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Comparison of the Reactivities of Baculovirus-Expressed Recombinant Norwalk Virus Capsid Antigen with Those of the Native Norwalk Virus Antigen in Serologic Assays and Some Epidemiologic Observations

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Since the discovery of the Norwalk virus (NV) by immune electron microscopy (IEM) in 1972, serologic studies with this virus have relied on particle-positive fecal material from infected volunteers as the source of antigen because it has not been possible to propagate this virus in cell culture. However, the recent cloning of the NV (strain 8FIIa) genome and expression of the capsid protein in a baculovirus system to form "virus-like particles" has provided a consistent source of antigen (designated rNV). The purpose of the present study was to compare the antigenicities of these rNV particles with those of native NV antigen derived from human fecal material by using well-characterized sera obtained from earlier studies. In IEM studies, the rNV antigen reacted with NV-specific antibodies in a manner similar to that observed previously when particle-positive fecal material was used as antigen. In addition, a direct enzyme-linked immunosorbent assay, in which the rNV antigen was used as antigen, proved efficient and specific for the detection of serologic responses to NV compared with the previously established techniques of IEM and blocking antibody immunoassays in which particle-positive fecal material was used as the antigen. The availability of an unlimited source of antigen will enable serologic studies that will greatly increase our understanding of the epidemiology of NV and its role in human enteric illness.

Norwalk virus (NV), considered a member of the *Caliciviridae* family, has been identified as an important etiologic agent of acute epidemic gastroenteritis, being associated primarily with outbreaks in adults, school-age children, and family contacts (16). The 27-nm virus was identified by immune electron microscopy (IEM) in 1972 (19) in fecal material derived from a 1968 outbreak of gastroenteritis in Norwalk, Ohio (1). Subsequently, other morphologically similar viruses were associated with outbreaks of gastroenteritis and were characteristically named according to the geographical location of the outbreak, e.g., Hawaii, Montgomery County, and Snow Mountain agents (16). Studies of the antigenic relationships among the 27-nm group of viruses by IEM or cross-challenge studies indicated the existence of different serotypes as well as certain antigenic relationships.

Classification and biochemical and molecular analyses of these viruses have proven difficult because they could not be adapted to growth in tissue culture. Although NV induced subclinical infection in chimpanzees, all attempts to develop a practical laboratory animal model have been unsuccessful (26, 27). Thus, volunteer studies that were instrumental for the initial identification of this 27-nm virus were continued not only to gain an understanding of the natural history of NV and related viruses but also to generate fecal material containing NV for use as antigen.

In this report, we describe the evaluation of a recently developed recombinant NV capsid antigen (designated rNV) for use in NV serologic assays. This antigen was tested by using well-characterized paired sera from early studies in our laboratory. The development of this rNV antigen resulted from the recent cloning of the NV genome by Jiang et al. (12) and the subsequent identification and insertion of the gene encoding the 58,000-molecular-weight viral capsid protein (the major structural component of the virion) into a baculovirus expression vector (13). The recombinant capsid protein self-assembles into "empty" virus-like particles that are similar in size to NV (13). The rNV particles were tested for use as an antigen in IEM and enzyme-linked immunosorbent assay (ELISA) in the present study to compare their reactivities with that of native NV antigen derived from stool material. Comparison of the rNV ELISA with previously established methods for the detection of serologic responses to Norwalk virus and the application of this test to epidemiologic studies of NV and related viruses are described.

MATERIALS AND METHODS

Sera used in the analyses. The sera used in the present analyses and results of assays for antibody to NV by previously described techniques were obtained from the following: (i) an NV challenge study with chimpanzees (9, 26), (ii) NV challenge studies with adult volunteers (6, 17, 19, 25), (iii) patients involved in a Henryton II, Md., outbreak of gastroenteritis (3, 15), (iv) patients involved in a Morgantown, W.Va., outbreak of gastroenteritis (15, 20), (v) a Hawaii virus challenge study with adult volunteers (7, 19, 23, 25), (vi) a Montgomery County agent challenge study with adult volunteers (19, 23, 25), (vii) a W (Wollan) agent challenge study with adult volunteers (4, 15, 17), (viii) an epidemiologic survey of diarrheal illnesses in U.S. military troops during Operation Desert Shield (11), and (ix) a study of the illness and microbial experience of healthy infants and

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young children in residence at a welfare facility in Washington, D.C. (2, 15).

