Viral Shedding and Fecal IgA Response after Norwalk Virus Infection

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Protection is not conferred by preexposure to Norwalk virus (NV). By use of an ELISA with baculovirus-expressed recombinant NV (rNV) capsid protein, the pattern of NV fecal shedding and the protective effect of rNV-specific fecal IgA (fIgA) were investigated in volunteers who were repeatedly challenged with NV. After the first challenge, ill volunteers were significantly more likely than well volunteers to have NV antigen in their stool ($P < .05$). After challenge, antigen shedding was detected on days 1–13; ill volunteers shed the antigen longer ($P = .02$). A higher prechallenge rNV-specific fIgA geometric mean titer was found in ill compared with well volunteers ($P < .05$) and in infected versus noninfected volunteers ($P < .05$). NV shedding was common after infection and was present up to 2 weeks after challenge. Preexisting rNV-specific fIgA, like serum IgG, is not protective and may be a marker for symptomatic disease.

Norwalk virus (NV) is a fastidious calicivirus [1–3] that has not been cultured and for which no animal model exists. NV is the prototype of a large group of small, round, structured viruses that cause acute, self-limited gastrointestinal illness. Norwalk virus is a major cause of acute nonbacterial gastroenteritis outbreaks in the United States [4] and elsewhere [5]. NV also is responsible for ~10% of traveler's diarrhea [6] and is a significant cause of infantile diarrhea in developing nations [7, 8]. Serologic evidence of past infection with NV is present in one-third to three-fourths of adults and varies according to the population studied [5, 9].

The duration of symptomatic NV illness is 48–72 h. Outbreaks may be caused by a common source [4] or person-to-person transmission and last an average of 5–9 days. However, in some instances, outbreaks caused by person-to-person transmission have lasted several weeks, suggesting that viral shedding may be prolonged. Initial studies indicated that shedding occurs in <50% of ill persons and does not persist beyond 100 h after initial infection [10]. Polymerase chain reaction analysis and new sensitive ELISA techniques indicate that viral shedding may be more prolonged than previously recognized [10, 11]. To further define the length of shedding and incidence in asymptomatic infection, we investigated NV shedding in healthy volunteers after repeated challenge with NV.

Host susceptibility to Norwalk virus is paradoxically related to the presence of specific serum antibodies against Norwalk virus. This has been demonstrated in volunteer challenge studies among US adults and US travelers to Mexico [9, 12, 13]. A distinct group of persons with persistent low or absent titers of serum antibodies to Norwalk virus do not experience illness even after several challenges [13]. On the basis of the hypothesis that immunity to NV might be mediated by local intestinal production of antibodies, we evaluated whether fecal IgA (fIgA) protects against Norwalk virus infection or stops viral shedding.

Materials and Methods

Subjects and fecal specimens. Fecal specimens were obtained from adults participating in volunteer challenge studies of Norwalk virus strain 8F IIa conducted at the University of Texas Clinical Research Center from September 1986 to October 1991. Volunteers were challenged twice, 6 months apart, and a third time 12 or 18 months after the first challenge, as described [13]. After challenge, patients were observed for enteric illness for 5 days; all fecal samples were collected. Thereafter, patients were followed as outpatients and were asked to submit stool samples at weekly intervals.

Stool samples collected prior to challenge and at several intervals thereafter (range, 1–28 days) were stored at −70°C. A 10% solution was made with 2 g of stool in PBS and mixed with one part trichlorotrifluoroethane (Fisher Scientific, Pittsburgh). Samples were clarified by centrifugation for 15 min at 1500 *g* at 5°C. Bovine lung protease inhibitor (1%; Sigma, St. Louis) was added to preserve fIgA. Extracts were aliquoted and frozen at −70°C until use. As a negative control, a fecal extract was obtained from a well 6-month-old child attending a day care center and similarly processed.

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The study was approved by the Committee for the Protection of Human Subjects, University of Texas–Houston Health Science Center. Informed consent was obtained from all participants.

