Norwalk Virus Infection of Volunteers: New Insights Based on Improved Assays

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Norwalk virus infection is a common cause of gastroenteritis in humans. The clinical features and virologic and immunologic responses following oral administration of Norwalk virus to 50 volunteers were monitored. New ELISAs using recombinant virus particles as the antigen source were used to assess the pattern of virus shedding and the specific immune responses. Forty-one subjects (82%) became infected; 68% were symptomatic and 32% were asymptomatic. The proportion of subjects infected was similar for those with and without preexisting antibody (82% vs. 60%; $P > .2$). The magnitude of seroconversion was highest in subjects who had vomiting. The peak of viral shedding was between 25 and 72 h, and virus first appeared in stool at 15 h. Specimens collected 7 days after inoculation remained positive. These results show a higher infection rate, more subclinical infections, and longer virus excretion following Norwalk virus inoculation than previously recognized.

Norwalk virus (NV) and the Norwalk-like viruses are important human pathogens that cause epidemic acute viral gastroenteritis [1, 2]. Viruses in this group are spread by the fecal-oral route, and outbreaks of water- and foodborne gastroenteritis are well documented. Previous studies have estimated that at least 42% of outbreaks of nonbacterial gastroenteritis in the United States are caused by NV or Norwalk-like viruses [3].

NV was first described in an outbreak of acute gastroenteritis in Norwalk, Ohio, in 1968 [4]. In a 2-day period during that outbreak, acute gastrointestinal illness developed in 50% of 232 students and teachers of an elementary school, and a secondary attack rate of 32% was observed among family contacts of primary cases. The disease lasted ~24 h and had an incubation period of ~48 h [4]. Although some of the patients had diarrhea, the predominant clinical manifestations were vomiting and nausea [4]. Subsequently, in 1972, virus particles were visualized in fecal material derived from the Norwalk outbreak by immune electron microscopy (IEM) [5]. NV has remained fastidious and noncultivatable in cell cultures and in readily available animal models. Chimpanzees were infected with NV and susceptible animals developed an antibody response, but only a few animals shed antigen [6]. Therefore, information on the pathogenesis, pathology, and immunity of NV infections has come from volunteer studies [2].

Dolin et al. [7] showed that a stool filtrate (called 8FIIa) from an affected adult in the original outbreak in Norwalk, Ohio, could reproduce the disease when administered orally to healthy adult volunteers. In subsequent studies, ~50% of adult volunteers developed illness, with diarrhea, vomiting, abdominal cramps, nausea, headache, myalgia, and fever as the major clinical symptoms. The incubation period ranged from 10 to 51 h (mean, 24) and illness usually lasted 24–48 h [8]. Viral shedding, as detected by IEM, coincided with the onset of illness and usually did not extend >72 h after the first symptoms [9]. Studies in the 1980s included cross-challenges of volunteers with NV and Snow Mountain or Hawaii agents (other Norwalk-like viruses) and sequential challenges of volunteers with NV. Such studies generated important information on the relationships of NV with other Norwalk-like viruses [8] and preexisting immunity with susceptibility to infection [10–12] as well as on the intestinal histopathology of infections [13, 14]. In the later studies, infection rates as determined by antibody increases measured by ELISAs were as high as 75% [15, 16]. Preexisting serum antibody to NV was not associated with protective immunity, and persons with higher levels of preexisting antibody were found to be more likely to experience symptomatic disease in most [11, 12, 16, 17] but not all studies [15].

Volunteer studies also provided reagents (pre- and postinfection serum and stools) for the development of immunologic assays for the detection of NV infection. The first immu-

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Informed consent was obtained from each volunteer. The study protocol adhered to guidelines of the US Department of Health and Human Services and was approved by the Institutional Review Board at Baylor College of Medicine and Methodist Hospital.

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nologic test developed for NV diagnosis was IEM [5]. Subsequently, an RIA to detect NV antigen, a blocking RIA to measure serologic responses to NV infection [12, 18], and ELISAs of different formats were developed to detect both antibody and virus [19–24]. Most recently, a Western blot assay was described for the detection of NV [25].

The application of these assays in volunteer studies and natural outbreaks helped elucidate the epidemiology, natural history, and basic characteristics of NV and NV-like infections. However, the results obtained were based on tests with possibly low and varied sensitivity because of the use of reagents from different adult volunteers with unknown infection histories. Unavailability of sufficient antigen also prevented large-scale epidemiologic or extensive laboratory studies.

Recently, we cloned the NV genome and expressed the viral capsid protein in insect cells infected with a recombinant baculovirus [26, 27]. This protein spontaneously assembles into recombinant Norwalk virus–like (rNV) particles that are immunoreactive when tested with convalescent serum from infected volunteers [27]. Using these highly purified rNV particles, we generated the first high-titered hyperimmune antiserum to NV in animals [27]. This report describes the use of these reagents to develop sensitive ELISAs for detection of NV-specific antigen and serum antibody; we used these ELISAs to analyze the infection and clinical features of volunteers experimentally inoculated with NV.