ELISA with baculovirus-expressed NV particles. The rNV ELISA was performed by the method of Jiang et al. (13), with slight modifications. Briefly, a 1:5,000 dilution of rNV particles (1 mg/ml in 0.01 M phosphate-buffered saline [PBS]) prepared as described previously (13) was made in PBS, and 75 μl was added to each well of a 96-well polyvinyl chloride, U-bottom microtiter plate (Dynatech). The antigen was allowed to adsorb for 4 h at room temperature. The plates were then washed once with 0.05% Tween 20–PBS, after which 200 μl of 5% BLOTTO in PBS was added to each well. After the plates were left overnight at 4°C, they were washed two times with 0.05% Tween 20–PBS. Serial twofold dilutions of pre- and postinfection sera were each added in duplicate wells, beginning at a 1:50 dilution in 1% BLOTTO–PBS in a volume of 75 μl per well. After 2 h at 37°C, the plates were washed six times with 0.05% Tween 20–PBS and 75 μl of a 1:5,000 dilution of conjugate (goat anti-human immunoglobulin G labelled with peroxidase and manufactured by Kirkegaard & Perry Laboratories, Inc.) in 1% BLOTTO–PBS was added to each well. After 1 h at 37°C, the plates were washed three times with 0.05% Tween 20–PBS and the signal was developed with 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) substrate (100 μl per well; Sigma). The A_{405} was determined with a Titertek spectrophotometer (Molecular Devices). In each experiment, a paired pre- and postinfection control serum sample with a previously demonstrated fourfold or greater increase in antibody to NV was titrated as a standard in at least one microtiter plate. An average optical density value from duplicate wells was calculated, and an average value of ≥0.200 (obtained on the basis of studies with known standards) was considered positive for NV-specific antibody. The rNV ELISA titer is reported as the reciprocal of the last dilution of serum that showed a positive antibody signal; a fourfold rise in antibody titer between preinfection (or acute-phase) and postinfection (or convalescent-phase) serum samples was considered a positive serologic response.

IEM. IEM was performed as described previously (17, 19), with some modifications, in a Siemens Elmiskop 1A electron microscope at a magnification of approximately ×50,000. Briefly, 100 μl of a 1:100 dilution of the rNV particles was made in PBS, and the mixture was incubated with an equal volume of a 1:5 dilution of pre- or postinfection serum for 1 h at room temperature. The mixture was then brought to a 1-ml volume with PBS and subjected to centrifugation for 1.5 h at 17,000 rpm in a Sorvall SS34 fixed-angle rotor. The supernatant was decanted and the pellet was suspended in approximately 10 μl of distilled water. The resuspended pellet was mixed with an equal volume of a 3% phosphotungstic acid negative stain and layered onto a carbon-coated Formvar 400 mesh grid; excess fluid was removed with the edge of a filter paper disk. Antibody ratings were scored from 1 to 4, and a response was considered to be a ≥1+ increase in rating between pre- and postinfection sera.

RESULTS

Jiang et al. (13) reported that the baculovirus-expressed NV capsid protein from strain 8FIIa forms virus-like particles. This was confirmed in our laboratory when a 1:100 dilution of the rNV capsid protein preparation was examined directly following negative staining (Fig. 1). The rNV particles appeared to be similar morphologically to the 27-nm NV, except that they were present almost exclusively as empty-appearing particles. In addition, they were abundant in number and were observed in most fields; this is in contrast to the native virus, which characteristically requires IEM and concentration by centrifugation for its visualization. It was noted that the outer capsid of certain rNV particles had a marked double capsid-like appearance. This feature has not been described with native NV, possibly because (i) it is almost always observed as a full particle and (ii) antibody was required for its recognition (19).

The reactivities of the rNV particles with reference paired sera obtained from a chimpanzee prior to and approximately 3 weeks following oral administration of NV were examined. A significant increase in antibody coating from 2+ to 4+ between the pre- and postinfection sera was demonstrated under code by IEM (Fig. 2). This final antibody rating was similar to that previously observed by IEM (1+ or 2+ to 4+) with these paired sera (from chimpanzee 14G) when the native NV was used as the antigen (9, 26). With the convalescent-phase serum, large and small aggregates, usually heavily coated with antibody, were observed readily; single particles and doublets, usually heavily coated with antibody, also were present. Aggregates, characteristically with lesser amounts of antibody, were observed with the prechallenge serum.