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Detection of NV in stools. Separate wells in ELISA polyvinyl plates (PVC; Dynatech Laboratories, Chantilly, VA) were coated with 50 μL of preinoculation and convalescent sera from a rabbit inoculated with recombinant NV (rNV) capsid proteins obtained by expression in insect cells using a baculovirus recombinant [11, 14]. Plates were incubated for 4 h at 37°C. Excess antibody was removed, wells were blocked for 1 h with 5% nonfat dry milk (Carnation, Los Angeles) in PBS, and washed twice with 0.05% PBS-Tween 20 (PBST). Fecal extracts were diluted in 2% milk, and 50 μL was placed in wells overnight at 5°C. After the wells were washed five times with PBST, 50 μL of convalescent serum from an rNV-inoculated guinea pig was added, and the plates were incubated for 2 h at 37°C then washed with PBST. Horseradish-labeled anti-guinea pig antibody (Hyclone, Durham, NC) was added, and the plates were incubated for 2 h at 37°C. After a final washing step, 50 μL of TMB (3,3,5,5 tetramethylbenzidine; Sigma) was added, and the color reaction was read 30 min later at A_{450} using an ELISA reader (MR 580; Dynatech Laboratories). Results were considered positive when the sample optical density exceeded twice the mean background reading of the wells coated with the sera from the rabbit prior to rNV inoculation. Known positive and negative samples were used as internal controls.

ELISA for rNV-specific fIgA detection. Flat-bottom PVC plates were coated for 4 h with 100 μL of 1 μg/mL rNV solution in PBS. Wells were blocked overnight with 200 μL of 5% low-fat dry milk in PBS at 5°C and then washed with PBST. Stool extracts obtained before and after challenge (range, 3–28 days) were added in twofold serial dilutions and incubated for 2 h at 37°C. After a washing step, peroxidase-conjugated goat anti-human IgA (α-chain specific, Hyclone) was added and the plates were incubated for 2 h at 37°C. After a wash step, TMB was added, and the plates were read 30 min later. Convalescent serum with a known end point–positive titer was used as a control on each plate.

ELISA for quantification of total IgA. ELISA plates were coated overnight with 100 μL of goat anti-human IgA (Sigma) in carbonate buffer, pH 9.6, at 5°C. After being blocked for 2 h with 5% milk at 37°C and after being washed, 50 μL of a known reference of human IgA was added to wells in duplicate twofold dilutions starting at 27 μg/mL. Stool extracts (100 μL) from volunteers were added in twofold dilutions to adjoining wells in duplicate and incubated for 1 h at 37°C. Peroxidase-labeled goat anti-human IgA was added, and the plates were incubated for 1 h and washed. TMB substrate was added, and the color reaction was read. Linear regression was used to determine the IgA content [15]. Samples were normalized to 1 mg to control for differences in titer variability between challenge dates and among volunteers.

Definitions. Clinical illness in volunteers was defined as the passage of two or more unformed stools or vomiting in a 24-h period associated with signs of enteric illness (nausea, abdominal cramping, malaise, or fever).

Infection with NV was considered to have occurred if levels of NV-specific fecal or serum antibodies rose fourfold or higher after challenge or if NV antigen was detected in postchallenge fecal samples.

Statistical analysis. Differences between NV-infected subjects according to illness outcome and number of challenges were determined by χ^2 analysis. Length of viral antigen shedding was established using Kaplan-Meier time survival analysis. Geometric mean titers (GMTs) were determined for all groups. Differences in IgA titers among subjects were determined by Student's *t* test. Differences in IgA titers between challenges were determined by analysis of variance with the Kruskal-Wallis test for groups.

Table 1. Identification, according to postchallenge clinical outcome, of NV-infected volunteers by capture recombinant ELISA of postchallenge stools.

Clinical outcome	Challenge		
	First	Second	Third
Ill	16/17* (94)†	5/6 (83)	2/5 (40)‡
Well	13/21 (62)	9/16 (56)	2/7 (29)
Total	29/38 (61)	14/22 (63)	4/12 (33)

NOTE. Data are no. positive/no. tested (%).

* 16 infected as determined by antigen shedding, development of immunologic response, or both.

