Subclass-Specific Serum Antibody Responses to Recombinant Norwalk Virus Capsid Antigen (rNV) in Adults Infected with Norwalk, Snow Mountain, or Hawaii Virus

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Subclass-specific antibody responses to the Norwalk virus capsid protein in adults challenged with Norwalk, Snow Mountain, or Hawaii virus were evaluated by solid-phase enzyme immunoassay using recombinant Norwalk virus capsid antigen (rNV). Fourfold or greater serum immunoglobulin G (IgG) antibody responses to rNV were detected in 15 of 20 volunteers challenged with Norwalk virus, and serum IgA and IgM antibody responses to rNV were seen in almost all subjects who had rNV IgG responses. Serum rNV IgG antibody responses also were detected in 6 of 15 volunteers challenged with Snow Mountain virus and 2 of 12 volunteers challenged with the Hawaii virus. However, the magnitude of antibody response and the geometric mean postchallenge rNV IgG antibody titers were lower in subjects challenged with Snow Mountain or Hawaii virus, and serum IgA and IgM responses generally did not occur.

The Norwalk viruses are a group of viruses that share certain morphologic and biochemical features and which are associated with acute gastroenteritis in humans (18). Norwalk virus, the most extensively characterized member of this group, is a small, round, structured virus (SRSV) with a single structural protein with an M_r of approximately 59,000 (11, 15) and a single-stranded positive-sense RNA genome of at least 7.5 kb (14). The Snow Mountain and Hawaii viruses are other SRSVs that have been detected in stool samples from outbreaks of gastroenteritis and which have been shown to cause a similar syndrome of acute gastroenteritis when administered orally to human volunteers (6, 28). The Snow Mountain virus also appears to have a single structural protein with an M_r of approximately 62,000 (21). Additional viruses associated with gastroenteritis and possibly related to Norwalk virus, including Taunton, Otofuke, and other SRSVs have been described previously (3, 8). The structural and genomic characteristics of Norwalk virus are similar to those of the *Caliciviridae* (16).

Further characterization of these viruses has been hampered by the lack of a system for in vitro propagation and the relatively small amounts of virus shed by infected individuals. As a result, the antigenic relationships between members within the group of Norwalk viruses are unclear. Norwalk, Snow Mountain, and Hawaii viruses can be distinguished from one another by immune electron microscopy, radioimmunoassay, and enzyme immunoassay (EIA) using postinfection human sera as reagents (1, 6, 22, 28-30). In addition, cross-challenge experiments with adult volunteers have demonstrated that Norwalk and Hawaii viruses are antigenically distinct, since individuals challenged with Norwalk, but not Hawaii, virus are protected from short-term rechallenge with Norwalk virus (33). However, several reports have suggested that cross-reactive antibody responses to Norwalk virus may occur in individuals infected with Snow Mountain virus or human calicivirus (4, 20) and

that similar antigenic cross-reactivity exists between Hawaii and Snow Mountain viruses and other SRSVs (13, 20). Responses to multiple SRSVs have also been noted in a number of outbreaks of gastroenteritis (12, 24, 31, 32). Each of these studies has used serologic assays in which unpurified and poorly characterized stool samples from infected individuals served as the source of antigen. The nature and potential significance of these cross-reactive or heterologous antibody responses are unclear.

The genome of the prototypic virus of this group, Norwalk virus, has been cloned, and the capsid protein has been expressed in a baculovirus expression system (14, 15). We used baculovirus-expressed recombinant Norwalk capsid antigen (rNV) to evaluate subclass-specific antibody responses to Norwalk virus in human subjects following challenge with Norwalk, Snow Mountain, and Hawaii viruses. Sera evaluated by EIA were obtained from adults who were challenged orally with Norwalk, Snow Mountain, and Hawaii viruses in several studies that have been reported previously (20). Sera were collected from subjects prior to challenge and 3 to 6 weeks after challenge. Subjects were observed daily for 7 days following challenge, and illness was defined as the presence of two or more episodes of vomiting or diarrhea during a 24-h period.

