Human susceptibility and resistance to Norwalk virus infection

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Infectious diseases have influenced population genetics and the evolution of the structure of the human genome in part by selecting for host susceptibility alleles that modify pathogenesis. Norovirus infection is associated with ~90% of epidemic non-bacterial acute gastroenteritis worldwide. Here, we show that resistance to Norwalk virus infection is multifactorial. Using a human challenge model, we showed that 29% of our study population was homozygous recessive for the α(1,2)fucosyltransferase gene (*FUT2*) in the ABH histo-blood group family and did not express the H type-1 oligosaccharide ligand required for Norwalk virus binding. The *FUT2* susceptibility allele was fully penetrant against Norwalk virus infection as none of these individuals developed an infection after challenge, regardless of dose. Of the susceptible population that encoded a functional *FUT2* gene, a portion was resistant to infection, suggesting that a memory immune response or some other unidentified factor also affords protection from Norwalk virus infection.

Noroviruses, or Norwalk-like viruses (NLVs), are the leading cause of epidemic acute, non-bacterial gastroenteritis in the United States, resulting in an estimated 23,000,000 infections, 50,000 hospitalizations and 300 deaths per year[1]. These heterogeneous human caliciviruses cause outbreaks in communities, families, nursing homes, schools, hospitals, cruise ships and the military, and are a leading cause of severe gastroenteritis in children[2–7]. NLVs are transmitted through ingestion of feces-contaminated food and water, exposure to contaminated surfaces and direct person-to-person contact.

Although Norwalk virus is highly infectious, volunteer studies have shown that some subjects remain uninfected even after challenge with high doses[8,9]. It is not clear whether these volunteers remain disease-free because of innate resistance or because of pre-existing immunity to Norwalk virus. Human ABH histo-blood group antigens may influence susceptibility to Norwalk virus. An increased risk of Norwalk virus infection was associated with blood group O (ref. 10), and Norwalk virus-like particles (VLPs) bound to gastroduodenal epithelial cells from individuals who were secretors (Se+), but not to cells from non-secretors (Se–)[11]. The gene responsible for the secretor phenotype, *FUT2* (ref. 12), encodes an α(1,2)fucosyltransferase that produces the carbohydrate H type-1 found on the surface of epithelial cells and in mucosal secretions. The form of H type-1 secreted depends on additional glycosyltransferases, including the Lewis, A and B enzymes found in epithelial and red blood cells[13]. Norwalk VLPs bind to H type-1 *in vitro*, supporting this association[14].

The *FUT2* gene is contained in a single 999-bp exon; several inactivating mutations responsible for the non-secretor phenotype have been identified[15]. The G428A nonsense mutation represents >95% of the inactivating mutations found in populations of European and African descent. The attenuating A385T missense mutation is found at high frequency in Asian populations. The attenuating mutations are responsible for the weak secretor phenotype (Se^w). Approximately 20% of Europeans are homozygous recessive for the inactivating G428A mutation and do not make H type-1 (ref. 11). Norwalk VLP binding data suggest that these individuals may be genetically resistant to Norwalk virus infection[11,14].

Previous Norwalk virus human challenge studies have suggested that protective immunity may occur after Norwalk virus challenge, but a specific protective immune component has never been identified. Five volunteers infected with Norwalk virus were all protected from subsequent Norwalk virus challenge 6–14 weeks later, indicating short-term immunity[16]. Long-term immunity has been difficult to prove, as some volunteers initially susceptible to Norwalk virus infection were re-infected 27–42 months later[17]. Interpretation of the results from these early human challenge studies is complicated by small sample size, diagnosis of infection based on symptoms, lack of refined diagnostic reagents to detect virus and virus-specific antibodies,

