equal volume of a 0.5% RBC solution (blood group type A_1, B, or O^-; CLB), incubating for 30 min at room temperature, and testing for HA.

Detection of NoV-specifi antibodies by ELISA. Serum samples were tested for IgG antibodies to NV (Hu/NV/I/Norwalk/1968/US) and Lordsdale virus (LV; Hu/NV/II/Bristol/1993/UK) by use of direct EIA with recombinant capsid antigens (provided by X. Jiang, Cincinnati Children's Hospital, and I. Clarke, Southampton General Hospital, respectively). Both rNV and recombinant LV capsid proteins were produced in a baculovirus expression system, as described elsewhere [25, 26]. Optimal working dilutions were determined by serial dilution of the antigens, as follows. Microtiter plates were coated overnight at 4°C with purifie recombinant capsid proteins in PBS (50 μL/well); after 3 rinses with 0.05% Tween 20–PBS, the wells were blocked with 5% Blotto (Pierce) in PBS, for 1 h at 37°C. The wells were then washed and incubated for 90 min at 37°C with a 1:100 dilution of serum samples in 1% Blotto in PBS (50 μL/well). After washing, 50 μL of a 1:1000 dilution (in 1% Blotto in PBS) of alkaline phosphatase–conjugated goat anti–human IgG (Sigma-Aldrich) was added to the wells, followed by incubation for 90 min at 37°C. After washing, 50 μL of p-nitrophenylphosphate substrate (Sigma-Aldrich) was added to the wells, to a concentration of 1 mg/mL in 0.1 mol/L glycine buffer (pH 10.4), for 30 min at room temperature. The absorption values were measured at 405 nm by use of an ELISA reader (Organon Teknika). Two positive and 2 negative control serum samples were included on every plate.

Previous testing of negative control wells coated with equivalent concentrations of wild-type baculovirus (AcNPV)–infected SF 9 antigen and 120 human serum samples showed no difference in absorption values with PBS; therefore, wells without VLP coating were included as negative controls. A sample was considered to be positive when the net absorbance (optical density [OD]) was greater than the set cutoff (mean + 3 SD of the OD of negative control serum samples).

Statistical analysis. Odds ratios (ORs) and 95% confidenc intervals (CIs) were calculated for blood group and secretor status. HBGA data were analyzed by use of Fisher's exact test (2-tailed).

RESULTS

Waterborne outbreak. A group of 231 children visited an interactive recreational water fountain. Information on clinical symptoms and presence of virus in stool samples was available for 29 children exposed to virus by playing in the water fountain, and saliva samples were collected from all of the children. Of the 24 children in whom NoV infection was diagnosed, 20 (84%) received the diagnosis on the basis of detection of virus in stool samples, whereas the remaining 4 children received the diagnosis on the basis of clinical symptoms.

Association of secretor status and outcome of infection. No H antigen was detected in the saliva samples from 7 (24%) of 29 children. Clinical symptoms combined with presence of virus in stool samples confi med infection in both secretors ($n = 20$) and nonsecretors ($n = 4$) (table 1). All 4 infected nonsecretors received a diagnose of NoV infection on the basis of detection of virus in stool samples. Although not significant there was a trend showing that nonsecretors were less likely to become infected with Hu/NV/I/Birmingham/93/UK (OR, 0.13 [95% CI, 0.02–0.94]; $P = .075$, Fisher's exact test).

Association of blood group distribution and outcome of infection. The histo–blood group phenotype could be determined by use of saliva samples from the 22 children secreting HBGA-ABH. The overall blood group distribution was comparable to the reference frequency in individuals from western Europe (table 2). Presence of HBGA type B was significantl correlated with protection against infection ($P = .004$, Fisher's exact test) (table 2).

Association of blood group distribution and presence of NoV-specifi antibodies. A total of 212 serum samples from a cohort of healthy adults were phenotyped for ABO histo–blood group. Overall, a normal frequency distribution of ABO histo–blood group phenotypes was observed, compared with the reference frequency distribution in individuals from western Europe (figu e 1). No difference in frequency distribution was

Table 2. Distribution of histo–blood group antigens (HBGAs) by outcome of exposure to Hu/NV/I/Birmingham/93/UK.

	Outcome of exposure		
HBGA type	Infected	Not infected	Overall
A	9 (45)	0	9 (41)
B	0	2 (100)[a]	2 (10)
AB	0	0	0
O	11 (55)	0	11 (50)

NOTE. Data are no. (%) of individuals.

[a] $P = .004$, Fisher's exact test.

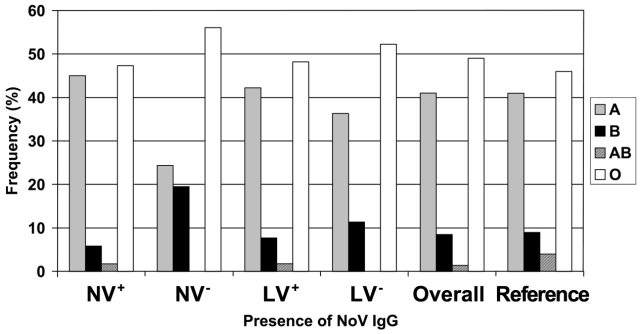

Figure 1. Distribution of ABO histo–blood group phenotypes among Norwalk virus (NV; genogroup I.1)–positive (NV^+) and NV-negative (NV^-) and Lordsdale virus (LV; genogroup II.4)–positive (LV^+) and LV-negative (LV^-) individuals, compared with that among a reference groups of western European individuals. Nos. of individuals are as follows: NV^+, $n = 171$; NV^-, $n = 41$; LV^+, $n = 168$; LV^-, $n = 44$; and overall, $n = 212$. The odds ratio (OR) for acquiring NV-specific IgG with histo–blood group antigen (HBGA) type A was 2.53 (95% confidence interval [CI], 1.19–5.42); the OR for acquiring NV-specific IgG with HBGA type B was 0.26 (95% CI, 0.10–0.66).

