JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1993, p. 2866–2872
0095-1137/93/112866-07$02.00/0

Vol. 31, No. 11

Detection of Antibody to Recombinant Norwalk Virus Antigen in Specimens from Outbreaks of Gastroenteritis

STEPHAN S. MONROE,[1]* SARAH E. STINE,[1] XI JIANG,[2] MARY K. ESTES,[2] AND R. I. GLASS[1]

Viral Gastroenteritis Section, Respiratory and Enteric Viruses Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333,[1] and Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030[2]

Received 22 April 1993/Returned for modification 22 June 1993/Accepted 3 August 1993

Norwalk virus and other small round-structured viruses are commonly associated with outbreaks of gastroenteritis. We used a recently described recombinant-expressed Norwalk virus (rNV) capsid protein in enzyme immunoassays to quantitatively measure immunoglobulin G (IgG) and IgA to Norwalk virus in serum pairs from patients involved in outbreaks of gastroenteritis. The outbreaks previously were classified, on the basis of the results of a blocking antibody assay, as Norwalk virus negative, serologically intermediate, or Norwalk virus positive. The rNV IgG assay was more sensitive than the blocking assay for detecting IgG to Norwalk virus in serum from patients in all outbreak classes. There was 79% concordance between seroconversions detected by the blocking antibody assay and those detected by the rNV IgG assay. The rNV IgA assay detected seroconversions to Norwalk virus primarily in patients involved in outbreaks previously classified as Norwalk virus positive.

Norwalk virus is one of the most common causes of outbreaks of acute nonbacterial gastroenteritis (12). Many other viruses, including the Snow Mountain, Hawaii, and Taunton agents and several strains of small round-structured viruses (SRSVs) with physical properties similar to those of Norwalk virus, have been described in association with outbreaks of gastroenteritis (12). The uniform inability to adapt any of these Norwalk-like viruses to growth in cell culture has limited progress in their characterization, and until recently, all reagents used to study them have been derived from experimentally infected volunteers or patients involved in outbreaks of gastroenteritis. The recent descriptions of the genome organization of Norwalk virus (8, 11) and a Norwalk-like SRSV (15) have established that these viruses should be classified in the family *Caliciviridae*.

Characterization of SRSVs by immune electron microscopy with specimens from patients with gastroenteritis has demonstrated several examples of one-way and two-way antigenic cross-reactivity (16, 21). Although there is no widely accepted system for classifying SRSVs, it is generally agreed that there are at least four major antigenic types, with Norwalk, Hawaii, Snow Mountain, and Taunton viruses as the prototype strains (15, 17). The precise antigenic relationships among these viruses, however, await the availability of type-specific reagents with defined antigenic reactivities.

Blocking radioimmunoassays (RIAs) and enzyme immunoassays (EIAs) have been developed for detecting antibody to Norwalk virus by using specimens from experimentally infected human volunteers (2, 6, 19). Widespread application of these assays, however, has been limited by the difficulty in obtaining and standardizing human-derived antigen and antibody reagents. Similar assays have been developed for the Snow Mountain and Hawaii agents (18, 19, 25), but they have not been widely applied to the study of specimens from humans involved in outbreaks of gastroenteritis.

* Corresponding author.

Recently, the expression of the Norwalk virus capsid protein by using a baculovirus vector system was described (10, 11). The recombinant-expressed Norwalk virus (rNV) capsid protein self-assembles into empty virus-like particles that can be purified by density gradient centrifugation (10). The usefulness of these particles in a highly sensitive EIA for detecting Norwalk virus antibodies in experimentally infected human volunteers has been demonstrated (4, 5, 10, 24). The relative ease with which these particles can be generated and purified makes possible the large-scale preparation of a standardized Norwalk virus capsid antigen reagent. This report describes the use of rNV antigen for detecting Norwalk virus antibodies in specimens from patients involved in outbreaks of gastroenteritis previously classified as either Norwalk virus negative, serologically intermediate, or Norwalk virus positive.