The efficiency and specificity of an ELISA in which the rNV is used as the antigen (13) were evaluated with a variety of reference sera previously studied by either IEM or blocking assays, or both, in which the native NV was used as antigen. As shown in Table 1, each of four chimpanzees challenged with NV had shown a serologic response by IEM and a radioimmunoassay-blocking test (RIA-BL) with the native NV as antigen (26). The ELISA in which the rNV was used as the antigen was as efficient as the established assays of IEM and RIA-BL in demonstrating a response. In addition, the rNV ELISA was more sensitive than the RIA-BL, with the titers obtained by the rNV ELISA reaching values 16 to more than 40 times greater than those obtained by RIA-BL (Table 1).

The efficiency of the rNV ELISA in detecting a serologic response to NV was compared with that of IEM (with 8FIIa in fecal material as the antigen) by using sera from eight human volunteers who participated in previously described NV challenge studies (6, 17, 19, 25). Sera from six of eight volunteers showed a serologic response to NV in the rNV ELISA (Table 2). Comparison of the serologic responses as determined by IEM or rNV ELISA indicated agreement in four of six determinations in which endpoints were available by IEM (i.e., volunteers 1 to 4, 6, and 7).

The rNV ELISA also proved to be specific when it was used to examine paired sera from individuals who developed gastroenteritis attributed to agents other than NV (as determined by IEM or challenge studies) (Table 3). Each of eight individuals who failed to develop a serologic response to NV as determined by IEM also did not respond as determined by the rNV ELISA. Of interest, volunteer 13, who was challenged with the Montgomery County agent, which was previously shown to be antigenically related to NV by IEM and in cross-challenge studies (19, 23, 25), developed a serologic response to NV by IEM and rNV ELISA.

Finally, 11 paired serum samples from U.S. military troops who were stationed in the Middle East during Operation Desert Shield and who had previously been tested for serologic responses to NV (11) by a blocking antibody immunoassay (BAI) similar to that described by Gary et al. (8) were examined by the rNV ELISA. There was total