Materials and Methods

Volunteer studies. Eight volunteer studies were done between July 1985 and January 1990. The study population comprised medical students or staff of the Texas Medical Center (21 women, 30 men; 19–39 years old; 43 white, 6 black, 1 Hispanic, and 1 East Indian). The NV inoculum (8FIIa; supplied by A. Kapikian, Laboratory of Allergy and Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD) originated from the outbreak of acute gastroenteritis in Norwalk, Ohio, and was prepared and safety tested by the National Institutes of Health for administration to volunteers [7, 8]. Two milliliters of a 1:100 dilution of the 8FIIa inoculum in sterile TRIS-buffered saline with 80 mL of autoclaved Milli-Q water (Millipore, Bedford, MA) was orally administered to each subject. Sodium bicarbonate solution was taken by each person 2 min before and 5 min after administration of virus. The virus was administered in the General Clinical Research Center, where subjects were hospitalized and under medical care for 4 days. Before admission and at the time of inoculation, subjects were interviewed and determined to be asymptomatic.

After inoculation, the frequency, weight, and composition (formed, unformed, watery diarrhea) of each stool were recorded and the occurrence of symptoms (abdominal cramps, chills, body ache and headache, nausea, vomiting, and fever) was recorded at 4-h intervals. Symptoms were graded using a five point score, with 0 denoting absence of a symptom and 5 denoting the most severe discomfort associated with a symptom. Individual scores were tabulated for the 72-h period after virus inoculation, and a composite symptom score (maximal score = 35) was determined for each subject. Symptom scores ranged from 0 to 24. Serum samples were collected before and 2 weeks and 1 month after virus administration. Stools were collected after virus administration during the hospitalization period and for 2–3 days thereafter. Preinoculation stools were collected from 22 subjects. A convalescent serum sample was not obtained from 1 subject, who was infected (as determined by clinical symptoms and virus excretion) but is not included in the data analyses herein.

Definitions. NV infection was defined as a ≥4-fold increase in serum antibody titer or excretion of virus. Diarrhea was defined as watery stools (unformed stools were not considered diarrhea). Asymptomatic infection was defined as no vomiting or diarrhea and a symptom score of ≤4 in an infected subject. Symptomatic infection was defined as a composite symptom score of ≥5 in an infected subject. All who vomited or had diarrhea had symptomatic infection.

ELISA to detect NV-specific antibodies. Baculovirus-expressed rNV particles were produced in *Spodoptera frugiperda* (Sf9) insect cells infected at a high MOI (10) with a baculovirus recombinant that contained the 3′ end of the NV genome [27]. Purified rNV particles were used as antigen for the ELISA to detect antibody in human sera. First, 96-well polyvinyl chloride plates (Dynatech, Chantilly, VA) were coated with 100 μL of rNV (1 μg/mL in 0.01 *M* PBS, pH 7.4) for 4 h at 37°C. The antigen in the plates was removed and the plates were blocked with 200 μL/well 5% Blotto (Carnation nonfat milk) in PBS overnight at 4°C. After the plates were washed two times with PBS containing 0.05% Tween 20 (PBS-T) (200 μL/well), test serum (100 μL/well diluted in 1% Blotto-PBS) was added to each well and the plates were incubated for 2 h at 37°C. After six washes with PBS-T, bound antibody was detected by addition of horseradish peroxidase (HRP)–conjugated goat anti-human IgG, IgM, and IgA (Organon Teknika Cappel, West Chester, PA) (1:5000 dilution in 1% Blotto-PBS, 100 μL/well) and the plates were incubated for 2 h at 37°C. After six washes with PBS-T, 100 μL of substrate 2,2′-azino-di(3-ethylbenz-thiazoline-6-sulfonate) (ABTS) was added per well. The plates were kept at room temperature for 30 min before the optical density was determined at 414 nm (Titertek Multiskan ELISA reader; Flow, McLean, VA). An alternate substrate, 3,3′,5,5′-tetramethylbenzidine (TMB, Microwell Peroxidase Substrate System; Kirkegaard & Perry, Gaithersburg, MD), was used in some experiments.

Each test plate included 4-fold dilutions of a positive and a negative control serum (each from volunteer 505). Fourfold dilutions of each serum pair were tested on the same plate. The titer of the positive control was uniform from plate to plate and did not vary by more than one dilution. Four serum pairs initially showed only a 4-fold dilution difference in titer; these increases were confirmed by retesting 2-fold dilutions of the serum pairs.

ELISA to detect NV antigen in stool. A sandwich-type ELISA was developed for the detection of NV antigen. For each sample to be tested, 2 wells of a 96-well polyvinyl microtiter plate were coated with 50 μL of a 1:10,000 dilution of rabbit serum (collected before or after hyperimmunization with puri-

fied rNV) and the plates were incubated for 4 h at room temperature. The plates then were blocked with 5% Blotto in PBS for 1 h at 37°C and washed two times with PBS-T. Test stool sample (50 μL), suspended in PBS and extracted once with trichlorotrifluoroethane (DuPont, Wilmington, DE) before being diluted in 1% Blotto-PBS, was added to each well. After the plates were washed four times with PBS-T (200 μL/well), hyperimmune guinea pig rNV serum (50 μL/well) was added at 1:5000 dilution in 1% Blotto-PBS and the plates were incubated for 2 h at 37°C. The plates were again washed four times with PBS-T, and HRP-conjugated goat anti-guinea pig serum (Hyclone, Logan, UT) was added at a 1:5000 dilution. After another incubation for 2 h at 37°C, the plates were washed a final four times with PBS-T, and 30 min after addition of the substrate (ABTS), the A_{414} of each well in the plate was measured (Titertek Multiskan Plus, Flow). Each test plate included a negative control stool sample and a positive control stool sample with a known titer.