MATERIALS AND METHODS

Blocking assay. Norwalk virus antibodies were measured by using a blocking biotin-avidin immunoassay (BAI) as described previously (2). Briefly, polyvinylchloride microtiter plates (Dynatech) were coated with convalescent-phase serum from an experimentally infected human volunteer (14) and were incubated successively with a stool specimen from a different volunteer, dilutions of the test serum, a biotin-conjugated convalescent-phase serum specimen from a third volunteer, horseradish peroxidase-conjugated strepavidin, and a chromogenic substrate. Antibody titers were expressed as the highest serum dilution that exhibited at least a 50% reduction in the specific absorbance value observed in the absence of test serum. A fourfold or greater increase in the blocking titer for paired specimens was considered to be evidence of seroconversion to Norwalk virus.

Recombinant antigen EIAs. Antibodies to Norwalk virus were measured by using a modification of a previously described method (10). Briefly, polyvinylchloride microtiter plates (Dynatech) were coated with either 1 μg of rNV per

ml in 0.01 M phosphate-buffered saline (PBS) (pH 7.4; positive antigen) or PBS alone (negative antigen) and were incubated successively with PBS–5% non-fat dry milk (Carnation), duplicate dilutions of the test serum in PBS–1% non-fat dry milk, alkaline phosphatase-conjugated goat anti-human immunoglobulin G (IgG) or goat anti-human IgA (Kirkegaard & Perry) in PBS–1% non-fat dry milk, and *p*-nitrophenylphosphate (Sigma) in diethanolamine buffer (pH 9.8). Plates were incubated in the dark for 3 h at room temperature, and absorbance was measured as the ratio of the values at 410 and 630 nm. Net absorbance ($P - N$) was calculated as the mean value in the antigen-coated wells (P) minus the mean value in the antigen-negative wells (N). Values with a net absorbance of ≥0.15 and a P/N value of ≥2.0 were considered positive. For the initial characterization of the assay, endpoint titers of the sera from volunteers were determined graphically by estimating the dilution that would result in a $P - N$ value of 0.15.

Serum specimens. Sera were collected from patient volunteers experimentally infected with Norwalk virus (14). The Norwalk virus reference serum specimen used in the present study (CDC DD0023) was obtained from a patient involved in a 1978 outbreak of gastroenteritis at a nursing home (outbreak 10 in Table 1 of reference 13). This outbreak was classified as Norwalk virus positive by blocking RIA, and a large volume of serum was obtained 15 weeks postonset from a patient with demonstrated seroconversion. We used serum specimen CDC DD0023 as a Norwalk virus assay reference serum because it was shown to have a high blocking titer by both RIA and BAI and it is available in a relatively large volume (~200 ml).

The Centers for Disease Control and Prevention (CDC) is involved in the investigation of approximately 25 outbreaks of nonbacterial gastroenteritis each year. Serum specimens from twenty outbreaks that occurred between 1985 and 1988 and that were previously analyzed by the Norwalk virus blocking antibody EIA were selected for the current study. These outbreaks included 5 previously classified as Norwalk virus negative (class I), 10 classified as serologically intermediate (class II), and 5 classified as Norwalk virus positive (class III) (3). Outbreaks were considered to be class I if less than 10 percent of the patients demonstrated a serologic response to Norwalk virus. Outbreaks were considered to be class II if 10 to 49% of the patients demonstrated a serologic response. Forty-two percent of the outbreaks classified in one survey were of class II (3), which probably includes outbreaks caused by SRSVs of other antigenic types that elicit a cross-reactive antibody response to Norwalk virus in some patients. Outbreaks were considered to be class III if at least 50 percent of the patients demonstrated a serologic response. Four serum specimen pairs were selected from each outbreak and included, when possible, both low- and high-level Norwalk antibody responses measured by the blocking EIA. The initial (acute-phase) and final (convalescent-phase) serum specimens were generally collected 1 to 7 and 21 to 35 days after the onset of symptoms, respectively. The sera were assayed at a 1:50 dilution for the rNV IgA assay and at a 1:2,000 dilution for the rNV IgG assay.