† $P < .05$ vs. well volunteers.

‡ $P < .05$ vs. first challenge.

Results

Antigen studies. Forty-nine volunteers were challenged once, 35 twice, and 23 three times. Only subjects in whom data from serologic and fecal specimens were available were studied further.

Fecal specimens from 38 volunteers after the first, 22 after the second, and 12 after the third challenge were studied for the presence of NV by capture ELISA. Gastroenteritis developed in 17 (44%) after the first challenge, 6 (27%) after the second, and 5 (42%) after the third. Five of the 6 subjects who became ill after the second challenge were also ill after the first; none of the well volunteers during the first challenge became ill after the second challenge.

NV was detected in both well and ill volunteers. It was found more frequently in unformed than formed stools, was maximal within the first 72 h after exposure, and was present up to 13 days after challenge. None of 17 stool samples tested beyond that time were positive for NV (postchallenge days 14–50; data not shown).

After the first challenge, 29 (61%) of 38 volunteers were positive for NV antigen. NV was detected more frequently in ill (16/17) than well (13/21) volunteers (94% vs. 62%; $P < .05$; table 1). One ill volunteer was negative for NV. This subject had a mild illness with two soft stools and mild abdominal cramping. Of 31 infected volunteers, 29 (96%) shed NV. Kaplan-Meier life table analysis showed that after the first challenge, ill subjects shed NV for a longer period than

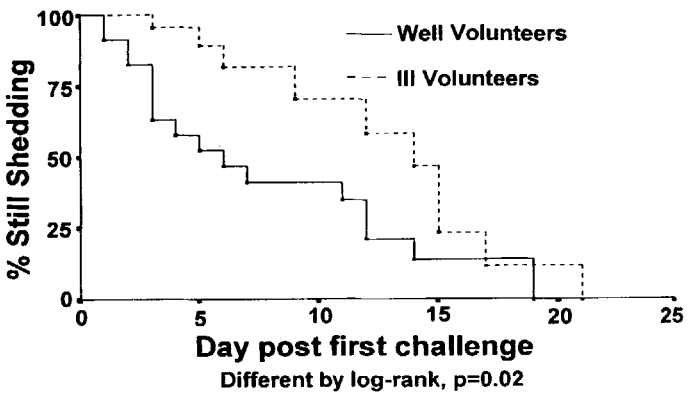

Figure 1. Duration (days), as determined by Kaplan-Meier survival analysis, of NV shedding after infection with NV 8 IIFa. Time to passage of last positive sample for NV antigen is plotted. Results are from 13 ill and 10 well subjects after challenge. Subjects with 1 positive sample or no shedding were excluded.

well subjects (figure 1). The duration of shedding was similar among groups after the second and third challenges.

In the rechallenge studies, 14 (63%) of 22 volunteers were infected after the second challenge. Eight and 4 subjects, respectively, were challenged 6 and 12 months after the second challenge; because of clinical and immunologic parameters similar to those seen in a previous serologic study [13], these subjects were considered as a single group in this analysis, and only 4 (33%) of these 12 subjects shed NV (table 1). There was a significant difference between the number who shed NV after the first and the third challenge ($P < .05$). After the second challenge, 5 (83%) of 6 ill volunteers and 9 (56%) of 16 well volunteers shed NV ($P > .05$). The ill volunteer who did not shed NV did not meet the definition for infection. Fourteen (77%) of 17 infected volunteers shed NV after the second challenge, and 4 (50%) of 8 shed NV after the third challenge.

Fecal IgA studies. Measurable anti-NV–specific fIgA titers were seen in 27 (71%) of 38 volunteers before the first challenge (table 2). Sixteen (94%) of the ill volunteers and 11 (52%) of the well volunteers had positive titers before the first challenge ($P < .01$). Of the 31 who became infected, 26 (84%) had positive prechallenge titers versus 2 (29%) of 7 who were not infected after first challenge ($P < .01$). Fifteen (84%) of 17 infected volunteers had positive titers before the second challenge versus 2 (40%) of 5 noninfected volunteers ($P < .05$).