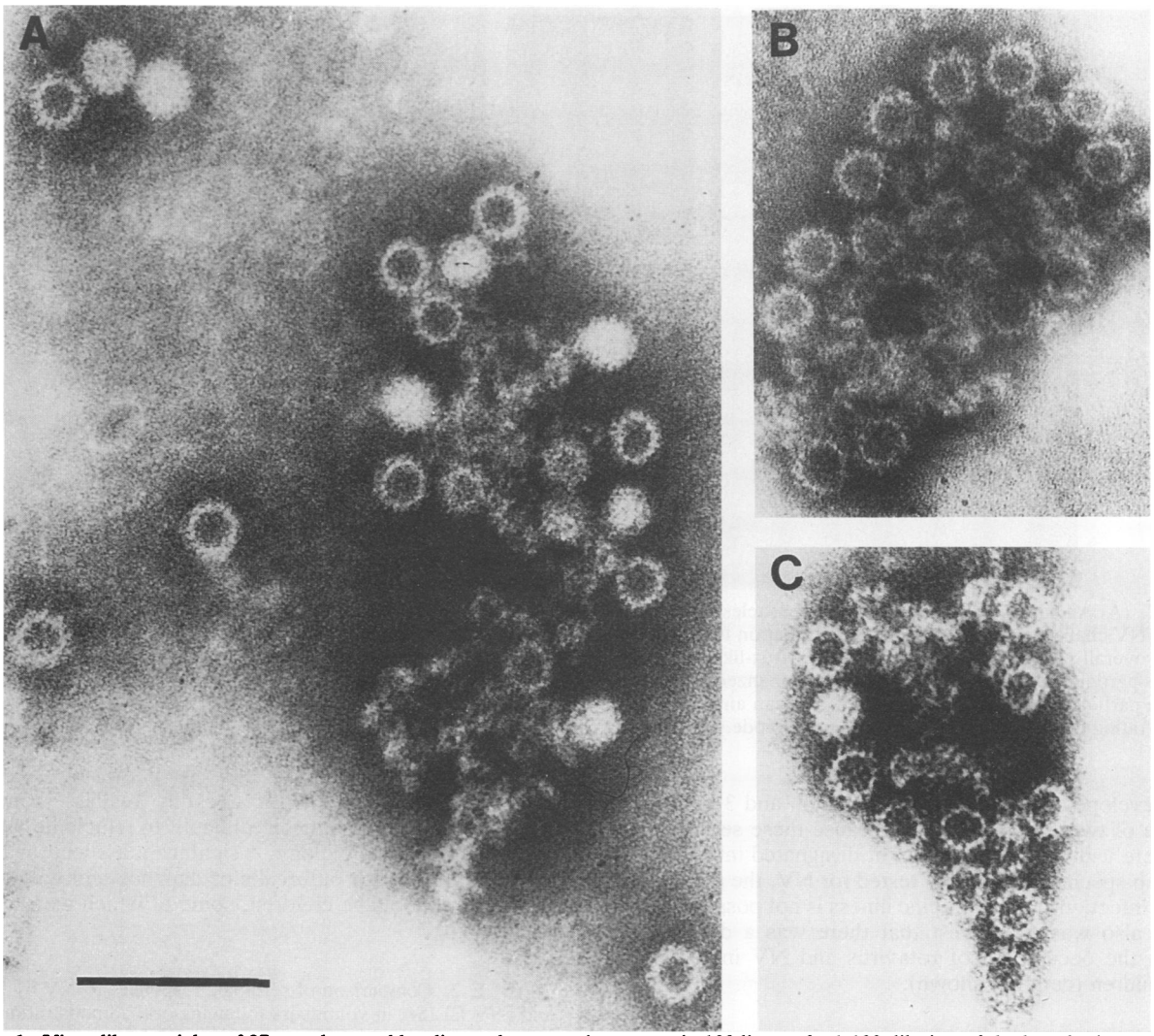

FIG. 1. Virus-like particles of 27 nm observed by direct electron microscopy in 100 liters of a 1:100 dilution of the baculovirus-expressed recombinant NV capsid protein after staining with 3% phosphotungstic acid. The virus-like particles were predominantly empty, readily seen in most fields, and similar to native NV, except that some had a double-shelled capsid appearance, a characteristic not reported with the native NV. Bar, 100 nm (for panels A, B, and C).

agreement in detecting serum antibody responses by the blocking assay in which NV was used as antigen and the rNV ELISA in that 9 of 11 (82%) individuals demonstrated a serologic response to NV in each of the assays (Table 4).

Thus, the recently developed rNV ELISA correlated to a high degree in efficiency and specificity with the established NV assays of IEM and blocking ELISA in which native NV was used as the antigen. There was total agreement in detecting the presence or absence of an antibody response in 28 of 30 paired serum samples ($P < 0.001$; Fisher exact test, two-tailed; the Kendall coefficient of association was +0.87). Seventeen serum samples had a fourfold or greater response by IEM, RIA-BL, BAI, and rNV ELISA; 2 serum samples did not have a fourfold or greater response by IEM, RIA-BL, or BAI but did have such a response by rNV ELISA; no samples had a response by IEM, RIA-BL, and BAI but no response by rNV ELISA; and 11 samples had no response by IEM, RIA-BL, BAI, or rNV ELISA.

The availability of an unlimited source of antigen for carrying out serologic assays for NV infection in various populations should enable the elucidation of the natural history of NV infection. In a preliminary study, we examined paired sera obtained during a longitudinal study of the illness and microbial experience of infants and young children in residence at a welfare facility in Washington, D.C. (Junior Village) (2, 15). A subset of 12 children who provided multiple serum samples was selected for this analysis, and a total of 51 serum samples were examined. The age at which the first serum sample was collected for this analysis, the number of sera collected from each child, the time interval for collection of the serum samples, and the number of fourfold or greater serologic responses to NV between sequential serum samples as determined by the rNV ELISA are presented in Table 5. The high rate of NV infection was surprising in that 8 of the 12 children (67%) developed at least one NV infection and 5 (42%) of these 12 individuals had evidence of two NV infections during a period of approximately 1 to 4 years, during which time the sequential sera were collected. Moreover, among 9 of these 12 children whose sequential sera were collected over a period of approximately 1 year, 6 of the 9