Data analysis. Serial dilutions of serum specimen CDC DD0023 were assayed on each plate, and the net absorbance as a function of antibody units was fitted to a four-parameter logistic-log curve (22) by using iterative fitting with the NonLin module of the SYSTAT program (SYSTAT, Inc.). The four-parameter logistic-log function was defined as follows: $ABS = d + \{(a - d)/[1 + (units/c)^b]\}$, where ABS is the net absorbance ($P - N$); parameters a and d are the lower and upper asymptotes, respectively, representing the theoretical ABS at time zero and infinite concentrations, respectively; c is the antibody level at the inflection point of the curve; and b is related to the slope of the curve. For the reference serum dilutions, units are calculated as the arbitrarily assigned stock concentration divided by the dilution factor. The resulting parameters then were used to extrapolate antibody units for each of the test sera, as follows: $units = c \times [(a - ABS)/(ABS - d)]^{(1/b)}$. The extrapolated units were multiplied by the appropriate dilution factor to calculate the total Norwalk virus antibody units in the test serum. A fourfold or greater increase in antibody units for paired serum specimens was considered to be evidence of seroconversion to Norwalk virus positivity.

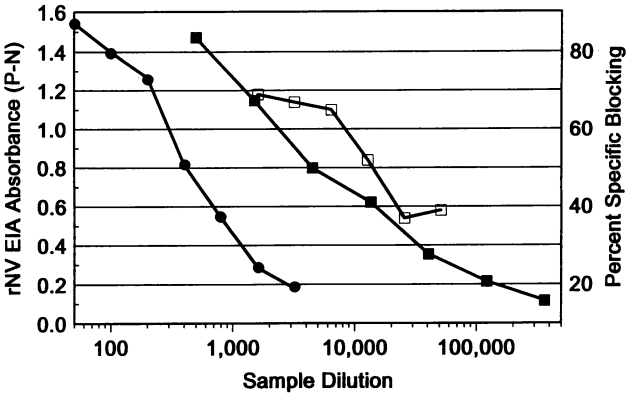

FIG. 1. Comparison of EIA formats. Reference serum specimen CDC DD0023 from a patient involved in an outbreak of Norwalk virus gastroenteritis was assayed by the blocking BAI (open squares), the rNV IgG EIA (solid squares), and the rNV IgA EIA (solid circles). Results are presented as percent specific blocking on the right axis for the BAI and as net absorbance ($P - N$) on the left axis for the rNV EIAs.

RESULTS

Standardization of the rNV IgG and IgA assays. A direct comparison of data obtained with reference serum specimen CDC DD0023 by the blocking BAI and the rNV IgG EIA demonstrated the wider working range and higher sensitivity of the latter test (Fig. 1). In the BAI, the specific blocking of the reference serum specimen fell sharply over the fourfold range between upper and lower plateau values at dilutions of 1:6,400 and 1:25,600, respectively, with a 50% blocking titer of 12,800. In contrast, when the rNV IgG test was used, this serum specimen gave a smooth response over the greater than 700-fold range from 1:500 to 1:364,500. At cutoff values of a $P - N$ of ≥0.15 and a P/N of ≥2.0, this reference serum had an IgG endpoint titer of greater than 200,000. In subsequent experiments, a different preparation of the rNV antigen and a slightly higher dilution of the goat anti-human IgG conjugate were used, resulting in an endpoint titer for serum specimen CDC DD0023 of approximately 50,000. In the rNV IgA test, the reference serum specimen gave a response from 1:50 to 1:3,200.

To validate the use of serum specimen CDC DD0023 as a Norwalk virus reference reagent, it was first necessary to compare the dose-response curves obtained in the rNV IgG assay with this serum specimen with those obtained with sera from experimentally infected volunteers (Fig. 2). Endpoint titers of convalescent-phase sera from four experimen-

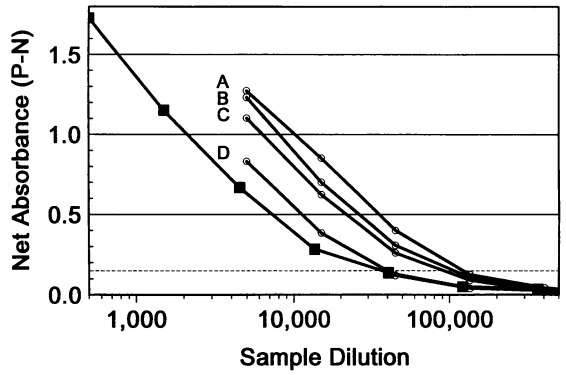

FIG. 2. Dose-response comparison of reference and test serum samples. Convalescent-phase sera from four volunteers (volunteers A to D; open circles) and the CDC DD0023 reference serum specimen (filled squares) were assayed by the rNV IgG EIA. Net absorbance ($P - N$) is plotted as a function of sample dilution. The dashed horizontal line at $P - N = 0.15$ indicates the cutoff value used to estimate endpoint titers.