Biotin-avidin ELISA to detect NV antigen in stool. This ELISA was similar to the sandwich-type ELISA described above for the detection of NV antigen in stools, except a biotin-labeled guinea pig IgG purified from the hyperimmune guinea pig serum was used as detector antibody. Briefly, the plate was coated with rabbit or guinea pig hyperimmune serum and stool samples were added. The plate was washed and the captured viral antigen was detected using the biotin-labeled hyperimmune guinea pig IgG followed by addition of HRP-conjugated avidin and substrate ABTS or TMB.

RIA to detect NV antigen in stools. The RIA using volunteer reagents for the detection of NV antigen was based on the method used by Greenberg et al. [18]. Briefly, 2 wells of a 96-well plate were coated with serum (from volunteer 505) collected before or after infection with NV, and the plate was incubated overnight at room temperature. The plate then was blocked with 1% BSA-PBS overnight at room temperature. After the plate was washed twice with PBS, 25 μL of a 10% stool suspension was added and the plate was incubated overnight at room temperature. After the plate was washed five times with PBS, human IgG (purified from volunteer 519) labeled with ^{125}I was added and the plate was incubated for 4 h at 37°C, then washed five times with PBS. Radioactivity was counted using a gamma counter. Positive-to-negative (P/N) ratios of the counts per minute were calculated for the reactivity of each sample with the post- and preinfection serum, respectively. Samples with P/N ratios of ≥2.0 were judged to be positive.

Reverse transcription–polymerase chain reaction (RT-PCR) and dot blot hybridization to detect NV. RT-PCR and dot blot hybridization were done as described [28].

Statistical analyses. Serum titers, expressed as the reciprocal of the dilution factor, were converted to $\log_{10}$ numbers for calculation of geometric means. Student's t test was used for comparison of two means of parametric data and the Wilcoxon rank sum for nonparametric data. Analysis of variance (ANOVA) was used for the comparison of three or more means. The χ^2 test was used for comparison of proportions [29].

Results

Establishment of New Assays to Detect NV Infection

ELISA using rNV to detect antibody responses to NV. An ELISA using rNV as a coating antigen was developed to detect antibody to NV in human serum. Optimization of the test conditions included evaluation of the concentration of rNV to be used as coating antigen and conditions for plate coating, washing, blocking, and concentration of second antibody. Duplicate serial 4-fold dilutions of each serum sample obtained from subjects before or 1 month after challenge were tested in wells with or without antigen. This initial screening showed that the background was very low in wells not containing antigen. To standardize the ELISA and determine the cutoff A_{414} to distinguish the presence from the absence of serum antibody, serum samples from 5 subjects containing different levels of antibody were tested in a blocking ELISA (figure 1).

The reactivity of each sample was tested either directly or after being premixed (incubated for 1 h at 37°C) with rNV antigen before being added to the plates. The reactivities in both the pre- and postinfection sera (except for the 1:10 and 1:40 postinfection samples from volunteer 519) were blocked to an A_{414} of <0.1. The background reactivity of these samples in wells not coated with rNV was similar to that obtained in the blocking experiment in which a maximum A_{414} was <0.1 (figure 1). The inability to block all of the antibody in the postinfection serum of volunteer 519 may have been due to use of an insufficient amount of antigen relative to the high titer of antibody (>1:40,960) present in this serum. As another negative control, uninfected insect cell lysates were used as antigen to coat the ELISA plate. No reactivity of the 5 samples tested was seen with this insect cell antigen (A_{414}, <0.1 at 1:10 dilution). On the basis of these results, a cutoff point of $A_{414} = 0.1$ was used in subsequent tests.

ELISA using hyperimmune anti-rNV sera to detect NV antigen. Two ELISA formats were compared for the detection of NV. The first used the rabbit hyperimmune serum as coating antibody and the guinea pig hyperimmune serum as detector, and the second used the guinea pig hyperimmune serum as the coating antibody and the rabbit hyperimmune serum as the detector. The first format gave lower backgrounds and a higher signal-to-noise ratio than the latter format. In addition, we tested the use of mouse hyperimmune serum and biotin-labeled guinea pig IgG as detector antibodies for the ELISAs. These reagents gave results comparable to those obtained with guinea pig serum. Because of the larger supply of rabbit and guinea pig hyperimmune sera, the format of rabbit hyperimmune serum as the coating antibody and guinea pig hyperimmune serum as the detector was used in this study.

To determine the cutoff for the antigen ELISA, we first tested preinoculation stools from 22 subjects (data not shown). The average A_{414} reading of the 22 stools in the wells coated with hyperimmune rabbit serum was 0.015 (SD, 0.008). Figure 2 shows the distribution of ELISA A_{414} readings of 341 stools from the 50 subjects. All had very low reactivities (A_{414} <0.05) with the preimmune rabbit serum