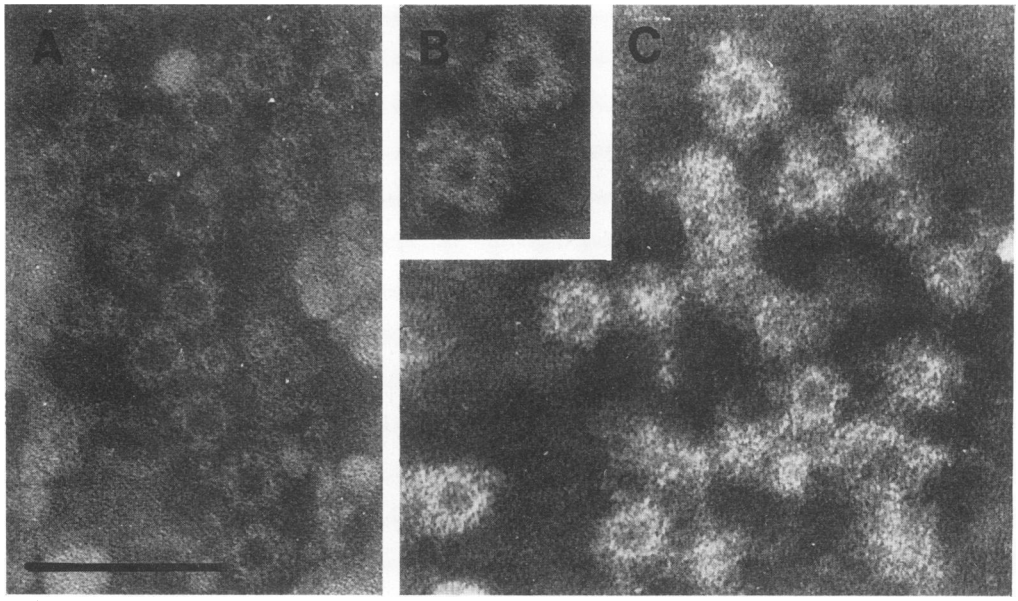

FIG. 2. (A) An aggregate of rNV virus-like particles observed after incubation of 100 μl of a 1:5 dilution of a chimpanzee's (chimpanzee 14G) pre-NV challenge serum and further preparation for IEM. The amount of antibody on this aggregate was rated 1+, and the serum was given an overall rating of 2+. (B) Two single virus-like particles. (C) More than 10 virus-like particles observed after incubation of rNV virus-like particles with the chimpanzee's (chimpanzee 14G) postinfection serum. These particles are heavily coated with antibody. The virus-like particles in panels B and C were given an antibody rating of 4+ and 3+ to 4+, respectively, and the serum was given an overall antibody rating of 4+. Ratings were made under code. Bar, 100 nm (for panels A, B, and C).

(67%) developed at least one NV infection and 3 (33%) had evidence of two NV infections. Because these serum specimens were usually obtained only at designated intervals and anal swab specimens were not tested for NV, the association of these infections with a specific illness is not possible at this time. It also was of interest that there was a dissociation between the occurrence of rotavirus and NV infections in these children (data not shown).

DISCUSSION

Large-scale epidemiologic studies as well as the development of routine laboratory diagnostic assays for NV have been hindered by the lack of a readily available source of antigen. Thus, it has been difficult to elucidate several aspects of NV epidemiology. A significant association of NV has been found with outbreaks of diarrhea and vomiting in adults and school-age children, some of which were related

TABLE 1. Serologic response of chimpanzees to NV following oral administration of a stool filtrate containing NV as determined by IEM, RIA-BL, and rNV ELISA[a]

Chimpanzee designation[b]	Serum sample	Serologic assay (antigen)		
		IEM rating (8FIIa)[c]	RIA-BL titer (8FIIa)[c]	ELISA titer (rNV)
14G	Preinfection	1+–2+	200	1,600
	Postinfection	**4+**	**≥5,000**	**102,400**
15G	Preinfection	3+	100	400
	Postinfection	**4+**	**10,000**	**405,600**
16G	Preinfection	1+	<25	<50
	Postinfection	**3+**	**400**	**6,400**
18G	Preinfection	1+–2+	200	1,600
	Postinfection	**4+**	**≥5,000**	**102,400**

[a] Data in boldface type indicate serologic responses as defined in the text.
[b] None of the chimpanzees developed illness following challenge.
[c] Previously reported, as described in the text.