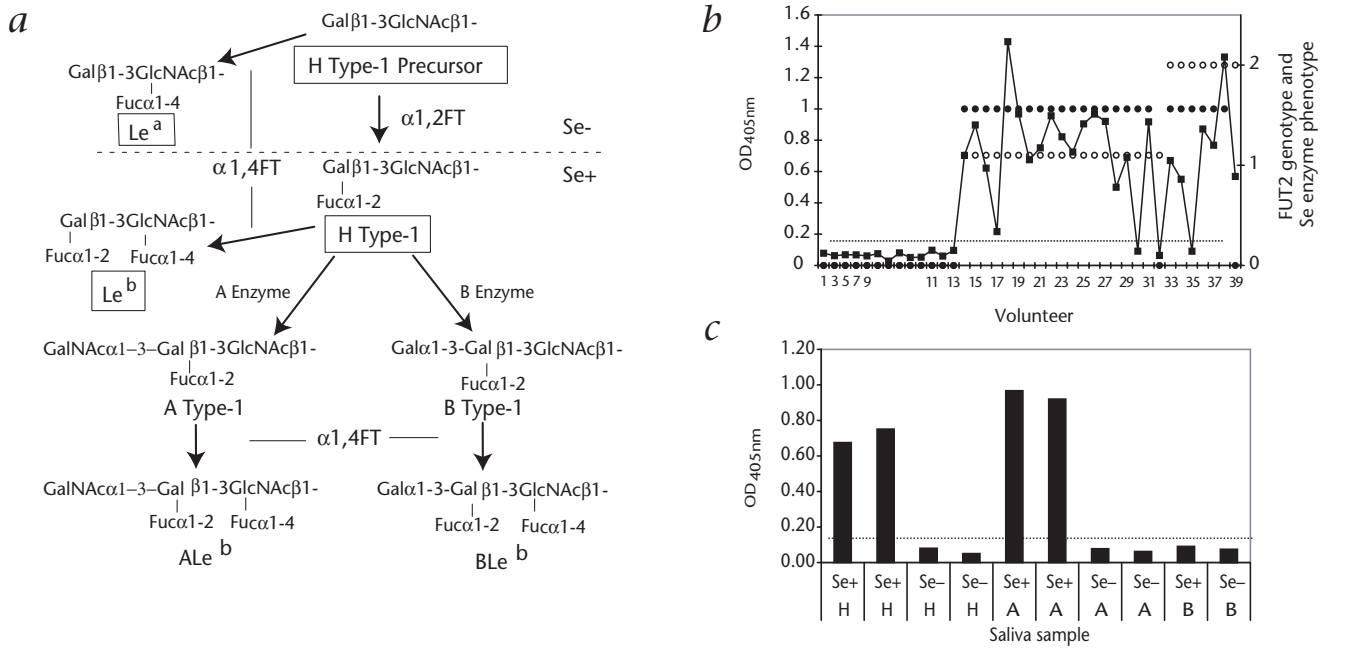

Fig. 1 Norwalk VLPs bind to saliva from Se+ volunteers. ***a***, Biosynthetic pathway for H type-1 and related carbohydrates (adapted from ref. 14). The secretor enzyme, an α(1,2) fucosyltransferase (FT), is encoded by *FUT2*. Certain polymorphisms in *FUT2* block expression of H type-1 and downstream carbohydrates (below dashed line) such as Le^b, A (blood group A) and B (blood group B) antigens. An individual who does not express A or B enzymes is blood group O. ***b***, Saliva samples from 39 of the Norwalk virus–challenged volunteers were secretor phenotyped by EIA (●; 0 = negative; 1.5 = positive), genotyped by PCR (○; 0.1 = $FUT2^{-/-}$; 1.1 = $FUT2^{+/-}$; 2 = $FUT2^{+/+}$), and assayed for ability to bind Norwalk VLPs in an *in vitro* binding assay (■; reported as $OD_{405\ nm}$). Dashed line represents background binding. Data points are connected for visual simplicity, not to denote relationship between points. ***c***, Saliva samples from 5 Se+ and 5 Se– volunteers matched for expression of H type-1 modifying enzymes (none (H), A enzyme (A) or B enzyme (B)) were assayed for ability to bind Norwalk VLPs in an *in vitro* binding assay as in (*b*).

lack of information on previous NLV exposure and cross-strain reactivity, and probable inclusion of volunteers with innate resistance to Norwalk virus.

We analyzed saliva samples collected during two Norwalk virus volunteer challenge studies to investigate the roles of secretor status and acquired immunity in Norwalk virus infection. We found that secretor status is an important predictor of susceptibility and that a memory mucosal immune response may also be associated with protection from Norwalk virus infection.