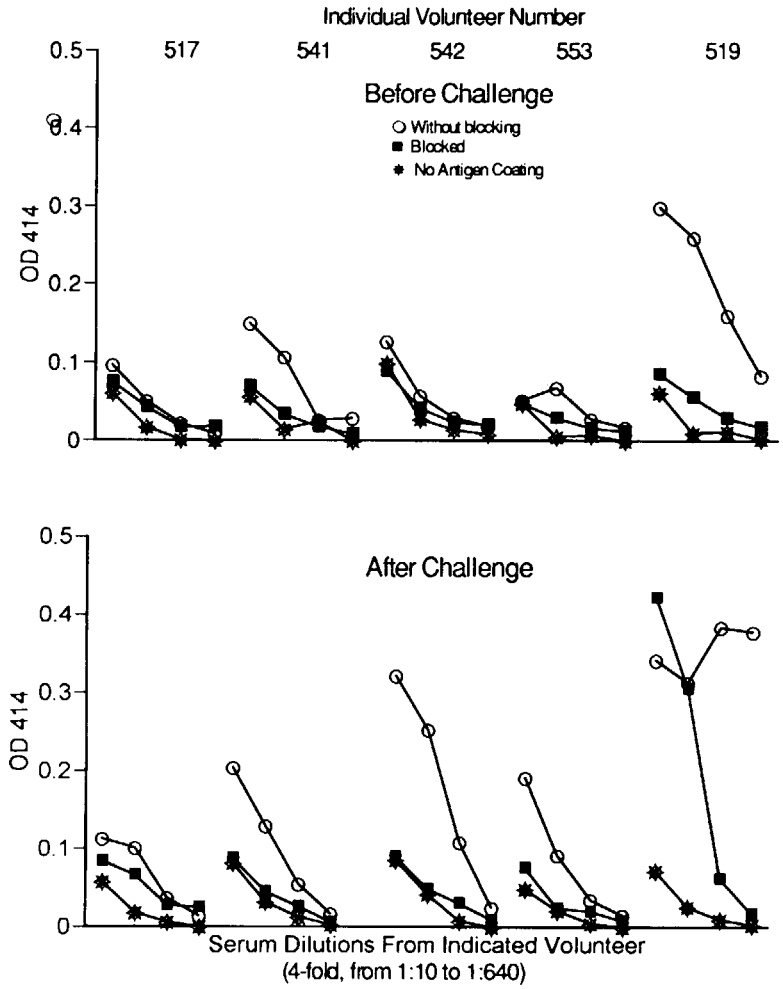

Figure 1. Determination of cutoff of ELISA for detection of Norwalk virus (NV)–specific antibody. Sera collected before and after inoculation with NV were tested by ELISA. Blocked = preincubated with equal volume of 1 μg/mL recombinant NV antigen. OD, optical density (absorbance).

(data not shown). Stools (n = 168) with A_{414} readings of <0.05 with the hyperimmune rabbit serum were considered negative, and stools (n = 155) with A_{414} readings of ≥0.1 positive. Eighteen stools (5%) with A_{414} readings between 0.05 and 0.1 and P/N ratios of >2.0 were considered as equivocal test results requiring confirmation by another criterion. Each of the 18 stool samples with low positive readings was the first or last in a series of stools that were positive; 50% of these samples were confirmed to be positive by the blocking assay, while another 20% were confirmed to be positive by RT-PCR.

The sensitivity of ELISA for Norwalk antigen detection was determined by testing serial dilutions of purified rNV capsid protein. A minimum of 0.025 ng of purified rNV/well was detectable. This represents $\sim 1.4 \times 10^6$ virions, because each particle contains 180 molecules of the capsid protein, which has an apparent molecular weight of 58,000 [27, 29a]. The sensitivity of the ELISA also was compared with that of RT-PCR, dot blot hybridization, and RIA for NV detection (table 1). NV was detected in stool dilutions as low as 1:100–1:10,000 by ELISA and RT-PCR but not by dot blot hybridization. These results indicated that the sensitivity of the ELISA was similar to that of RT-PCR and the ELISA was more sensitive than dot blot hybridization. We also tested stool samples (n = 124) by ELISA and RIA (data not shown); 24 were positive and 73 were negative for both tests (agreement, 78.2%). However, 26 samples were positive by ELISA and negative by RIA; only 1 sample was positive by RIA but not by ELISA. The ELISA-positive, RIA-negative stool samples were positive by RT-PCR, indicating true positive results. These results indicated the ELISA was significantly more sensitive than RIA ($\chi^2 = 21.3$; $P < 4 \times 10^{-6}$).

The NV antigen ELISA was specific for NV and did not detect viruses in duplicate tests of stools known to be positive for Snow Mountain agent, Hawaii agent, or 2 other strains of human caliciviruses (HuCV Sapporo and HuCV UK) and astrovirus (data not shown). The specificity of the test also was shown by the lack of detection of cell culture–grown feline calicivirus and other enteric viruses such as rotavirus serotypes 1–4, hepatitis A virus, poliovirus, coxsackieviruses A9 and B1, and echovirus types 5 and 9. All stool samples were shown to contain the test agent by ELISA or IEM [15, 30, 31, 32], and the cultured viruses were examined by electron microscopy. Further evidence for the specificity of the test was shown by blocking experiments in which positive reactions were blocked by preincubation of the test stools

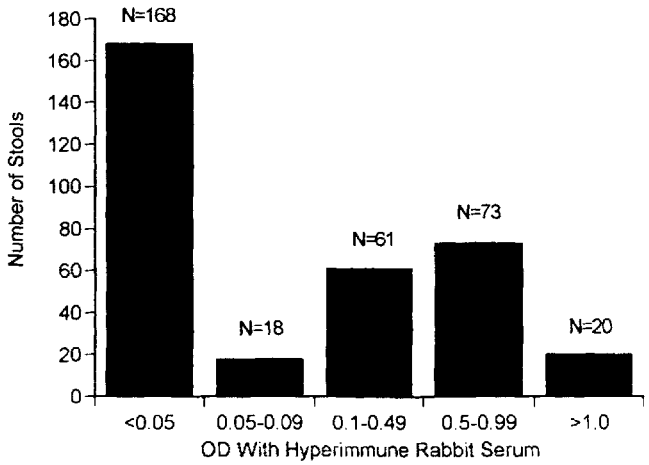

Figure 2. Distribution of ELISA optical density (OD) reading of 341 stools obtained from 50 subjects after inoculation with Norwalk virus.

