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Detection of Immunoglobulin M (IgM), IgA, and IgG Norwalk Virus-Specific Antibodies by Indirect Enzyme-Linked Immunosorbent Assay with Baculovirus-Expressed Norwalk Virus Capsid Antigen in Adult Volunteers Challenged with Norwalk Virus

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Pre- and postexposure sera collected from 17 adult volunteers challenged with Norwalk virus as described previously (D. Y. Graham, X. Jiang, T. Tanaka, A. Opekun, P. Madore, and M. K. Estes, J. Infect. Dis. 170:34–43, 1994) were examined for Norwalk virus-specific immunoglobulin M (IgM), IgA, and IgG by indirect enzyme-linked immunosorbent assays with recombinant Norwalk virus antigen bound to the solid phase. Sixteen of the 17 volunteers had evidence of past infection, all presenting with preexisting IgG antibody of high avidity; only one volunteer had no evidence of previous infection. Virus infection was detected in 14 of the 16 volunteers with evidence of past infection, and 9 of the infected volunteers had symptomatic illness. A significant rise in both virus-specific IgA and IgG titers was detected after challenge in all of the volunteers who became ill. Five of the asymptomatic volunteers who were infected had rising titers of virus-specific IgG, but only two of the five had a concomitant rise in their virus-specific IgA antibody titers. Antibody rises were detectable in eight of nine ill volunteers 8 to 11 days after challenge but in the asymptomatic volunteers only after more than 15 days had elapsed. Virus-specific IgM was detected after challenge in all 14 infected volunteers. Between symptomatic and asymptomatic volunteers there were no significant differences in titers of virus-specific IgG and IgA in serum before challenge; however, there were significantly higher titers in symptomatic volunteers between 8 and >90 days after challenge for virus-specific IgG and 8 and 24 days after challenge for virus-specific IgA.

Norwalk virus infection is a major cause of epidemic gastroenteritis and is associated with outbreaks in schoolchildren and adults (16). Infection can be transmitted via contaminated water, contaminated shellfish, or contact with infected individuals (9, 11, 17, 20).

The protective effect of antibody produced after infection with Norwalk virus is poorly understood. Seroepidemiological studies carried out in the developing world indicated that antibody titers in the sera of >100 in children were associated with protection against subsequent Norwalk virus infections (1, 21). By contrast, volunteer studies among young adults in the United States suggest that a high antibody titer in serum before challenge may predispose the individual to illness (2). The presence of preexisting Norwalk virus-specific immunoglobulin A (IgA) does not either protect against infection or reduce the severity of symptoms. Although specific IgM has been detected in volunteers after challenge with Norwalk virus, the reported responses were weak and short-lived (4).

The majority of seroepidemiological studies have been performed with Norwalk virus antigens derived from fecal material collected from infected individuals (4, 15). Success in cloning and characterizing Norwalk virus-specific cDNA (13, 18) and in the subsequent production of recombinant Norwalk virus (rNV) antigen particles in the form of empty capsids (14) has provided a ready supply of antigen free of fecal contamination. Seroepidemiological studies performed with recombinant antigens indicate that they are immunologically reactive and specific for Norwalk virus antibody (7, 8). When rNV was used in an enzyme-linked immunosorbent assay (ELISA), antibody titers which were 16 to 40 times greater than those obtained by radioimmunoassays with native Norwalk virus were measured (8).

In the study described here, pre- and postexposure sera collected from 17 volunteers challenged with Norwalk virus as described previously (5) were examined for Norwalk virus-specific IgM, IgA, and IgG by indirect ELISAs with rNV particles bound to the solid phase. The Norwalk virus-specific IgG antibody avidity was determined in order to distinguish between primary infection and reinfection, as has been successfully demonstrated in other systems with pedigree sera (6, 10, 12, 22).