Table 2. Numbers of volunteers with measurable prechallenge NV-specific fecal IgA titers.

Clinical status	Challenge		
	First	Second	Third
Ill	16/17 (94)*	4/6 (66)	5/5 (100)
Well	11/21 (52)	13/16 (81)	6/7 (86)
Infected	26/31 (83)†	15/17 (88)‡	8/8 (100)
Noninfected	2/7 (29)	2/5 (40)	3/4 (75)
Total	27/38 (71)	17/22 (77)	11/12 (91)

NOTE. Data are no. positive/no. tested (%).
$P <$ * .01 vs. well volunteers, † < .01 and ‡ < .05 vs. noninfected volunteers.

Table 3. Fecal IgA geometric mean titers (GMT) in volunteers before and after challenge with NV 8 IIFa.

Clinical status	Challenge		
	First	Second	Third
Ill, before	2782	388	2170
After	2566	592	6071
Well, before	165*	1338	1209
After	207*	834	1599
Infected, before	725	860	2951
After	913	738	6211
Noninfected, before	50†	192	448†
After	74†	50	486†

* $P < .05$ compared with prechallenge ill GMT or † compared with prechallenge infected GMT.

The reciprocal GMTs of fIgA before and after each challenge are shown in table 3 for the ill and well and infected and noninfected volunteers. No significant increases in NV-specific fIgA were seen after one or more challenges. However, a significantly higher baseline fIgA titer was seen in ill versus well volunteers before the first challenge (GMT, 2782 vs. 207; $P < .05$) and in infected versus noninfected volunteers (GMT, 725 vs. 50; $P < .05$). While these differences were not present in the second challenge, a higher prechallenge titer was also seen in the infected versus noninfected volunteers before the third challenge (GMT, 2951 vs. 448; $P < .05$; table 3).

Discussion

Previous studies using immune electron microscopy in ill volunteers determined that shedding of NV occurred in about half of the ill volunteers, was infrequent after 72 h, and undetectable after 100 h after challenge [10]. Our study provides evidence that NV shedding occurs in >90% of ill volunteers. The difference in findings in this study compared with those in previous ones may reflect the high test sensitivity of the new ELISA. We were unable to demonstrate the presence of NV in only 1 of 23 volunteers with presumed NV-induced illness after the first challenge. This patient, who may have had a gastrointestinal illness unrelated to NV, had low levels of preexisting serum anti-NV antibodies (a common finding in NV-resistant individuals [13]) and passed only 2 unformed stools. The present study also provides evidence

that shedding of NV is more prolonged than previously thought and detectable up to 2 weeks after challenge.

Our study confirms and expands on a prior report on the prolonged shedding of NV antigen [11]. In that study, however, the absolute length of shedding could not be determined [11]. In our study, NV shedding occurred in over half of the well volunteers and persisted up to 2 weeks. Among the group of well volunteers studied in the first challenge, we separately analyzed a subgroup of 12 volunteers who were characterized by low pre- and postchallenge serum antibodies as previously determined by biotin-avidin immunoassay [13]. Viral antigen was detected in 6 (50%) convalescent fecal specimens, confirming that asymptomatic infection may occur in the absence of preexisting antibodies in serum.

The secretory immune response to infection with NV is complex and incompletely understood. A pilot study evaluated the intestinal immune response to NV, measuring the blocking activity of duodenal fluid in 14 volunteers by an RIA that did not distinguish between specific antibody classes [9]. In that study, Blacklow et al. found that although NV-specific IgA was not measured by the assay, the titers of duodenal IgA for ill and well persons were similar, and none of the volunteers developed a fourfold increase in titers. Furthermore, all persons with blocking activity in their duodenal fluid before NV challenge became ill after challenge, while only 46% of those without blocking activity became ill after challenge. Newer sensitive techniques derived from the knowledge of the NV genome have allowed us to study the intestinal immune response to NV. Although a stepwise increase in prechallenge fIgA was observed as volunteers were reexposed to NV, we were unable to prove a significant increase in convalescent titers after exposure to NV. Our study demonstrates that the presence of high titers of specific anti-NV IgA in volunteers before challenge correlates with the likelihood of development of clinical illness and the failure of protection to subsequent challenge.