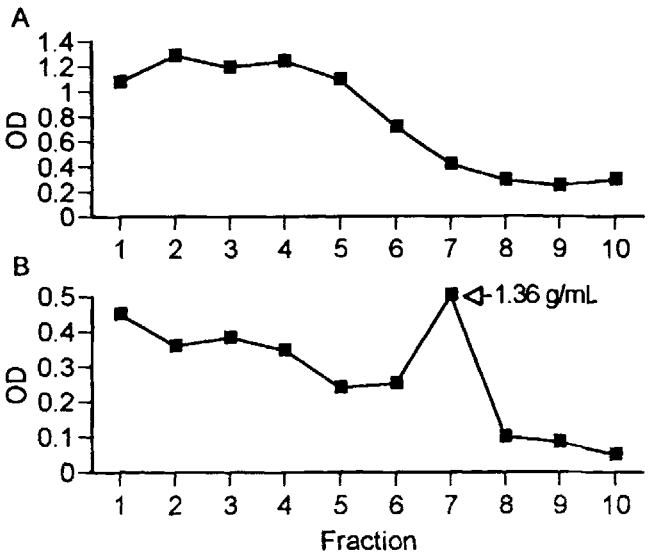

Figure 3. Detection of Norwalk virus (NV) in stool by antigen ELISA after CsCl gradient centrifugation: 30 mL of 10% stool suspension from volunteer 551 was extracted with 1,1,2-trichloro-1,2,2-trifluoro-ethane (DuPont, Wilmington, DE) and centrifuged 90 min in 50.2 Ti rotor at 36,000 rpm in tube containing 5 mL of 40% sucrose cushion. Pellet was saved, and supernatant above cushion was repelleted for 5 h in 50.2 Ti rotor at 40,000 rpm. Pellet from second centrifugation (**A**) and pellet from first centrifugation (**B**) each were mixed with CsCl to obtain final density of 1.36 g/mL, and centrifuged in SW 50.1 rotor for 24 h at 35,000 rpm. Gradients were fractionated and tested for viral antigen by antigen ELISA. Soluble antigen was unlikely to have resulted from processing because ELISA optical density (OD) readings of original 10% stool suspension were extremely high but virus particles were not detected by direct electron microscopy.

with volunteer convalescent serum before adding these mixtures to the plates (data not shown).

To determine if the ELISA detected both virus particles and soluble antigen, we assayed viral antigen in a volunteer's stool that was fractionated on an isopycnic density gradient of CsCl. The highest ELISA reactivity was seen in the fractions with a density of <1.31 g/mL (figure 3A), but fractions with the density of virions (1.36 g/mL) also clearly were positive (figure 3B). These results indicated that both virions and soluble antigen were detected in this stool sample and the concentration of soluble antigen was much higher than that of virion antigen.

Table 1. Comparative sensitivity of detecting Norwalk virus in stools using ELISA, reverse transcription–polymerase chain reaction (RT-PCR), and dot blot hybridization.

	Dilution					
Stool, method	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
502A-03						
ELISA	NT	+	+	−	−	−
RT-PCR	NT	+	−	−	−	−
Dot blot	±	−	−	−	−	NT
505A-05						
ELISA	NT	+	+	+	+	−
RT-PCR	NT	+	+	+	+	−
Dot blot	+	+	±	−	−	NT
544A-04						
ELISA	NT	+	+	−	−	−
RT-PCR	NT	+	+	−	−	−
Dot blot	−	−	−	−	−	NT
544A-05						
ELISA	NT	+	+	+	−	−
RT-PCR	NT	+	+	+	−	−
Dot blot	−	−	−	−	−	NT

NOTE. NT, not tested; +, strong signal; −, no signal; ±, weak but clear signal.

Biotin-avidin ELISA using hyperimmune anti-rNV serum to detect NV antigen. A biotin-avidin ELISA showed results similar to those of the sandwich ELISA described above. In the biotin-avidin ELISA, wells coated with rabbit serum also gave lower background than the wells coated with guinea pig serum. The biotin-avidin and sandwich ELISAs were directly compared using a set of stools from NV-infected volunteers. Of 17 stools tested, all 3 collected before challenge were negative and all 14 collected after challenge were positive by both tests. These two tests were more sensitive than previous ELISAs using human serum (only 5 of the 14 stools collected after challenge were positive by ELISA using human reagents). Because the biotin ELISA was the second antigen detection ELISA established, it was not used for evaluation of the volunteer study.