The wells of 96-well flat-bottom Falcon microtiter plates (Becton Dickinson UK Ltd., Oxford, United Kingdom) were coated with 100 μl of baculovirus-expressed rNV (1 μg/ml in 0.05 M carbonate-bicarbonate buffer [pH 9.6]), and the plates were incubated at room temperature for 4 h. The wells were then washed once with phosphate-buffered saline (PBS; pH 7.2), containing 0.05% Tween 20 and were blocked at 4°C overnight with 200 μl of PBS containing 5% skim milk powder. The wells were again washed two times as described above. In the IgG assay, 100 μl of doubling dilutions from 1 in 100 to 1 in 12,800 were added to the plate. An initial dilution of 1 in 100

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TABLE 1. Detection of Norwalk virus-specific IgM, IgA, and IgG antibodies in serial samples collected from adult volunteers to whom Norwalk virus was administered[a]

Volunteer[b]	Days after ingestion sample collected	Infected	Ill	IgM (IgG and IgA adsorbed)	IgA titer (IgG adsorbed)	IgG titer	Significant IgG rise within 8 to 11 days after infection
502A	0	Y	Y	Neg	<100	800	
	10			Neg	1,600	3,200	Yes
	16			Pos	>12,800	6,400	
	157			Neg	400	>12,800	
503A	0	Y	Y	Neg	200	6,400	
	10			Neg	>12,800	>12,800	Yes
	17			Pos	>12,800	>12,800	
505A	0	Y	Y	Neg	<100	100	
	11			Neg	400	1,600	Yes
	17			Pos	1,600	6,400	
	104			Neg	400	6,400	
509A	0	Y	Y	Neg	<100	800	
	11			Neg	>12,800	>12,800	Yes
	16			Pos	>12,800	>12,800	
	104			Pos	400	>12,800	
510A	0	Y	Y	Neg	<100	1,600	
	10			Neg	3,200	6,400	Yes
	24			Pos	>12,800	>12,800	
512B	0	Y	Y	Neg	<100	100	
	8			Neg	>12,800	6,400	Yes
	15			Pos	>12,800	6,400	
	96			Neg	200	6,400	
514B	0	Y	Y	Neg	<100	400	
	10			Pos	400	800	No
	18			Pos	6,400	>12,800	
	97			Pos	200	>12,800	
516B	0	Y	Y	Neg	<100	400	
	9			Pos	800	3,200	Yes
	16			Pos	3,200	>12,800	
520B	0	Y	Y	Neg	<100	400	
	10			Pos	1,600	1,600	Yes
	18			Pos	>12,800	>12,800	
	98			Pos	200	6,400	
501A	0	Y	N	Neg	<100	100	
	9			Pos	<100	100	No
	16			Pos	<100	400	
504A	0	Y	N	Neg	<100	3,200	
	9			Pos	200	3,200	No
	24			Pos	6,400	>12,800	
511B	0	Y	N	Neg	<100	800	
	8			Neg	<100	800	No
	15			Pos	<100	3,200	
	96			Neg	<100	1,600	
515B	0	Y	N	Neg	<100	200	
	10			Pos	<100	200	No
	17			Pos	800	6,400	
	98			Neg	<100	1,600	
518B	0	Y	N	Neg	<100	100	
	68			Pos	200	400	NA
	75			Pos	3,200	>12,800	
	160			Neg	<100	6,400	

Continued on following page

TABLE 1—*Continued*

Volunteer[b]	Days after ingestion sample collected	Infected	Ill	IgM (IgG and IgA adsorbed)	IgA titer (IgG adsorbed)	IgG titer	Significant IgG rise within 8 to 11 days after infection
507A	0	N	N	Neg	<100	400	
	11			Neg	<100	400	NA
	17			Neg	<100	400	
508A	0	N	N	Neg	<100	400	
	11			Neg	<100	400	NA
	16			Neg	<100	400	
	157			Neg	<100	400	
517B	0	N	N	Neg	<100	<100	
	68			Neg	<100	<100	NA
	75			Neg	<100	<100	
	157			Neg	<100	<100	

[a] Y, yes, N, no; NT, not tested; NA, not applicable.
[b] Numbering as given by Graham et al. (5).

has previously been determined to be the optimal lowest dilution (7).

In the IgA assay, sera were initially diluted 1 in 10 in goat anti-human IgG (5 μl of serum in 45 μl of anti-IgG [Incstar Corp., Stillwater, Minn.] treated for 30 min at room temperature), in order to remove blocking IgG (3), before being diluted from 1 in 100 to 1 in 12,800 in PBS containing 1% skim milk powder. In the IgM assay, sera were tested at a dilution of 1 in 80 after being pretreated either with goat anti-human IgG alone as described above or with both goat anti-human IgG and goat anti-human IgA (40 μl of anti-IgG-treated serum in 40 μl of anti-IgA [Sigma Chemical Co. Ltd., Poole, United Kingdom] overnight at 4°C) to reduce IgG and IgA; sera were then made up to a final dilution of 1 in 80 in PBS containing 1% skim milk powder. Selected sera were treated twice with anti-IgG and anti-IgA in order to estimate the efficiency of treatment. Positive and negative antibody controls, conjugate controls, and a substrate blank were included in each assay.