tally infected volunteers were determined by serial dilution. Serum specimen CDC DD0023 was less reactive than the convalescent-phase serum specimens from volunteers and had an endpoint titer of 37,000 in this experiment (Fig. 2). Importantly, the log-linear portion of the dose-response curve with serum specimen CDC DD0023 was roughly parallel to those obtained with the serum specimens from volunteers. These parallel dose-response curves indicated that antibody values could be determined by comparing the EIA response at a single test dilution with a standard curve generated by serial dilution of the reference serum.

Four-parameter logistic-log curve fitting. Many methods have been used to approximate the sigmoidal dose-response typical of EIAs (23). We choose to use the four-parameter logistic-log model since it fits the experimental data very accurately ($r^2 = 0.996$) (Fig. 3) and does not require prior knowledge of the maximum and minimum asymptotes (22). On the basis of the endpoint titers determined during initial optimization of the rNV assays, the reference serum specimen CDC DD0023 was assigned arbitrary values of 200,000 IgG units and 4,000 IgA units per ml. To avoid the high variation inherent in making estimates from net absorbance values near the asymptotes, we restricted the extrapolation to $P - N$ values of between 5 and 90% of the total assay range (upper asymptote minus lower asymptote). This procedure generally resulted in about a 200-fold useful assay range from 4 to 800 IgG units (Fig. 3).

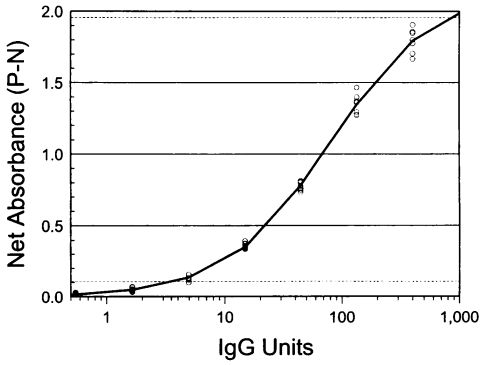

FIG. 3. Curve fitting for calculation of antibody units. Reference serum specimen CDC DD0023 was serially diluted beginning at 1:500 and assayed in parallel on seven plates by the rNV IgG EIA. The net absorbance of each replicate at each dilution is represented by open circles. A four-parameter logistic-log curve was fit to the datum points as described in Materials and Methods and is plotted as a solid line. The dashed horizontal lines at 5 and 90% of the theoretical assay range (upper asymptote minus lower asymptote) mark the limits of the absorbance values used for extrapolation of unknown antibody levels.

To assess the validity of the curve-fitting approach, we calculated IgG units for each dilution that was considered EIA positive, using the sera from four experimentally infected volunteers. The calculated IgG units were proportional to the endpoint titers estimated by visual inspection (Fig. 2), and the standard deviation between the values determined at different dilutions of the same serum was 16% or less (data not shown).

Analysis of sera from patients involved in outbreaks of gastroenteritis. We examined the responses in the rNV EIAs of selected serum pairs from patients involved in outbreaks previously characterized as class I, II, and III to address three questions: (i) Do the rNV EIAs detect Norwalk virus antibodies in sera from patients in all three types of outbreaks? (ii) Is there a correlation between the values obtained with an individual serum specimen by using the blocking BAI and the rNV IgG EIA? (iii) Is there a correlation between seroconversions measured by the blocking BAI and those measured by the rNV IgG EIA?

Comparison of assay results for individual sera. A comparison of the results obtained with 78 convalescent-phase serum specimens from representative serum specimens of each gastroenteritis outbreak class showed a general correlation between the blocking titers measured by the BAI and the antibody units calculated from the rNV IgG EIA (Fig. 4). There was, however, more scatter in the calculated antibody units for sera with low blocking titers. This is due, in part, to the higher sensitivity of the rNV EIA, which detects IgG antibodies in serum specimens that were at or below the limit of detection of the blocking BAI.