Infectivity of Norwalk virus

Volunteers received doses of Norwalk virus inoculum ranging from 10 to 3×10^8 PCR-detectable units (PDU), as determined by endpoint titration RT-PCR[18]. Different doses of inoculum were used to reflect the dose variation seen in natural settings and to allow determination of the median infectious dose (ID_{50}) in humans. The study population ($n = 77$) was 49% male, 71% white, 23% black and 6% other races, with an average age of 30 (range 20–49). Demographic characteristics and infection rates did not differ between the inoculum groups. After challenge, 44% of the volunteers developed Norwalk virus infection.

Table 1 Infection[a] among Se+ and Se– volunteers by Norwalk virus dose

PDU NV	≤10^4	10^5	10^6	10^7	10^8	Total
Se+	9/13[b] (69%)	3/6 (50%)	2/3 (67%)	7/14 (50%)	13/19 (68%)	34/55 (62%)
Se–	0/6 (0%)	0/4 (0%)	0/2 (0%)	0/6 (0%)	0/4 (0%)	0/22 (0%)

NV, Norwalk virus. [a]Viral RNA detected in stool ($n = 34$) or a ≥4-fold increase in Norwalk virus–specific serum IgG ($n = 32$). [b]Number of volunteers infected/number of volunteers dosed. PDU, PCR-detectable units.

Secretor type influences Norwalk VLP binding and infection

To investigate the relationship between secretor status and susceptibility to Norwalk virus infection, saliva samples from volunteers were assayed for Lewis a (Le^a), Lewis b (Le^b), A and B antigens by enzyme immunoassay (EIA) and hemagglutination inhibition. Se+ volunteers were identified in 55 samples (71%) containing Le^b, A or B (Fig. 1*a*). Se– volunteers were identified in 22 samples (29%) containing only Le^a. Secretor status in 39 (51%) of the volunteers was evaluated by *FUT2* genotyping of the most common inactivating *FUT2* mutation, G428A. The phenotypes and genotypes agreed for 37 of the 39 (95%) volunteers (Fig. 1*b*). Two volunteers had a Se^w phenotype; one had both the G428A and the attenuating A385T *FUT2* alleles by genotype, and no mutations were identified in the other. Both volunteers were classified as Se+. No samples were typed as Se+ phenotype and Se– genotype, and only 5 of 22 Se– phenotype volunteers were not genotyped.

Norwalk VLPs bound to saliva from 41 (75%) of the 55 Se+ volunteers but did not bind to any of the saliva samples from the 22 Se– volunteers in a Norwalk virus binding assay (Fig. 1*b*)[11,14]. Norwalk VLP binding to saliva was strongly associated with secretor status ($P < 0.01$). Norwalk VLPs bound to saliva from group O and A secretors, but bound much less to saliva from group B secretors ($P = 0.04$; Fig. 1*c*). Eight of the 14 Se+ samples that did not bind Norwalk VLPs expressed B antigen.

Table 2 Association between blood type and Norwalk virus infection by secretor status

Blood type[a]	Se+			Se–			Total (Se+ and Se-)			
	No.	% inf.[b]	RR[d]	No.	% inf.	RR	No.	% inf.	% sympt.[c]	RR
O	28	75	1.56[e]	7	0	ND	35	60	71	1.89[e]
A	19	53	0.79	14	0	ND	33	44	44	0.54[e]
B	7	43	0.66	1	0	ND	8	33	33	0.82
AB	1	0	ND	0	ND	ND	1	0	ND	ND
Total	55	62		22	0		77	44		

No., number of volunteers; ND, not determined. [a]A, B or H (blood type O) antigen expression on red blood cells mediated by *FUT1*. [b]Viral RNA detected in stool or a ≥4-fold increase in Norwalk virus–specific serum IgG (percent volunteers infected per volunteers dosed). [c]Percent infected subjects with vomiting or diarrhea (>2 unformed stools in 24 h). [d]Relative risk of infection between blood groups. [e]Significant RR values ($P < 0.05$), based on Fisher's 2-tail test comparing infection rates between blood groups.