Evaluation of NV Infection by the New ELISAs

Infection status measured by serum antibody responses. The antibody ELISA was used to determine pre- and post-challenge serum antibody titers against NV in the 50 subjects

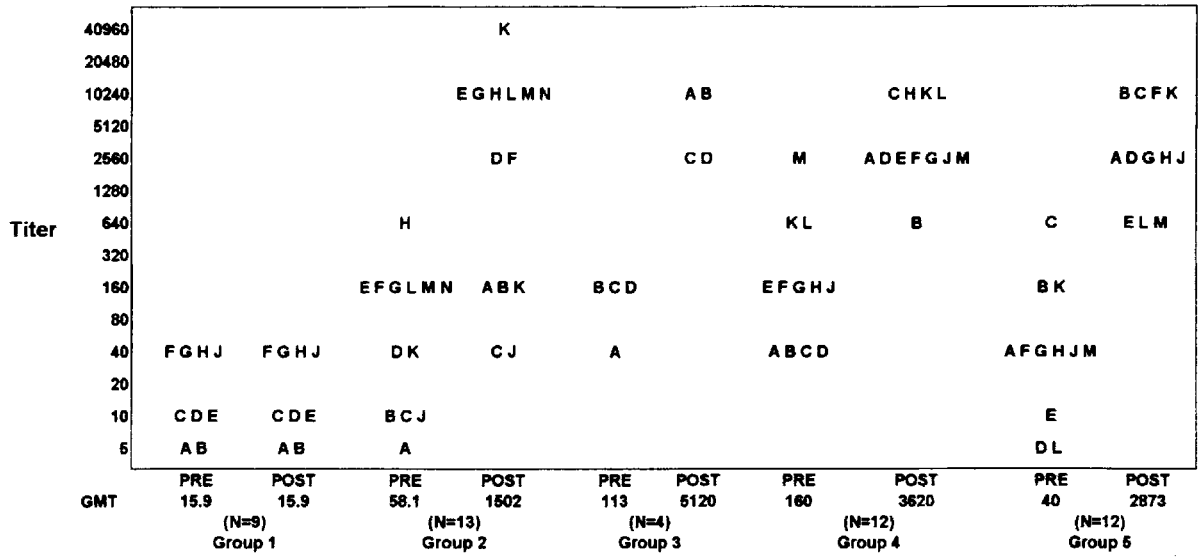

Figure 4. Serologic status of volunteers inoculated with Norwalk virus relative to clinical illness: group 1, uninfected (no seroconversion and no antigen shedding); group 2, asymptomatic or mildly symptomatic (no vomiting and no diarrhea); group 3, symptomatic (vomiting but no diarrhea); group 4, symptomatic (no vomiting but watery diarrhea); group 5, symptomatic (vomiting and watery diarrhea). Pre- and postinoculation titers for each subject are shown (individual letters); geometric means (GMT) are at bottom. Cutoff was A_{414} nm of 0.1.

(figure 4). Nine (18%) did not show an antibody response or shed virus after NV inoculation (i.e., remained uninfected after NV challenge). Forty demonstrated ≥4-fold increases in serum antibody titers to NV (mean, 35-fold), and 1 excreted virus but did not show a seroresponse. Therefore, 41 subjects (82%) were classified as infected.

Subjects were divided into five groups based on infection status and on the clinical outcome and severity of their infection (group 1, uninfected; group 2, asymptomatic or mildly symptomatic [no vomiting and no diarrhea]; group 3, symptomatic [vomiting but no diarrhea]; group 4, symptomatic [no vomiting but watery diarrhea]; and group 5, symptomatic [vomiting and watery diarrhea]). Uninfected subjects were more likely to have lower preexisting antibody titers than infected subjects ($P < .001$, Wilcoxon rank sum). When preexisting antibody titers of uninfected subjects were compared with those of each infected group (groups 2–5), only group 4 subjects had significantly higher preexisting antibody titers than did uninfected subjects ($P = .004$, ANOVA). For all infected groups, there were significant increases in geometric mean titers after infection ($P < .01$, paired t test). Among the infected groups, the increases in antibody titers in convalescent sera were significantly higher in subjects who had vomiting (groups 3 and 5 vs. 2 and 4, $P = .016$, Wilcoxon rank sum) and in those who had vomiting and diarrhea (group 5 vs. 2–4, $P = .02$, Wilcoxon rank sum).

We next examined the infection outcome of subjects relative to their preexisting antibody status (table 2). The frequency of seroconversion was 60% in subjects lacking preexisting serum antibody (titer of <10). Therefore, the lack of detectable serum antibody did not correlate with protection from infection. Titers of preexisting antibody were higher in subjects who excreted virus ($P < .001$), and there was a trend for higher titers of preexisting antibody in those who seroconverted ($P = .065$).

Virologic parameters of infection. Table 3 shows the time course of NV detection in stools from subjects according to their infection status. The most highly positive samples (based on A_{414}) were in stools from infected, symptomatic subjects (data not shown). The percentage of stools that contained antigen was higher in subjects with symptomatic infection (64%) than in those with asymptomatic infection (32%). Very few positive samples (18%) were collected the first day after challenge. A peak of viral shedding in stools was observed 25–72 h after inoculation. Most infected volunteers shed viral antigen continuously from their first positive sample until the last sample obtained. The earliest positive sample was detected 15 h after inoculation. Not all subjects provided samples after hospital discharge (day 3), so the absolute length of time of viral shedding could not be determined in this study. However, the longest time of antigen shedding was 7 days after inoculation, and 1 asymptomatic subject was still shedding antigen 6 days after infection. Evaluation of the relationship of antigen shedding and seroresponse following infection showed a correlation between the magnitude of the antibody response and antigen shedding (table 4).

Clinical features of subjects relative to infection status. The median incubation time to onset of symptoms ranged from 24 to 38 h, and the duration of illness ranged from 2 to 3 days (table 4). Onset of diarrhea occurred as early as 15 h and persisted for as long as 55 h after inoculation.

Table 2. Infection outcome relative to preexisting Norwalk virus antibody status.