Comparison of assay results for serum specimen pairs. We found good agreement between the results obtained by the blocking BAI and those obtained by the rNV IgG EIA using the definition of a fourfold or greater increase in antibody titer or IgG units as an indicator of seroconversion to Norwalk virus positivity (Fig. 5). Twenty-two serum specimen pairs showed seroconversions by both assays and 40 were negative by both assays, yielding a total of 62 (79%) concordant results. Five serum specimen pairs (6%) showed seroconversions by the rNV IgG EIA but not by the blocking BAI. Eleven serum specimen pairs (14%) showed seroconversions by the blocking BAI but not by the rNV IgG EIA.

An examination of the initial and final Norwalk virus IgG levels in the serum specimen pairs suggested explanations for some of the discordant seroconversion results (Fig. 6). In Fig. 6, each serum specimen pair is represented by a symbol at the intersection of the IgG units in the initial serum specimen (abscissa) and the IgG units in the final serum specimen (ordinate). Points which fall along the solid line indicate no change in antibody units from the initial to the final serum specimens. Points on or above the dashed line indicate a fourfold or greater increase in antibody units and represent seroconversions.

Most of the serum specimen pairs in which no seroconversion was detected by the blocking BAI fell close to the line of identity by the IgG EIA, indicating no change in the level of IgG to rNV antigen. Eleven serum specimen pairs

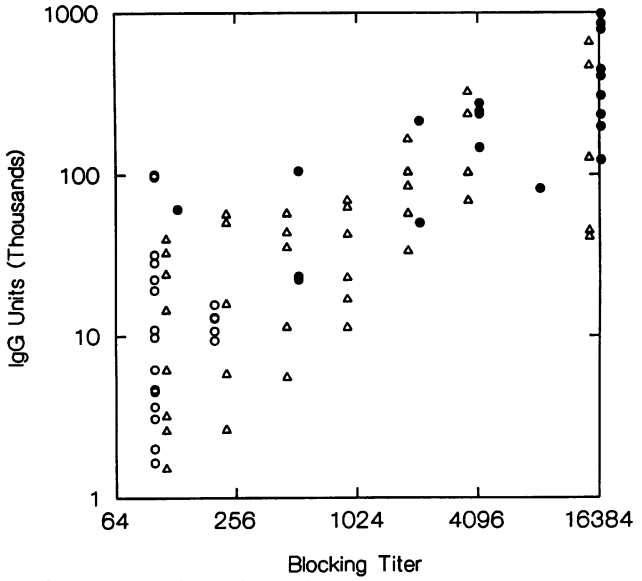

FIG. 4. Comparison of blocking titer and rNV IgG units for 78 convalescent-phase serum specimens from patients involved in 20 outbreaks of gastroenteritis. Each symbol represents a single convalescent-phase serum specimen assayed by both tests. The symbol type indicates the classification of the outbreak as determined by the blocking BAI, as follows: open circles, sera from patients involved in Norwalk virus-negative outbreaks (class I); open triangles, sera from patients involved in serologically intermediate outbreaks (class II); solid circles, sera from patients involved in Norwalk virus-positive outbreaks (class III). The triangles and solid circles are offset slightly along the x axis to minimize overplotting of symbols.