Among Se+ volunteers, 34 of 55 (62%) developed an infection after Norwalk virus challenge (Table 1). At each Norwalk virus dose level, 50–69% of Se+ volunteers became infected. Assignment to dose was randomized and secretor status was not known at the time of challenge. Even though the Se– volunteers were distributed among all dose levels, none of them became infected (Table 1), suggesting that susceptibility to Norwalk virus is highly dependent upon *FUT2* expression ($P < 0.01$). Among Se+ volunteers, blood group O was associated with an increased risk of Norwalk virus infection ($P = 0.04$; Table 2), supporting previous reports[10], but was not associated with an increased likelihood of symptomatic infection ($P = 0.14$). In the whole study population, blood group A seemed to be associated with decreased risk of infection, but this was not significant after controlling for the effect of secretor status ($P = 0.31$).

Norwalk virus immunity

Even at the highest dose, only 68% of the Se+ volunteers became infected, suggesting the existence of additional mechanisms that prevent Norwalk virus infection. Approximately 76% of the Se+, 74% of the Se–, and 89% of the infected volunteers had pre-challenge Norwalk virus–specific serum IgG (data not shown). The risk of infection was higher among all volunteers ($P = 0.02$) and Se+ volunteers ($P = 0.02$) with pre-challenge Norwalk virus–specific IgG.

To examine mucosal immunity, total and Norwalk virus–specific salivary IgA titers were measured in a subset of 50 volunteers for whom there were complete sample sets; the response in infected Se+, uninfected Se+ and Se– volunteers was compared. Saliva from infected (40%) and uninfected (60%) volunteers from all dose levels was included. Demographic characteristics, dose and infection rates were similar to those of the original study population. Norwalk virus–specific salivary antibody titers were normalized for total salivary IgA titers (Norwalk virus–specific IgA/total IgA) to indicate Norwalk virus–specific antibody production and to account for total IgA variation between samples.

Three different patterns of Norwalk virus–specific salivary IgA production were observed ($P < 0.01$; Fig. 2). The infected Se+ volunteers ($n = 20$) showed an increase in antibody production only after day 5 after challenge ($P < 0.01$). For this group, Norwalk virus–specific IgA production peaked around day 14 after challenge with a greater than seven-fold increase above baseline titer (Fig. 2*a*). In contrast, the uninfected Se+ volunteers ($n = 15$) had an increase in Norwalk virus–specific IgA production only during days 1–5 after challenge ($P < 0.05$). On day 3 after challenge, the group titer was approximately two-fold above baseline titer. The increase above baseline was significant on days 2 and 3 ($P < 0.01$). Norwalk virus–specific IgA production remained high through day 5 and returned to baseline by day 8, indicating that there was exposure and a rapid immunological response to Norwalk virus challenge. Daily variation in Norwalk virus–specific salivary IgA was not detected in the group of Se– volunteers ($P = 0.97$; Fig. 2*b*).

The two distinct patterns of change in salivary IgA titers over time identified in the two groups of Se+ volunteers suggests that acquired immunity may explain the difference between the susceptible volunteers who developed infection after challenge and those who did not. None of the individual volunteers with an 'early-only' (days 1–5 after challenge) increase in IgA became infected after challenge ($P < 0.01$). However, only 4 of 15 had IgA increases greater than four-fold and six individuals did not respond, suggesting that protection is multifactorial in the uninfected Se+ volunteers. In contrast, 83% of those individual volunteers who had a Norwalk virus–specific salivary IgA increase after day 5 after challenge became infected, suggesting that the timing of the Norwalk virus–specific mucosal IgA increase is a good predictor of the risk of Norwalk virus infection among susceptible volunteers ($P < 0.01$).