Subclass-specific rNV EIA was performed essentially as described elsewhere (15), except with substitution of subclass-specific reagents for detection of bound human antibody. The production of rNV by a baculovirus recombinant that contains the 3' end of the Norwalk virus genome has been described previously (15). rNV particles used as the antigen were purified by centrifugation in sucrose and CsCl gradients (reference 15 and unpublished data). Briefly, the sequence of reagents used in the EIA, from the polyvinylchloride plate (Dynatech Laboratories, Chantilly, Va.) outward, was rNV at a concentration of 100 ng per well in 0.1 M phosphate-buffered saline (PBS) (pH 7.2), blocking reagent (5% Carnation nonfat dry milk in PBS), the sera to be tested, and affinity-purified alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG), IgM, or IgA (Tago Laboratories, Burlingame, Calif.). Each dilution of serum

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was tested in two wells with and one well without rNV antigen. The end point titer was defined as the greatest dilution giving an optical density reading twice that in antigen negative wells and ≥ 0.1 optical density unit. IgM capture assay was performed by adsorption of unlabeled goat anti-human IgM (μ chain specific; Tago Laboratories) to polyvinylchloride plates, followed by the sera to be tested, rNV at 100 ng per well, biotin-labeled rabbit anti-rNV antibody, and avidin linked to alkaline phosphatase. End point titers were determined as described above. The results of these assays are compared with those previously obtained by blocking assay using human reagents (20). For sera that were tested multiple times by blocking EIA, the results reported in this paper are those which were most representative of the results for that serum pair overall.

A total of 15 of the 20 subjects who were challenged with Norwalk virus manifested a fourfold or greater serum IgG antibody response to rNV (Table 1). Serum rNV IgG responses were detected in all 15 of the Norwalk virus-challenged subjects in whom blocking antibody responses to antigen-containing stool had been detected previously (20) and in none of the 5 subjects who had not had a blocking EIA response to Norwalk virus challenge. There also was a good correlation between the two tests in the magnitude of antibody responses in subjects challenged with Norwalk virus, as assessed by the increase in titer ($r = 0.83$; $P < 0.001$). Serum rNV IgG responses occurred in 13 of 15 (86.7%) subjects who developed gastrointestinal illness after challenge and in 2 of 5 (40%) subjects who remained well.

Serum rNV IgM responses were detected by direct binding assay in 14 of 15 subjects who had serum IgG responses and in none of the subjects who did not have IgG responses following Norwalk virus challenge. We found the IgM capture assay to give results qualitatively similar to those of the direct assay for IgM. All 14 subjects with IgM antibody responses following Norwalk virus challenge also responded by the IgM capture assay. In addition, the IgM capture assay detected an IgM response in the single individual who had an rNV IgG EIA response without an IgM response by direct binding. The correlation between postinfection IgM titers as measured by the two assays was 79% ($P < 0.001$), although titers by the IgM capture assay were four- to eightfold higher than those by the IgM direct binding assay. Serum rNV IgM responses by either test occurred regardless of the presence of prechallenge serum IgG, i.e., in individuals both with and without evidence of prior Norwalk virus infection. To confirm that the responses seen by IgM rNV EIA represented true IgM responses, sera were tested before and after treatment with the reducing agent dithiothreitol (5). Treatment with dithiothreitol reduced or abolished the reactivity of sera in the IgM rNV direct binding EIA and in the IgM capture assay but did not decrease the titer of IgG antibody to rNV (data not shown). Serum rNV IgA responses were detected in all 15 subjects who developed serum IgG responses to Norwalk virus challenge and in none of the 5 subjects who did not have such a response.

Low-level serum Norwalk virus blocking EIA and rNV IgG responses were also detected in some subjects challenged with Hawaii and Snow Mountain viruses. The apparently heterologous rNV IgG EIA responses of these subjects were less frequent and of significantly lower magnitude than the homologous responses seen in subjects challenged with Norwalk virus. Among those in whom an rNV IgG response was detected, the mean $\log_2$ postinfection titer was 15.0 ± 1.6 in those who were challenged with Norwalk virus and 9.8 ± 0.8 in those challenged with Hawaii or Snow Mountain

TABLE 1. Norwalk virus blocking and subclass-specific rNV response to challenge with Norwalk, Snow Mountain, or Hawaii virus