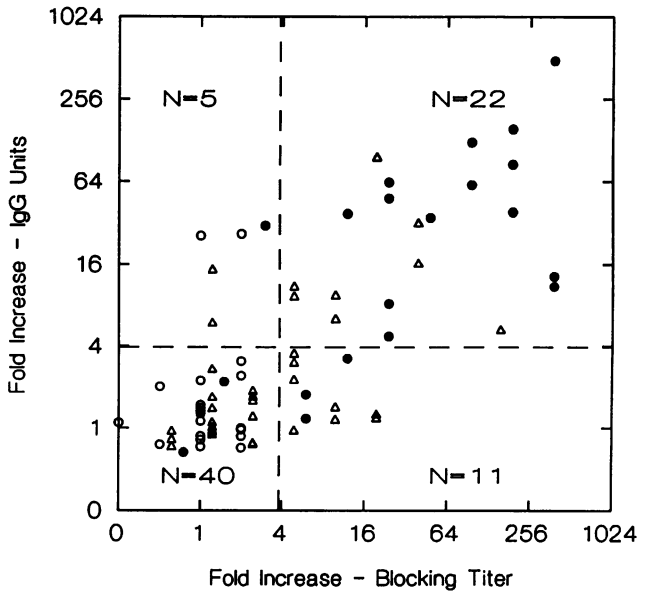

FIG. 5. Comparison of seroconversions by blocking titer and rNV IgG EIAs. Each symbol represents the fold increase in antibody response for a single serum specimen pair assayed by both tests. The symbol type indicates the classification of the outbreak as described in the legend to Fig. 3. The dashed vertical and horizontal lines indicate the fourfold minimum increases that define a positive seroconversion for the blocking BAI and rNV IgG EIA, respectively. Points to the right of the vertical dashed line are seroconversions defined by a greater than or equal to fourfold increase in blocking titer. Points above the horizontal dashed line are seroconversions defined by a greater than or equal to fourfold increase in IgG units. The N values are the number of serum pairs represented in each quadrant of the plot.

were considered positive in the blocking test but negative in the rNV IgG test (Fig. 6). Four of these pairs, with IgG increases of between two- and fourfold, were examined in greater detail. Of these four pairs of serum specimens, three were from patients from whom the acute-phase serum was collected 4 or more days after the onset of symptoms. In such patients, a previous exposure to a Norwalk-like virus may have resulted in an increase in antibody to rNV before collection of the acute-phase serum specimen, thereby artificially depressing the magnitude of the rise for the serum specimen pair. For the other serum specimen pair in this group, the acute-phase specimen was collected on the day that symptoms began but the calculated IgG level was higher than that in the CDC DD0023 reference serum specimen. The magnitude of the increase for this patient's serum may have been limited by the high level of antibody in the acute-phase serum. The remaining seven serum specimen pairs that were seroconversions by the blocking assay but not by the rNV IgG assay were close to the line of identity in the IgG test, indicating no change in Norwalk virus-specific antibody when using the rNV antigen.

Five seroconversions were detected by the IgG EIA, but not by the blocking BAI (Fig. 6). All of these serum specimen pairs had very low or no detectable antibody in the initial serum specimen and relatively low levels (<100,000 units) of antibody in the final serum specimen. Thus, these pairs correspond to weak seroconversions that were below the level of detection of the blocking antibody test.

Two aspects of the unusual nature of the human serologic response to Norwalk virus are demonstrated by the data in Fig. 6. First, there was a wide distribution of antibody levels in the initial serum specimens from patients involved in outbreaks of gastroenteritis, from less than 2,000 to greater than 240,000 IgG units. Second, the absence or presence of antibodies in the initial serum specimen did not correlate with protection against the occurrence of a subsequent seroconversion.

Comparison of results for specimens from Norwalk virus-negative, serologically intermediate, and Norwalk virus-positive outbreaks. The samples we chose for analysis were originally divided into three classes on the basis of the percent seroconversions detected by the blocking BAI (3). Since only four serum specimen pairs were selected from each outbreak for the current analysis, we examined not only the number of seroconversions but also the median fold increase in antibody units within each outbreak (Fig. 7). Of the 20 serum specimen pairs from patients involved in class I outbreaks (outbreaks A to E), only two had seroconversions that were detectable by the recombinant IgG assay (Fig. 7A). Both of these pairs were from outbreak C, and in each case, the IgG units in the initial serum specimen were very low and the final serum specimen had moderate IgG levels, resulting in weak positive seroconversions. Sera from the 10 patients involved in class II outbreaks (outbreaks F to O) typically had one or more seroconversions to Norwalk virus, but the median IgG increase for each outbreak was generally less than fourfold. Most of the serum pairs from the five patients involved in class III outbreaks showed seroconversions by the rNV IgG EIA, with a median increase for the group of 36-fold.