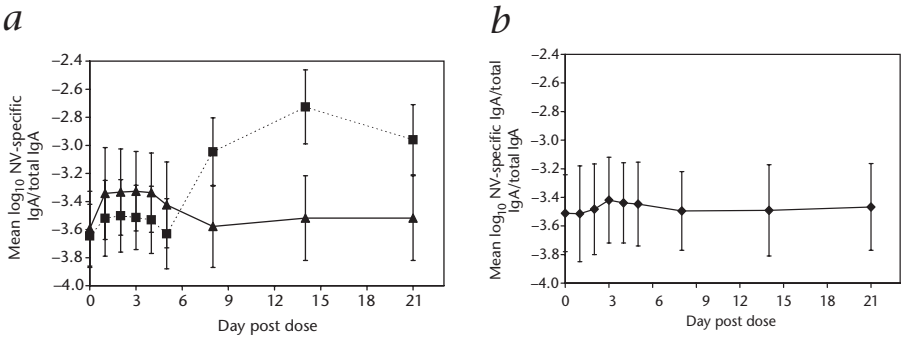

Fig. 2 Norwalk virus–specific salivary IgA response is predictive of infection. Saliva samples were collected from Norwalk virus-challenged volunteers on days 0–5, 8, 14 and 21 after challenge, and assayed for Norwalk virus–specific and total IgA by EIA. The 3 IgA patterns identified were significantly different over the 21 d ($P < 0.01$). ***a***, Log of the ratio of Norwalk virus–specific IgA to total IgA in the group of uninfected Se+ (▲; $n = 15$) and infected Se+ (■; $n = 20$) volunteers. ***b***, Log of the ratio of Norwalk virus–specific IgA to total IgA in Se– volunteers (◆, $n = 15$). Error bars represent 95% confidence intervals.

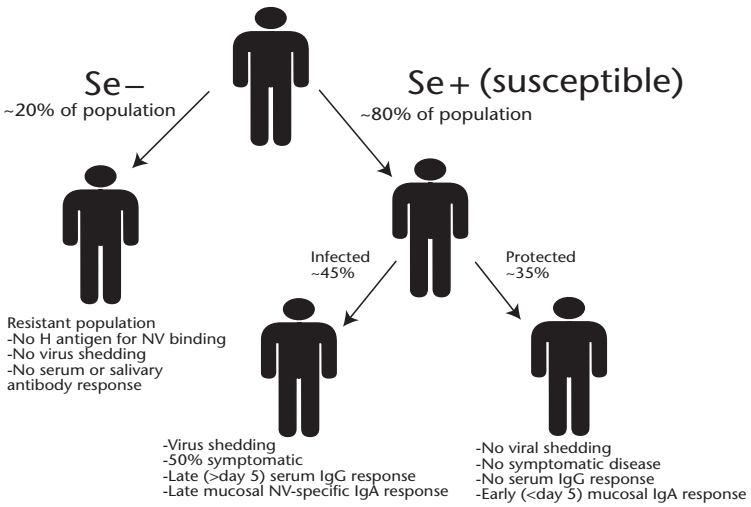

Fig. 3 Model of Norwalk virus challenge outcomes. The percentage of Se–, infected Se+ and protected Se+ individuals is probably influenced by age, race and previous exposure history.

Discussion

NLV-related gastroenteritis is responsible for considerable morbidity worldwide. Earlier reports[9,16,17] have described three responses that follow Norwalk virus challenge in humans: (i) resistance despite repeated challenge, (ii) development of protective immunity, and (iii) susceptibility despite repeated challenge. Our findings are consistent with those reports. The large number of subjects in our study and the application of more sensitive and specific assays to diagnose infection and identify Norwalk virus–resistant volunteers allowed us to suggest at least two mechanisms of Norwalk virus resistance (Fig. 3): (i) innate genetic resistance mediated by inactive *FUT2* alleles and (ii) acquired immunity characterized by rapid Norwalk virus–specific mucosal IgA production in some Se+ volunteers. Both resistance mechanisms seemed to provide complete protection at every dose level studied, suggesting that both mechanisms are dose-independent. Compared to earlier studies, our 44% infection rate probably reflects the high percentage of resistant individuals and the older population used in this study.

Innate resistant alleles that alter virus-receptor interactions have been described for HIV, where homozygous recessive *CCR5* alleles provide some protection against infection and disease progression[19,20]. Human blood group antigens may serve as receptors for *Campylobacter jejuni*[13]and *Helicobacter pylori*[21] that bind to Se+ cells and for uropathogenic *Escherichia coli*[22]and *Staphyloccocus aureus*[23] that bind to Se– cells. Polymorphic ABH genes influence susceptibility to infection with these agents. This study supports the hypothesis that the *FUT2* gene and secretor status are susceptibility markers for Norwalk virus infection. As transfection of the *FUT2* gene enhances Norwalk virus binding to non-permissive cells[11], it is likely that H type-1 or a related blood group antigen is functioning as a receptor for Norwalk virus docking. NLVs are genetically diverse and have different ABH binding patterns[14]. Further studies are needed to determine whether ABH antigens serve as receptors for other NLVs and whether variability in ABH histo-blood group antigen expression influences binding and disease progression in humans.