TABLE 2. Comparison of serologic responses to NV by IEM and rNV ELISA in volunteers following oral administration of a stool filtrate containing NV[a]

Volunteer no.	Illness	Serum sample	IEM rating[b]	rNV ELISA titer
1	Yes	Preinfection	1+–2+	800
		Postinfection	**4+**	**12,800**
2	Yes	Preinfection	3+	3,200
		Postinfection	3+–4+, 4+	**≥102,400**
3	Yes	Preinfection	1+	400
		Postinfection	1+–2+	**1,600**
4	No	Preinfection	1+–2+	400
		Postinfection	**4+**	**12,800**
5	No	Preinfection	3+–4+	1,600
		Postinfection	4+	**25,600**
6	No	Preinfection	2+	1,600
		Postinfection	**4+**	**25,600**
7	No	Preinfection	1+, 1+–2+	800
		Postinfection	1+–2+	800
8	No	Preinfection	1+, 1+–2+	<50
		Postinfection	NR[c]	<50

[a] Data in boldface type indicate serologic responses as defined in the text.
[b] IEM rating previously reported, as described in the text.
[c] NR, not rated.

TABLE 3. Comparison of serologic responses to NV by IEM and rNV ELISA in adults infected with various agents of gastroenteritis[a]

Individual	Source of serum	Serum sample	IEM rating (8FIIa)[b]	rNV ELISA titer
1	Henryton II outbreak	Preinfection	1+, 1+–2+	3,200
		Postinfection	1+, 1+–2+	3,200
2	Henryton II outbreak	Preinfection	1+	3,200
		Postinfection	1+	3,200
3	Morgantown, W.Va., outbreak	Preinfection	1+	800
		Postinfection	1+, 1+–2+	800
4	Morgantown, W.Va., outbreak	Preinfection	1+	3,200
		Postinfection	1+–2+	1,600
5	W (Wollan) agent challenge	Preinfection	1+–2+	400
		Postinfection	1+, 1+–2+	200
6	W (Wollan) agent challenge	Preinfection	1+	1,600
		Postinfection	1+–2+, 2+	1,600
7	W (Wollan) agent challenge	Preinfection	1+, 1+–2+	200
		Postinfection	1+	200
8	Hawaii agent challenge (stool filtrate 21)	Preinfection	NT[c]	3,200
		Postinfection	NT	6,400
9	Hawaii agent challenge (stool filtrate 21)	Preinfection	NT	800
		Postinfection	NT	800
10	Hawaii agent challenge (stool filtrate 21)	Preinfection	2+	3,200
		Postinfection	2+, 2+–3+	3,200
11	Hawaii agent passage challenge (stool filtrate 21K1)	Preinfection	NT	1,600
		Postinfection	NT	800
12	Hawaii agent passage challenge (stool filtrate 21K1)	Preinfection	NT	400
		Postinfection	NT	400
13	Montgomery County agent challenge (stool filtrate 22)	Preinfection	3+	400
		Postinfection	4+	**3,200**
14	Montgomery County agent challenge (stool filtrate 22)	Preinfection	NT	1,600
		Postinfection	NT	800

[a] Data in boldface type indicate serologic responses as defined in the text.
[b] IEM rating previously reported, as described in the text.
[c] NT, not tested.

to consumption of contaminated food or water (16). However, the importance of NV in acute gastroenteritis—especially in infants and young children and elderly and immunocompromised individuals—is not clear. The natural history of the disease and the reservoir for the virus remain unknown. The mechanisms responsible for resistance or susceptibility to illness with the virus are not clear, and, interestingly, levels of NV-specific antibodies in serum have not been associated with long-term immunity to illness in challenge studies (5, 10, 14, 20, 21). The extent and role of antigenic diversity among NV and Norwalk-like viruses also remain to be established.

The use of the recently available rNV as a tool for the study of NV epidemiology was evaluated in the current study. An excellent correlation was observed between the efficiency of detecting a serologic response to NV by the rNV ELISA and the established techniques of IEM, RIA-BL, and BAI. Furthermore, the lack of detection of cross-reactive antibody responses in eight adult volunteers following challenge with the Hawaii or W (Wollan) agent was consistent with the specificity of the rNV ELISA for NV. The rNV particles functioned effectively as an antigen for IEM, demonstrating the presence of antigens on the baculovirus-expressed particles that react with specific serum antibodies. In summary, in our experiments rNV antigen performed similarly to native NV antigen derived from fecal material.