The prolonged excretion of NV after resolution of clinical symptoms in ill volunteers and the shedding of NV in well volunteers may be important in continuing outbreaks, which have been known to persist well beyond the symptomatic stages of initial cases. However, it remains to be determined whether the antigen detected at later times represents infectious virus or soluble antigen.

The lack of protection afforded by preexisting serum IgG and IgA antibodies [13], the unexplained paradoxical association of high levels of serum antibodies with clinical illness after exposure, the development of short-term immunity [16], and the occurrence of long-term immunity suggest that resistance to infection with NV is mediated by cellular mechanisms, probably in the gastrointestinal tract.

References

1. Jiang X, Wang K, Graham DY, Estes MK. Norwalk virus genome cloning and characterization. Science **1991**;250:1580–3.
2. Matsui SM, Kim JP, Greenberg HB, et al. The isolation and characterization of a Norwalk virus-specific cDNA. J Clin Invest **1991**;87:1456–61.
3. Jiang X, Wang J, Graham DY, Estes MK. Detection of Norwalk virus in stool by polymerase chain reaction. J Clin Microbiol **1992**;30:2529–34.
4. Kaplan JE, Gary GW, Baron RC, et al. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. Ann Intern Med **1982**;96:756–61.
5. Lew J, Valdesuso J, Vesikari T, et al. Detection of Norwalk virus or Norwalk-like infections in Finnish infants and young children. J Infect Dis **1994**;169:1364–7.
6. Keswick BH, Blacklow NR, Cukor GC, DuPont HL, Vollet JL. Norwalk virus and rotavirus in travellers' diarrhoea in Mexico [letter]. Lancet **1982**;1:109–10.
7. Black RE, Greenberg HB, Kapikian AZ, Brown KH, Becker S. Acquisition of serum antibody to Norwalk virus and rotavirus and relation to diarrhea in a longitudinal study of young children in rural Bangladesh. J Infect Dis **1982**;145:483–9.
8. Ryder RW, Singh N, Reeves WC, Kapikian AZ, Greenberg HB, Sack RB. Evidence of immunity induced by naturally acquired rotavirus and Norwalk virus infection on two remote Panamanian islands. J Infect Dis **1985**;151:99–105.
9. Blacklow NR, Cuckor G, Bedigian MK, et al. Immune response and prevalence of antibody to Norwalk enteritis virus as determined by radioimmunoassay. J Clin Microbiol **1979**;10:903–7.
10. Thornhill TS, Kalica AR, Wyatt RG, Kapikian AZ, Chanock RM. Pattern of shedding of the Norwalk particle in stools during experimentally induced gastroenteritis in volunteers as determined by immune electron microscopy. J Infect Dis **1975**;132:28–34.
11. Graham D, Jiang X, Tanaka T, Opekun A, Madore H, Estes M. Norwalk virus infection of volunteers: new insights based on improved assays. J Infect Dis **1994**;170:34–43.
12. Johnson PC, Hoy J, Mathewson JJ, Ericsson CD, DuPont HL. Occurrence of Norwalk virus infections among adults in Mexico. J Infect Dis **1990**;162:389–93.
13. Johnson PC, Mathewson JJ, DuPont HL, Greenberg HB. Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults. J Infect Dis **1990**;161:18–21.
14. Jiang X, Wang M, Graham D, Estes MK. Expression, self assembly, and antigenicity of Norwalk virus capsid protein. J Virol **1992**;66:6527–32.
15. Conner ME, Gilger MA, Estes MK, Graham DY. Serologic and mucosal Immune response to rotavirus infection in the rabbit model. J Virol **1991**;65:2562–71.
16. Parrino TA, Schreiber DS, Trier JS, Kapikian AZ, Blacklow NR. Clinical immunity in acute gastroenteritis caused by the Norwalk agent. N Engl J Med **1977**;297:86–9.