Virus challenge	No. of subjects tested	Norwalk virus response by blocking EIA						rNV antibody response of:					
		log ₂ titer $\pm$ SD		No. of subjects with rise in titer	log ₂ titer $\pm$ SD		No. of subjects with rise in titer	log ₂ titer $\pm$ SD		No. of subjects with rise in titer	log ₂ titer $\pm$ SD		No. of subjects with rise in titer
		Prechallenge	Postchallenge		Prechallenge	Postchallenge		Prechallenge	Postchallenge		Prechallenge	Postchallenge	
Norwalk	20	6.7 $\pm$ 1.3	11.0 $\pm$ 3.1 ^a	15	9.6 $\pm$ 2.7	13.2 $\pm$ 3.6 ^a	15	4.9 $\pm$ 1.4	8.9 $\pm$ 3.6 ^a	14	5.2 $\pm$ 1.6	10.3 $\pm$ 4.0 ^a	15
Hawaii	12	5.9 $\pm$ 0.5	6.7 $\pm$ 1.1	3	8.0 $\pm$ 2.1	8.5 $\pm$ 2.0	2	3.8 $\pm$ 1.1	3.8 $\pm$ 1.1	1	5.1 $\pm$ 1.1	5.2 $\pm$ 1.1	1
Snow Mountain	15	6.0 $\pm$ 0.7	5.9 $\pm$ 0.6	0	8.7 $\pm$ 2.0	9.9 $\pm$ 1.3	6	3.9 $\pm$ 0.9	3.8 $\pm$ 0.8	0	4.9 $\pm$ 0.8	5.2 $\pm$ 1.0	1

^a $P < 0.001$ comparing titers in subjects challenged with Norwalk virus with those challenged with Hawaii or Snow Mountain virus.

virus ($P < 0.0001$, Student t test). In addition, the geometric mean increase in rNV IgG antibody was 28.6-fold in the 15 subjects who mounted an rNV IgG response following Norwalk virus challenge and only 6.2-fold in the 8 subjects who mounted an rNV IgG response following Hawaii or Snow Mountain virus challenge.

There was essentially no correlation between the magnitude of response seen previously by blocking EIA (20) and rNV IgG EIA in subjects who were challenged with Hawaii or Snow Mountain virus ($r^2 = 0.001$; $P = 0.90$), and the heterologous responses occurred in different subjects by the two tests. However, heterologous responses by either blocking or rNV EIAs were detected only in subjects who were ill and who had a homologous response to the challenge Hawaii or Snow Mountain virus by the previously described blocking EIAs for these viruses (data not shown).

In contrast to the results in subjects challenged with Norwalk virus, IgM or IgA rNV antibody increases were detected infrequently in individuals challenged with Hawaii or Snow Mountain virus. None of the subjects with rNV IgG responses following Snow Mountain or Hawaii virus challenge had an accompanying IgM or IgA response. A single subject challenged with Hawaii virus manifested an eightfold rise in rNV IgM antibody. This subject also had a fourfold increase in serum rNV IgA antibody but did not have an rNV IgG antibody response. The rNV IgM response following Hawaii virus challenge also was confirmed by IgM capture assay. No other rNV IgM responses were detected by IgM capture assay in any of the subjects challenged with Hawaii or Snow Mountain virus who manifested an IgG rNV response. An additional subject manifested a serum rNV IgA response, but not an IgG or IgM response, following challenge with Snow Mountain virus.

We also evaluated the relationship between prechallenge serum rNV antibody levels and subsequent illness or infection status in individuals challenged with Norwalk virus. The relationship between the presence or absence of detectable rNV antibody and the subsequent development of gastrointestinal illness is shown in Fig. 1. The risk of illness was higher in individuals with preexisting antibody of any of the three subclasses. In addition, the mean $\log_2$ prechallenge serum rNV IgG titer was 10.2 ± 2.5 in subjects who became ill and 7.7 ± 2.6 in those who remained well ($P = 0.11$), and the mean level of prechallenge serum IgM and IgA was also higher in ill individuals. However, relatively few subjects remained well after Norwalk virus challenge, making assessment of the relationship between prechallenge antibody and illness difficult, and none of the differences seen were statistically significant. The risk of infection with Norwalk virus, as defined by significant antibody response or virus shedding, also was somewhat higher in subjects with evidence of prechallenge antibody (data not shown).

This study demonstrates that adults experimentally infected with Norwalk virus manifest serum IgG, IgM, and IgA responses to recombinant Norwalk virus capsid (rNV) antigen. Serum IgG EIA responses to rNV in volunteers challenged with Norwalk virus were highly correlated with responses measured previously by blocking EIA using antigen-containing stool samples from experimentally challenged volunteers (20). This confirms the validity of the previously reported blocking EIA for the Norwalk agent (20, 22) and suggests that epitopes present on intact Norwalk virion are largely represented in the second open reading frame of the cloned Norwalk virus 8FIIa genome.