The rNV particles were used as antigen for the testing of sera from a long-term longitudinal epidemiologic study with infants and young children in Washington, D.C. (Junior Village). Screening of sequentially collected sera from these infants and young children demonstrated that NV infection

TABLE 4. Serologic response to NV in a subset of troops involved in Operation Desert Shield with illness characterized by vomiting as a predominant symptom as determined by a BAI and rNV ELISA[a]

Desert Shield troop no.	Serum sample	Norwalk BAI titer (8FIIa)[b]	rNV ELISA titer
1	Acute phase	20	50
	Convalescent phase	**320**	**400**
2	Acute phase	40	400
	Convalescent phase	80	800
3	Acute phase	20	100
	Convalescent phase	**80**	**400**
4	Acute phase	40	100
	Convalescent phase	40	100
5	Acute phase	40	100
	Convalescent phase	**1,280**	**3,200**
6	Acute phase	80	400
	Convalescent phase	**320**	**3,200**
7	Acute phase	10	<50
	Convalescent phase	**640**	**3,200**
8	Acute phase	40	100
	Convalescent phase	**1,280**	**12,800**
9	Acute phase	20	<50
	Convalescent phase	**1,280**	**12,800**
10	Acute phase	10	<50
	Convalescent phase	**1,280**	**6,400**
11	Acute phase	<10	<50
	Convalescent phase	**40**	**100**

[a] Data in boldface type indicate serologic responses as defined in the text.
[b] BAI data were previously reported by Hyams et al. (11).

TABLE 5. Summary of serologic responses to NV determined by the rNV ELISA in children from whom serum was periodically collected in the Junior Village, Washington, D.C., study

Individual	Age (mo) of individual when first serum sample was obtained for analysis	Total no. of serum samples from each individual tested for NV-specific antibody[a]	Total no. of ≥fourfold serum antibody responses to NV
1	7	6	2
2	9	3	0
3	13	3	0
4	21	3	1
5	7	6	2
6	9	5	2
7	20	3	2
8	9	5	2
9	7	5	1
10	20	4	0
11	17	3	0
12	10	5	1

[a] The total intervals between the time of examination of the first and last serum specimens were as follows: for individuals 3, 4, 5, 6, 8, 9, 10, 11, and 12, approximately 1 year; for individuals 2 and 7, approximately 2 years; and for individual 1, approximately 4 years.

may occur at a higher rate in this age group than has previously been reported (18, 22). Another finding with interesting implications was the observed NV reinfection rate (42%) in infants and young children in Junior Village. This may reflect high transmission rates because of close living conditions. Whether these infections and reinfections correlate with illness in this young age group will be examined elsewhere (8a).

The cloning of the NV genome and expression of the capsid protein have opened the way for rapid progress in the elucidation of NV epidemiology. For example, an association of NV infection with a vomiting illness in U.S. troops involved in Operation Desert Shield was demonstrated by examining by BAI 11 paired serum samples from the large military population. The availability of the rNV ELISA has allowed the screening of 404 additional paired serum samples from troops involved in Operation Desert Storm in order to determine the incidence of NV infection in the troops (11a). This analysis illustrates the utility of the assay for large-scale epidemiologic studies. In addition, the assay may offer insight into NV epidemiology when specimens obtained from efficacy trials for vaccines designed for the prevention of severe diarrheal disease caused by rotavirus and other enteric pathogens are examined. For example, in a D × RRV or DS1 × RRV reassortant rotavirus vaccine study in Finland described by Vesikari et al. (24), 77% of the diarrheal cases in rotavirus vaccinees or placebo recipients were considered to be nonrotavirus diarrhea. The rNV ELISA allowed analysis of several hundred serum samples from infants and young children (four sequential serum samples from each individual) enrolled in that vaccine study. Preliminary analysis indicates a high NV infection rate (approximately 49%) in these children over a 2-year period, consistent with the high infection rate (67%) reported in the limited study of U.S. children described here. Analysis of the role of NV infection in the rotavirus vaccine study is ongoing and will be reported elsewhere (20a).

Progress in the study of NV has lagged behind that of rotaviruses, both of which were discovered approximately 20 years ago. This has been attributed primarily to the lack of ability to cultivate NV in tissue culture and the relatively small quantities of NV virus particles shed in feces during infection (16). The molecular approach of using rNV for the elucidation of NV epidemiology is a major advance in the study of enteric viruses and will provide a new framework on which to establish the role of NV and other human enteric caliciviruses in acute gastroenteritis.

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