As Norwalk virus binds to H type-1, H type-3 and Le^b *in vitro*[14,24], it is likely that the available ABH antigens strongly influence Norwalk virus binding and human susceptibility. Our data and that of others suggest that B modifications influence ligand availability, Norwalk virus binding efficiency and susceptibility to infection[10,11,14]. We observed that Norwalk VLPs bound efficiently to O and A antigens but less efficiently to B-antigen saliva specimens. As three B-antigen-positive volunteers became infected, B modifications must not completely prevent Norwalk virus binding. Although the numbers are small, our findings support earlier work suggesting that the B allele may be weakly penetrant against Norwalk virus infection[10]. In agreement with Norwalk VLP binding data, after controlling for the effect of secretor status, blood group A volunteers were no less likely to become infected than volunteers of other blood groups ($P = 0.31$), indicating the importance of controlling for secretor status when considering the effect of other risk factors for infection such as dose and blood group. Group O individuals, however, are clearly more susceptible than group A or B individuals. One possible explanation is that the addition of terminal oligosaccharides to H type-1 may subtly alter or modify Norwalk virus ligand interactions, binding efficiency and infection. Alternatively, the overall availability of ligands that bind Norwalk virus, such as H type-1 or Le^b (refs. 14, 26), may be increased in group O individuals.

Genetic differences in susceptibility to Norwalk virus infection did not entirely explain the different responses to challenge, indicating that susceptibility is multifactorial. In agreement with earlier studies[25,26], we also identified an association between pre-challenge Norwalk virus–specific serum IgG and infection. This paradox was independent of secretor phenotype. Many Se– volunteers had pre-challenge Norwalk virus–specific IgG but they were resistant to infection, suggesting that other NLVs probably infect Se– populations and elicit cross-reactive antibodies against Norwalk virus. Among the Se+ volunteers, we identified two distinct patterns of Norwalk virus–specific salivary IgA increase after infection. Infected individuals showed an IgA response after day 5, corresponding to the timing of a primary immune response. In contrast, uninfected Se+ volunteers who had a rapid increase in salivary IgA, which was resolved by day 8 after challenge, seemed to be immune to Norwalk virus infection. Among these Se+ volunteers, we have not identified a pre-challenge factor that is associated with decreased susceptibility. Nevertheless, most uninfected Se+ volunteers (9 of 15) showed increases in secretory IgA on two or more early days after exposure, suggesting that a memory immune response prevented infection although the exact mechanism remains undefined. The uninfected Se+ volunteers that did not develop mucosal IgA responses may lack some other cellular factor that was critical for virus infection.

Stool specimens from the three-week follow-up period were also tested for fecal IgA. Because of sample degradation, only 40% had detectable IgA; this was insufficient to examine fecal IgA variation over time. For the volunteers with measurable fecal and salivary responses, the patterns were equivalent (data not shown). Because other groups have noted similar difficulties in detecting fecal Norwalk virus–specific IgA[25,27], we suggest that salivary IgA samples are more robust measures of mucosal immunity after Norwalk virus infection.

It is not clear whether the protective immunity described in this study represents short-term or long-term immunity, why some Se+ volunteers developed acquired immunity, what frequency of repeated challenge induces protective immunity, and whether these observations extend to other NLVs. Norwalk virus infection accounted for ~5% of the detected NLV outbreaks in the 1990s[2], suggesting that long-term protective immunity to Norwalk virus is possible, but conclusive proof will require re-challenge studies of the Se+ volunteers. Although additional human challenge studies with other NLVs are needed, our data suggest that NLV vaccines may elicit protective immunity and reduce the substantial disease burden associated with these viruses[27,28].