After the addition of the diluted serum to the antigen-coated plates the plates were incubated at 37°C for 2 h and were then washed five times as described above. One hundred microliters of horseradish peroxidase (HRPO)-conjugated rabbit anti-human IgG (DAKO Ltd., High Wycombe, United Kingdom) at a dilution of 1 in 1,000 in PBS containing 1% skim milk powder (conjugate diluent) or HRPO-conjugated goat anti-human IgA (Sigma) or HRPO-conjugated goat anti-human IgM (Sigma), both at a dilution of 1 in 2,000 in conjugate diluent, were added to all wells except the substrate blank. The plates were incubated at 37°C and were then washed five times as described above. One hundred microliters of tetramethylbenzidine (0.1 mg/ml in 0.05 M phosphate citrate buffer [pH 5.0] containing 2 μl of 30% hydrogen peroxide per 10 ml of buffer) was added to each well. After incubation at room temperature for 10 min, the reaction was stopped by the addition of 100 μl of 2 M sulfuric acid to each well, and the optical density (OD) was read at a wavelength of 450 nm. Serum dilutions with an OD greater than or equal to three times the mean OD of the negative control were regarded as Norwalk virus antibody positive.

Norwalk virus-specific IgG antibody avidity was determined by testing the samples in a modified ELISA in which 8 M urea was included in the first wash step in order to elute low-avidity antibody (12). Serum dilutions were added to each of two wells in a microtiter plate coated with rNV particles as described above. After incubation, the serum was aspirated and 300 μl of wash fluid containing 8 M urea was added to one well and 300 μl of wash fluid was added to the other well. The plate was then incubated at room temperature for 5 min. The wash fluid was aspirated and an additional 300 μl of wash fluid or wash fluid containing 8 M urea was added to the appropriate wells and the plate was again incubated at room temperature for 5 min. All wells of the plate were then washed three times with wash fluid and the assay was completed as described above. Two Norwalk low-avidity virus-specific IgG control serum samples obtained from young children and one high-avidity Norwalk virus-specific IgG control serum sample obtained from a healthy blood donor were included in each assay run. The OD values obtained in the presence of 8 M urea were compared with the values obtained when no urea was added, and samples that gave a reduction in the OD value of >50% in the presence of 8 M urea were regarded as containing a significant amount of low-avidity antibody.

TABLE 2. Difference in Norwalk virus-specific IgG antibody responses between infected volunteers with illness and volunteers with no illness

Days after challenge	Symptomatic volunteers			Asymptomatic volunteers			Difference in arithmetic means and significance[a]
	No. of serum samples	Arithmetic mean ± SD of log titers	Geometric mean titer	No. of serum samples	Arithmetic mean ± SD of log titers	Geometric mean titer	
0	9	2.73 ± 0.56	537	6	2.56 ± 0.51	447	0.17 ($P < 0.1$)
8–11	9	3.63 ± 0.52	4,266	6	2.65 ± 0.51	447	0.98 ($P = 0.003$)
15–24	9	4.20 ± 0.30	15,849	6	3.18 ± 0.84	1,513	1.02 ($P = 0.005$)
68–75	0			2	3.18 ± 0.84	1,513	
>90	6	4.10 ± 0.32	12,589	4	3.20 ± 0.48	1,584	0.90 ($P = 0.007$)

[a] *P* values were determined by the *t* test.

TABLE 3. Difference in Norwalk virus-specific IgA antibody responses between infected volunteers with illness and volunteers with no illness

Days after challenge	Symptomatic volunteers			Asymptomatic volunteers			Difference in arithmetic means and significance[a]
	No. of serum samples	Arithmetic mean ± SD of log titers	Geometric mean titer	No. of serum samples	Arithmetic mean ± SD of log titers	Geometric mean titer	
0	9	1.75 ± 0.20	56	6	1.69 ± 0.00	49	0.06 ($P > 0.1$)
8–11	9	3.46 ± 0.75	2,884	6	1.79 ± 0.24	62	1.67 ($P < 0.001$)
15–24	9	4.10 ± 0.47	12,589	6	2.24 ± 0.90	174	1.86 ($P < 0.001$)
68–75	0			2	2.90 ± 0.84	794	
>90	6	2.75 ± 0.82	562	4	2.30 ± 1.20	199	0.45 ($P > 0.1$)

[a] P values were determined by the t test.