observed for LV-positive and LV-negative serum samples or for NV-positive serum samples. However, the frequency distribution of NV-negative serum samples was different, with an increased number of HBGA type B–positive serum samples. Individuals with HBGA type B were significantl less likely to acquire NV-specifi IgG (OR, 0.26 [95% CI, 0.10–0.66]; $P = .0098$, Fisher's exact test). In addition, individuals with HBGA type A were significantl more likely to acquire NV-specifi IgG (OR, 2.53 [95% CI, 1.19–5.42]; $P = .021$, Fisher's exact test).

DISCUSSION

In the present retrospective study, the association between NoV infection and presence of HBGAs was investigated in a group of schoolchildren involved in a waterborne outbreak and in a cohort of healthy adults. In addition, the association between secretor status and susceptibility to Hu/NV/I/Birmingham/93/UK infection was investigated. We have shown that presence of HBGA type B is associated with a lack of susceptibility to Hu/NV/I/Birmingham/93/UK (GGI.3) infection. In addition, presence of HBGA type B was associated with an absence of antibodies against NV (GGI.1) but not against LV (GGII.4).

Recent studies of volunteers have shown a correlation between susceptibility to NV infection, the prototype GGI virus, and presence of antigens of the ABH histo–blood group system [8]. Harrington et al. speculated whether the associations between ABH phenotype and NV infection could be generalized to other NoV lineages and concluded that additional volunteer challenge studies with other NoV strains are needed.

A trend of association between being a secretor and susceptibility to Hu/NV/I/Birmingham/93/UK infection was found, although 4 individuals with a nonsecretor phenotype became infected. This findin suggests that protection from infection in nonsecretors, reported elsewhere for this genogroup [27], may not be complete and that other receptors, possibly H type 3 or 4 (which are expressed independent of the status of being a secretor [28]), are likely to be involved. However, secretor status was determined by phenotyping of saliva samples, which is not as sensitive as genotyping, and the concentration of H antigen in saliva may have been below the limit of detection. Unfortunately, no materials were available for confi mation of secretor status by genotyping [29]. Interestingly, in agreement with the observations of decreased susceptibility to NV infection in individuals with HBGA type B [20], none of the individuals with HBGA type B became infected after exposure to the Hu/NV/I/Birmingham/93/UK strain. Although this findin is based on only 2 observations, it suggests that presence of HBGA type B may interfere with attachment of virus and be a more common property of GGI viruses [8, 20, 27].

Two limitations of the present study are acknowledged. First, diagnosis of NoV infection was not based entirely on the detection of virus in stool samples but also included, for 16% of individuals, diagnosis based on the presence of clinical signs. Although asymptomatic infections do occur in a small number of cases [30] and although we previously found that virus shedding can be missed by testing too early [31], the chance that both occurred at the same time in 1 individual is very low and, therefore, unlikely to have had a significan effect on the present study. Second, even though a response rate of only 13% is low and a selection bias may therefore have occurred for both secretor status and histo–blood group phenotype, the percentage of nonsecretors represented in the present study (24%) is in agreement with the distribution of nonsecretors in the general population (20%). Also, the overall histo–blood group distribution is not significantl different from the distribution in the general population. This suggests that the low response rate did not induce a selection bias.

To investigate whether this association is true for other NoV strains, the association between antibodies and HBGAs was studied. Presence of specifi antibodies indicates that an individual had been infected by that (or a related) virus in the past and is therefore susceptible to that strain. Several studies have shown significan cross-reactivity between different NoV strains, and this cross-reactivity was mainly restricted to NoV strains within a genogroup [32, 33]. By use of the current serological assays, no distinction can be made between antibodies directed against different NoV strains within a genogroup. Therefore, we assumed that presence of antibodies against NV (GGI) or LV (GGII) is indicative of previous infection with GGI or GGII NoV, respectively, and not necessarily the specifi NoV type tested for. This assumption is further substantiated by the presence of antibodies directed against NV (GGI.1). The NV

strain has never been found in outbreaks or endemic infections; however, high seroprevalence against this virus has been found, suggesting that these antibodies are cross-reactive and were produced after infection with a related GGI virus. Since antibodies within a genogroup are cross-reactive, this observation may be true for several genotypes within the genogroup.

Individuals with HBGA type B were less likely to have antibodies against NV (GGI), suggesting that they are less susceptible to NV infection. That these same individuals were not less likely to have antibodies against GGII is in accordance with reports that LV (GGII) can bind to all HBGAs [10]. This difference may, in part, explain why GGI infections are less frequent than GGII infections [34].

Our data show that being a secretor is a correlate of protection against GGI NoV infection, although this is not the sole mechanism in nonsusceptibility. Our finding do support the concept of a decreased risk of infection by another GGI virus when HBGA type B is present, and this findin may be generalized for GGI (but not for GGII) viruses. Further research on the susceptibility and resistance to NoV infections should be conducted by the study of both volunteer challenge experiments and well-documented outbreaks of other NoV strains. It should be noted that future investigations of outbreaks that study the association between presence of HBGAs and susceptibility to infection should account for possible selection biases when multiple family members are involved in the outbreak, since secretor status and histo–blood group phenotype are genetically determined. In addition, both saliva and blood samples should be collected for determination of secretor status and for histo–blood group phenotyping and genotyping.

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