Methods

Study design. Volunteers (n = 77) were dosed with Norwalk virus 8FIIa[17,26,29,30] or 8FIIb (prepared from the stool of a volunteer infected with Norwalk virus 8FIIa) in 2 randomized, double-blind clinical trials. Stool, serum and saliva specimens were collected before challenge (day 0) and daily during days 1–5, 8, 14 and 21 after challenge. Volunteers were classified as infected if Norwalk virus RNA was detected by RT-PCR in any stool sample after day 0 (ref. 31) or if a 4-fold increase above day 0 Norwalk virus–specific serum IgG titer was detected by EIA[32]. Baseline serum samples with ≥2-fold background binding at 1:100 dilution were considered positive for Norwalk virus–specific IgG. Red blood cell histo-blood group antigen expression (blood type) was determined from serum by the clinical microbiology lab at UNC Hospitals. All study protocols and data collection tools were reviewed and approved by the University of North Carolina School of Medicine Committee for the Protection of Human Subjects. Informed consent was obtained from all subjects before their participation.

Secretor phenotype, genotype and VLP binding. Saliva samples were secretor phenotyped as described[33,14]. DNA was extracted from saliva cell pellets using the QUIamp DNA mini-kit (Qiagen, Valencia, California). A fragment of the *FUT2* gene was PCR-amplified with GAGGAATACCGCCACATCCCGGGGGAGTAC (forward) and ATGGACCCCTACAAAGGTGCCCGGCCGGCT (reverse) primers and digested with *Ava*II. The G428A mutation abrogates this restriction site[34]. Norwalk VLP binding to saliva was determined as previously reported[11,14]. The presence of A and B enzymes in Se– volunteers was inferred from blood type.

Saliva collection and measurement of total and Norwalk virus–specific IgA. Volunteers expelled saliva into sterile tubes. Samples were inactivated for 60 min at 56 °C and pelleted, and the clarified supernatants were stored at –20 °C[35]. Of the 77 sample sets collected, 17 were excluded because 2 or more consecutive samples were missing, and 10 because of poor sample quality (consecutive samples with total IgA titers < 50 μg/ml).

Norwalk virus–specific salivary IgA titers were determined by EIA. Plates were coated with 0.1 μg/well of recombinant Norwalk virus capsid protein[36] diluted in Tris-buffered saline for 4 h at room temperature, blocked overnight at 4 °C in 5% Blotto and Tris-buffered saline before the addition of processed saliva samples diluted 1:4, and incubated overnight at 4 °C. Samples were then incubated for 2 h at 37 °C with alkaline phosphatase–conjugated antibody against human IgA (Sigma Chemicals, St. Louis, Missouri) and Sigma Fast *p*-nitrophenyl phosphate (Sigma). Known concentrations of human IgA (Sigma) were included on each plate to construct a standard curve. Samples below the limit of detection were assigned a titer equal to one-half of this value for analyses[37,38].

Total salivary IgA titers were determined as described, except that plates were coated with 2 μg/ml antibody against human IgA (Sigma) and saliva was added in 2-fold serial dilutions beginning at 1:200. Total salivary IgA was determined as the average value from 3 dilutions within the linear range defined by the standard curve. Fold increase above baseline was defined as the daily (Norwalk virus–specific IgA/total IgA) divided by this ratio on day 0.

Statistical Analysis. General linear model methods appropriate for incomplete longitudinal data were used to represent mean $\log_{10}$ IgA response as a function of group (infected Se+, uninfected Se+ and Se–), day (9 levels), and group-by-day interaction terms. An unstructured covariance matrix was assumed. The model was applied to the total IgA values ($\log_{10}$ μg/ml scale) and then separately to $\log_{10}$ (Norwalk virus–specific IgA/total IgA) to examine differences in temporal variation between the 3 groups. The Fisher 2-tail exact test was used to test the association between risk of infection and other factors. In Table 2, the comparator was A+B blood group volunteers for blood group O, O+B volunteers for blood group A, and O+A volunteers for blood group B. An IgA responder was defined as a volunteer with ≥2 consecutive samples with ≥1.5-fold increase in (Norwalk virus–specific IgA/total IgA) above the baseline ratio. Norwalk VLP binding was compared between blood groups using the Wilcoxon rank sum test. All computations were made using SAS System Software (version 8.1; SAS Institute, Cary, North Carolina) or Epi Info 2002 (Centers for Disease Control and Prevention, Atlanta, Georgia).

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Competing interests statement

The authors declare that they have no competing financial interests.

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