Serum titer	No.	Symptom, no. (%) of subjects								
		Seroconversion*	Viral shedding†	Diarrhea	Vomiting	Nausea*	Cramps	Headache	Chills	Fever
<10‡	5	3 (60)	2 (40)	2 (40)	2 (40)	2 (40)	2 (40)	4 (80)	1 (20)	1 (20)
10	7	4 (57)	2 (29)	1 (14)	1 (14)	1 (14)	1 (14)	3 (42)	0	0
40	17	13 (76)	12 (70)	10 (59)	7 (41)	11 (65)	12 (70)	12 (70)	5 (29)	4 (23)
160	16	16 (100)	16 (100)	7 (44)	5 (31)	10 (62)	10 (62)	9 (56)	3 (19)	3 (19)
640	4	4 (100)	3 (75)	3 (75)	1 (25)	4 (100)	2 (50)	3 (75)	1 (25)	1 (25)
2560	1	0	1 (100)	1 (100)	0	0	0	0	0	0

* $P = .065$; χ^2 for trend = 3.4.
† $P = .0012$; χ^2 for trend = 10.5.
‡ Subjects did not have preexisting antibody; blocking also was consistent with lacking antibody (see serum 517, figure 1).

We also evaluated the occurrence of virus excretion, diarrhea, or other clinical symptoms relative to the magnitude of the seroresponse (table 4). The presence of vomiting and nausea ($P \leq .02$) and headache or body aches ($P = .04$) correlated with the magnitude of the seroresponses.

The results of detection of antigen and antibody responses are summarized in table 5. Of 28 subjects who had composite symptom scores of ≥5, 26 shed virus and developed an immune response following challenge. One subject (volunteer 536) with a clinical score of 5 shed virus but did not seroconvert. This subject had an exceptionally higher titer (1:2560) of preexisting antibody against NV and low viral shedding in stool. An additional 13 subjects were infected as determined by antigen detection or antibody response, for an overall infection rate of 82%. Asymptomatic infections without any clinical symptoms (clinical score = 0) were seen in 8 subjects (10%) and mild asymptomatic infections (clinical scores 1–4) in 5.

Correlation of responses in both the antigen and antibody ELISA showed that 34 subjects (68%) both shed virus and mounted an immune response to NV. Nine were negative for both viral shedding and an immune response, and 6 seroconverted but had no viral shedding detected. These results indicate that antibody detection is more sensitive than antigen detection for measuring NV infection. The importance of diagnosing infection by use of specific assays rather than symptoms was emphasized by the finding that of the 9 subjects who were uninfected, 5 had mild symptoms (clinical scores 1 or 2) unrelated to NV infection (data not shown); none had diarrhea or vomiting.

Discussion

Here we describe the development of two highly sensitive and specific ELISAs for the detection of NV-specific antibody and antigen and the application of these tests to study the outcome of inoculation of volunteers with NV. Because a highly purified rNV capsid antigen was used in the antibody ELISA and high-titer hyperimmune sera from animals were used in the antigen ELISA [27], previously unrecognized

Table 3. Timing of Norwalk virus shedding in stools from infected and uninfected volunteers.

Day after infection	Uninfected ($n = 9$)		Infected (asymptomatic) ($n = 13$)		Infected (symptomatic) ($n = 28$)	
	No. positive/ no. tested	Mean no. stools/person/day	No. positive/ no. tested (%)	Mean no. stools/person/day	No. positive/ no. tested (%)	Mean no. stools/person/day
0	0/5	0.6	0/7	0.5	0/10	0.4
1	0/6	0.7	0/16	1.2	12/51 (24)	1.8
2	0/7	0.8	9/17 (53)	1.3	81/109 (74)	3.9
3	0/13	1.4	5/9 (56)	0.7	40/44 (91)	1.6
4	0/1	NC	2/3 (67)	NC	16/22 (73)	NC
5	0/4	NC	1/3 (33)	NC	1/2 (50)	NC
6	0/2	NC	1/1 (100)	NC	5/5 (100)	NC
7	0/1	NC	NS —	NC	2/2 (100)	NC
Total	0/39	NC	18/56 (32)	NC	157/245 (64)	NC

NOTE. NC, not calculated because not all stools were collected after subjects were released from hospital; NS, no samples received.

Table 4. Clinical status in relation to magnitude of seroresponse to Norwalk virus.

Antibody response (-fold)	No. of cases	No. antigen positive	No. (%) of subjects with symptom						
			Diarrhea (38 [15–55])	Vomiting* (24 [23–31])	Nausea† (25 [15–51])	Cramps (28 [7–55])	Headache/ body ache‡ (29 [3–55])	Chills (27 [19–55])	Fever >37.8°C§ (33 [15–55])
0	10‖	1‖ (10)	1‖ (10)	0	1 (10)	0	4 (40)	0	0
4	3	0	0	0	0	1 (33)	0	0	0
16	15	13 (87)	9 (60)	4 (27)	10 (67)	10 (67)	11 (73)	4 (27)	3 (20)
64	17	17 (100)	11 (65)	9 (53)	13 (76)	12 (71)	12 (71)	5 (29)	3 (18)
256	5	5 (100)	3 (60)	3 (60)	4 (80)	4 (80)	4 (80)	1 (20)	3 (60)
Total	50¶	36 (88)	24 (59)	16 (39)	27 (66)	27 (66)	27 (66)	10 (24)	9 (22)