While the antibody responses detected by the rNV IgG

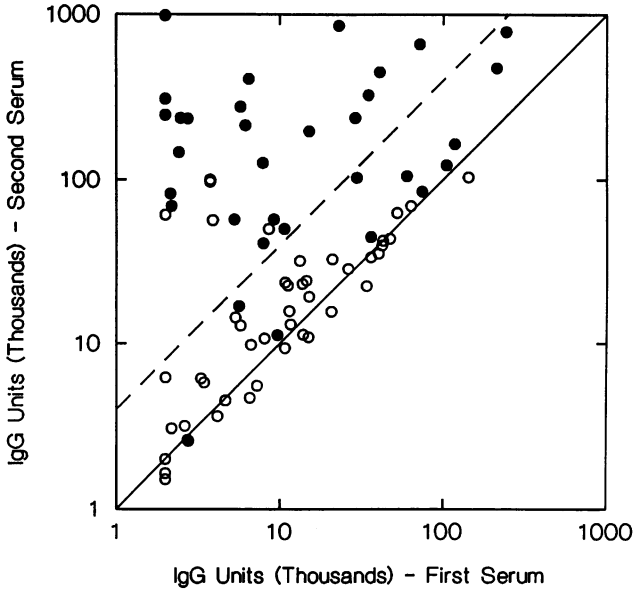

FIG. 6. Comparison of acute- and convalescent-phase IgG levels in 80 serum specimen pairs from patients involved in 20 outbreaks of gastroenteritis. Each symbol is plotted at the intersection of the IgG units calculated for the acute-phase (abscissa) and the convalescent-phase (ordinate) serum specimens. The solid diagonal line corresponds to identical values in the two serum specimens. The dashed diagonal line corresponds to final values that were fourfold greater than the initial values. Open and filled circles are serum specimen pairs that were negative and positive seroconversions, respectively, as determined by the blocking BAI.

EIA were similar to those obtained by the blocking assay, a different pattern of immune reactivity was detected with the rNV IgA EIA. Few of the serum specimen pairs from either the class I or class II outbreak showed IgA seroconversions to rNV antigen. Most of the serum specimen pairs from patients involved in the class III outbreaks, in contrast, had IgA seroconversions, with a median increase of 14-fold (Fig. 7B).

DISCUSSION

The widespread application of the previously available blocking assays for measuring antibodies to Norwalk virus was limited by the difficulty in obtaining and standardizing the necessary human-derived reagents. The recent cloning and expression of rNV capsid protein by using baculovirus recombinants (10) has provided a replenishable supply of purified antigen for developing serologic assays that are far simpler in design than the blocking EIAs. The wide dose-response range and excellent reproducibility of the rNV EIAs enabled us to quantitatively determine antibody levels from the absorbance obtained at a single test serum dilution rather than determining endpoint titers by multiple serial dilutions. This procedure increases the number of samples that can be analyzed and allows for automation of the calculation of results.

Although we have previously shown the rNV EIA to be highly sensitive for detecting antibodies to Norwalk virus in serum specimens from experimentally infected volunteers (10), it was important to determine whether this assay format also would detect antibodies and seroconversions in sera from patients involved in outbreaks of gastroenteritis previously associated with Norwalk and Norwalk-like SRSVs.