Serum IgM responses to rNV were detected in all of the subjects who manifested serum IgG responses following

FIG. 1. Relationship between the presence of detectable prechallenge serum antibody and subsequent development of clinical gastroenteritis following oral challenge with Norwalk virus. Detectable antibody was defined as a titer of $\geq 1:40$ for IgG and $\geq 1:20$ for IgM and IgA. ■, ill subjects; ▨, well subjects.

Norwalk virus infection, including individuals with evidence of prior Norwalk virus infection. Serum IgM responses were more common in ill individuals than in those who had subclinical infection, but we also noted IgM responses in those with asymptomatic Norwalk virus infections. Similar results have been reported previously following both experimental and natural Norwalk virus infections by using Norwalk virus-containing stool samples as a source of antigen in a blocking immunoassay of IgM-containing serum fractions or by IgM capture assay (1, 5, 9). Serum IgA responses to rNV also were detected in all of the subjects who manifested serum IgG responses following Norwalk virus infection. Serum IgA responses to Norwalk virus infection have previously been demonstrated by capture EIA using Norwalk virus-containing stool as a source of antigen and appear to occur in most individuals who manifest blocking antibody responses to infection (10). Recently, fecal IgA responses to rNV after Norwalk virus infection have been reported (25).

While 83% of the subjects overall had detectable prechallenge serum IgG rNV antibody (i.e., a titer of $\geq 1:80$), only 21% of the subjects had serum IgM antibody titers of $\geq 1:40$ and only 40% had serum IgA antibody titers of this level prior to challenge. These results are consistent with the suggestion that the duration of the serum IgA and IgM response following infection is less than that of the IgG response (5, 9, 10). These findings also allowed us to evaluate the relationship of preexisting serum antibody to illness and infection following Norwalk virus challenge. We found that the risk of both infection and illness following challenge was somewhat higher in individuals with prechallenge antibody and that the level of prechallenge serum IgG, IgA, and IgM antibody was higher in those who subsequently developed illness or infection. These results are consistent with the suggestion that serum antibody is not protective against Norwalk virus infection (17, 26). If serum IgM antibody is relatively short-lived following infection,

then these data would suggest that even relatively recent infection with Norwalk virus or an antigenically related virus does not protect against symptomatic reinfection. Further studies of the duration of the IgM and IgA response as measured by this technique may help clarify this issue. It should also be noted that conflicting results regarding the protective effect of serum antibody have been obtained in studies of naturally acquired Norwalk virus infection in children living in the third world (2, 27).

Heterologous rNV IgG responses were detected in adults infected with Snow Mountain and Hawaii viruses, suggesting that some degree of antigenic relatedness, based at least in part on epitopes present on the virus capsid, exists between these viruses. Heterologous responses to rNV were more frequent than those seen by blocking EIA against Norwalk virus-containing stool samples and occurred in different subjects. The reasons for this discrepancy are unclear, but one potential explanation is that the heterologous responses seen by blocking assay are directed against epitopes that are not present on the rNV antigen and that the rNV IgG assay is more sensitive at detecting heterologous capsid responses than the blocking assay. Epitopes present in the nonstructural proteins encoded by Norwalk virus are immunoreactive (16, 23).

In contrast to the findings in subjects infected with Norwalk virus, serum rNV IgG antibody responses in subjects infected with Snow Mountain and Hawaii viruses were generally not accompanied by serum IgA and IgM responses to rNV. It is possible that this finding merely reflects a slightly decreased sensitivity of the serum IgA and IgM rNV assays, but IgA and IgM responses were detected even in those Norwalk virus-infected subjects who had modest rNV IgG responses comparable to those in Snow Mountain and Hawaii virus-infected subjects. Alternatively, it is possible that IgM and IgA, but not IgG, secondary responses are directed predominantly towards type-specific epitopes on the virus capsid. It has previously been suggested that IgM antibody to SRSVs is more type specific than IgG antibody, and this has been the basis of an IgM capture immune electron microscopy method of serotyping SRSVs (19). If the suggestion is true, then measurement of IgM antibody may represent a relatively specific marker for Norwalk virus infection in evaluation of gastroenteritis outbreaks.

The development of methods of cloning, sequencing, and expression of the genome of the Norwalk viruses will allow performance of further studies which should completely characterize the antigenic relationships between members of this group and clarify the currently confusing information regarding resistance to infection with these viruses.

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