NOTE. Symptom headings show (median h of incubation [range]).
* $P = .02$, χ^2 for trend = 5.4.
† $P \leq .02$, χ^2 for trend = 5.9.
‡ $P = .04$, χ^2 for trend = 4.2.
§ $P = .08$, χ^2 for trend = 3.2.
‖ Includes 1 infected subject with no antibody response but who had watery diarrhea and excreted virus and had antibody titers of 1:2560.
¶ Norwalk infection was confirmed in 41 subjects by either antigen excretion or seroconversion; %s were calculated on 41 as total.

features of the disease were obtained. The high sensitivities of the two ELISAs and the unlimited supply of the reagents now should allow large-scale epidemiologic studies to address important questions such as the prevalence, pathogenesis, pathology, and immunity of NV infection and transmission and the antigenic relationship of NV with other small round structured viruses often detected in stools and associated with gastroenteritis.

The new findings of this study included an overall higher infection rate than previously recognized, a high rate of subclinical infection, prolonged viral shedding, and a significant correlation of the magnitude of antibody responses with vomiting or vomiting and watery diarrhea. These new data add to our understanding of the clinical manifestations of NV-induced acute gastroenteritis and have implications for the diagnosis of NV infections and the natural history and epidemiology of NV. For example, the findings of prolonged viral shedding and a higher rate of subclinical infections are important for understanding virus transmission and for planning intervention measures to control disease spread. Previously, it had been thought that viral shedding began with the onset of clinical illness and usually did not last >72 h [7, 9]. Our results indicate that while virus excretion peaks at 3 days, virus was still being shed in the latest samples available for testing (7 days after inoculation). This suggests that additional studies of virus excretion with these new, more sensitive assays are needed. The high rate of asymptomatic infection, with 1 subject still shedding virus at day 6, also may facilitate virus transmission. However, it will be important to determine whether the viral antigen excreted at late times is infectious. If it is, this information is important for planning outbreak control efforts in hospitals, nursing homes, and the food industry [33].

Table 5. Summary of antigen excretion and antibody responses in 50 volunteers.

Clinical score	Antigen response/antibody response			
	Positive/ positive	Negative/ positive	Positive/ negative	Negative/ negative
0–2*	0	0	0	9
0†	4	4	0	0
1–4‡	4	1	0	0
5–24	26	1	1	0
Total	34	6	1	9

NOTE. Positive antibody response, ≥4-fold.
* Uninfected.
† Asymptomatic infection.
‡ Mild asymptomatic infection.

In our previous rNV expression work, we reported the observation of both soluble and virion proteins in the recombinant-infected insect cells [27]. This observation was consistent with a previous report that soluble and virion antigens were detected in stools of patients infected with NV [18, 34]. In the present study, two peaks of NV-specific antigens (representing soluble and virion antigens) clearly were detected by antigen ELISA. Our data suggest that a higher concentration of soluble than of virion antigen is present in stools. It is not known whether the soluble antigen is simply a degradation product of the capsid protein whose appearance may be variable or if it plays a specific biologic function in viral replication. It will be of interest to determine whether the low-density antigen is directly related to the 34-kDa cleavage product observed in the baculovirus expression system [27] or to the 30-kDa soluble protein in stools [34] or both. The low concentration of particulate virion antigen in the stools at least partially explains why investigators have had diffi-

culty finding virus particles in the stools of NV-infected patients. The large concentration of soluble antigen (apparently lacking virion nucleic acid) also may explain why the ELISA showed a similar sensitivity to RT-PCR for virus detection in stools.

The finding of higher preexisting antibody titers against NV in volunteers who became infected compared with those who remained uninfected was consistent with previous reports that compared antibody titers and illness [11, 12, 16, 17]. However, our study also shows that the lack of preexisting antibody does not necessarily preclude susceptibility to infection and illness, as symptomatic infections were observed in 2 of 7 subjects who had no evidence of preexisting antibodies to NV. While some subjects with preexisting serum antibody titers of <1:50 (determined by the human reagent RIA) have been reported to become ill [12], it was not clear if they really lacked antibody or simply possessed low titers. The lack of antibody in our subjects could be confirmed by adsorption of the prechallenge sera with rNV particles. In our study, there was a significant correlation of the magnitude of antibody responses with vomiting or vomiting and watery diarrhea. Therefore, the higher antibody responses in persons with these clinical signs would be expected to persist longer, increasing the likelihood of detection of NV infection. This may explain the close association of vomiting with NV infection documented by seroconversion in Desert Shield troops [35]. Specifically, high antibody responses in persons who have vomiting may prolong the time after which such infections can be detected. Unfortunately, we did not have a sufficient number of subjects with vomiting alone to determine if antibody response correlated only with vomiting. However, these results confirm that the interactions between host immunity and NV infection remain a paradox, and clinical immunity to NV infection fails to fit immunologic concepts traditionally associated with common human viral illnesses [12].

The antigen ELISA described in this study was highly specific and has confirmed the previous conclusion that NV is antigenically distinguishable from the Snow Mountain and Hawaii agents [8, 24, 36]. In addition, this highly specific ELISA showed that some virus isolates previously identified as Norwalk-related viruses by tests using human reagents were clearly distinct from NV. The lack of reaction of these samples with the antigen ELISA was not due to a low virus concentration in the sample or a degradation of viral antigen during storage of the stools. This was demonstrated by RT-PCR analysis of these samples, which showed that viruses were easily detected and the viruses found were genetically distinct from, yet related to, NV [37–39].

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