The results of the indirect ELISAs for detecting Norwalk virus-specific IgM, IgA, and IgG antibodies are shown in Table 1. Sixteen of the 17 adult volunteers had evidence of past infection, 15 with preexisting IgG and one with preexisting IgG and IgA. Virus infection was detected in 14 of the 16 volunteers with evidence of past infection, and 9 of the infected volunteers were ill. Virus infection was not detected in the one volunteer (volunteer 517B) who had no detectable virus-specific antibody before challenge.

A significant rise in both virus-specific IgA and virus-specific IgG titers was detected after challenge in all the volunteers who became ill. Although all five of the asymptomatic volunteers who were infected had rising virus-specific IgG titers, only two of the five had a concomitant rise in their virus-specific IgA antibody titers. Significant rises (fourfold or greater) in virus-specific IgG titers were detected in samples collected during the first 8 to 11 days after challenge in eight of nine ill volunteers but were not detected in the asymptomatic volunteers until more than 15 days had elapsed (Table 1).

The Norwalk virus-specific antibodies detected before and after infection (10 to 160 days) were all high avidity (the geometric mean percentage reduction in titer in the presence of 8 M urea was 12.4%, with a range of 0 to 50%), whereas controls of recent infections (two children of 1 and 2 years of age) had reductions of 75 to 79%, indicating low-avidity antibodies, and a control of past infection (a healthy blood donor) had only a 12% reduction in titer (data not shown).

Virus-specific IgM antibody was detected in serum samples adsorbed with anti-IgG from 11 of 14 infected volunteers and 14 of 14 infected volunteers after adsorption with both anti-IgG and anti-IgA (Table 1). Virus-specific IgM antibody was detected earlier in five volunteers when adsorption with anti-IgG and anti-IgA was performed twice; however, in three of five patients detection was just borderline (within 10% of the cutoff OD value) (data not shown), suggesting that one cycle of adsorption was adequate in most circumstances, provided that adsorption was with both anti-IgG and anti-IgA.

Tables 2 and 3 show the differences in antibody titers of Norwalk virus-specific IgG and IgA antibodies, respectively, between infected volunteers with illness and those who remained asymptomatic. The titers of virus-specific IgG and IgA were significantly higher from 8 days to >90 days and from 8 days to 24 days postchallenge, respectively, in symptomatic volunteers compared with the titers in asymptomatic volunteers.

Detectable virus-specific IgM in the presence of high titers of IgA and IgG was a recurring feature in the majority of volunteers who became ill after challenge with Norwalk virus. Blacklow et al. (2) reported that volunteers who had preexisting antibody titers in serum detected in a radioimmunoassay with native Norwalk virus antigen were more likely to become ill after challenge with Norwalk virus. In the present study with rNV antigen, there was no significant difference, prechallenge, between infected volunteers who became ill and those with no symptoms in either virus-specific IgA or IgG titers (Tables 2 and 3, respectively). Two volunteers (volunteers 507A and 508A) with preexisting antibody and one volunteer (volunteer 517B) with no preexisting antibody failed to become infected after challenge (Table 1). This is in line with previous reports which described small subgroups of susceptible individuals who did not become infected, even upon repeated challenge, for unknown reasons (2, 15). Also, the kinetics of the antibody response to infection with Norwalk virus, particularly virus-specific IgG, were more rapid in volunteers who became ill than in those who were asymptomatic.

The presence of high-avidity Norwalk virus-specific IgG after challenge with Norwalk virus confirms reinfection in all of the volunteers who were infected (22) and suggests that antibodies detected before challenge were Norwalk virus specific and did not represent cross-reacting antibodies produced as a result of a previous infection with a different Norwalk-like virus. Similar findings have been reported for the serology of herpesviruses, in particular human herpesvirus 6 (23).

The results of the present serological study with rNV antigen in ELISAs for the detection of virus-specific IgM, IgA, and IgG antibodies are comparable to those obtained in studies with antigen derived from feces collected from individuals (2, 15, 19). The presence of preexisting virus-specific antibodies, whether IgG or IgA, does not protect against infection with Norwalk virus and may predispose an individual to reinfection. Infected individuals who become ill mount a more vigorous and rapid humoral response than individuals who have asymptomatic infections.

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