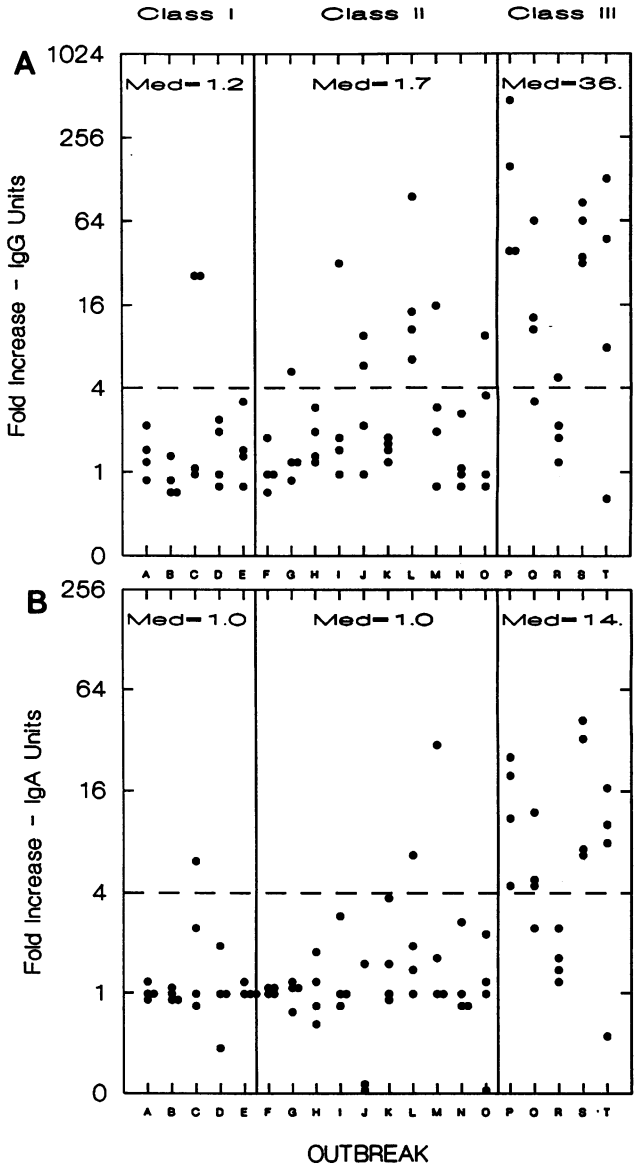

FIG. 7. Analysis of antibody rises by outbreak category. The letters along the abscissa are arbitrary designations for each of the 20 outbreaks analyzed. The filled circles represent the antibody rises for an individual serum specimen pair in either the IgG EIA (A) or the IgA EIA (B). The horizontal dashed line indicates a fourfold increase used as the minimum rise for defining seroconversions. The vertical lines divide the outbreaks into Norwalk virus-negative, serologically intermediate, or Norwalk virus-positive categories on the basis of the percent seroconversions detected in the blocking test. The median antibody increase for each outbreak category is indicated.

Our results show that for antibody levels in individual serum specimens and seroconversions, the agreement between the results obtained by the blocking BAI and the recombinant antigen IgG EIA is good. Seven serum specimen pairs that demonstrated seroconversions by the blocking test showed no change in antibody units in the rNV IgG EIA (Fig. 6). These discordant results may indicate that the blocking test detects antibody responses to epitopes not present in the recombinant antigen EIA format. In particular, the impor-

tance of antibodies to the products of the first and third open reading frames (4, 8, 20), which are not present in the current antigen preparation, needs to be addressed. Other studies indicated that epitopes in the 2C-like region of the first open reading frame are immunoreactive (11, 20). It also is possible, however, that these results are due to nonspecific blocking activity originally detected in these specimens by using the human reagent-dependent assay. The five serum specimen pairs that were negative by the blocking test and positive by the rNV IgG EIA all had acute-phase antibody levels of <10,000 IgG units, at or near the lower limit of the newer assay, with convalescent-phase antibody levels of ≤100,000 IgG units. The relatively low convalescent-phase antibody levels and the incremental nature of the blocking assay titers may have resulted in the classification of relatively weak seroconversions as negative by the original test.

The lack of standardized reagents has restricted progress in the elucidation of the antigenic relatedness between Norwalk virus and other viruses classified by electron microscopy as SRSVs. Previous work has demonstrated serologic cross-reactivity between several of these viruses by either blocking EIA (18), immune electron microscopy (16, 21), or Western blot (immunoblot) assays (7). We have somewhat arbitrarily used 50% of patients with seroconversions as a cutoff for concluding that an outbreak of gastroenteritis is associated with Norwalk virus infection (class III). However, our analysis of blocking antibody results from 100 outbreaks demonstrated that the distinction between the class II and class III outbreaks is not clear (3). Rather, there is a fairly continuous distribution of the antibody response when either the percentage of patients with seroconversions or the geometric mean titer rise is used as a measure of antigenic relatedness (data not shown). In the current analysis, by using the rNV IgG EIA with a more limited serum sample set, the median IgG increases in patients involved in the class III outbreaks tended to be higher than those in patients involved in the class II outbreaks (Fig. 6A). The values obtained with any given outbreak, however, overlap between the groups, and classification of outbreaks on the basis of either percent seroconversion or median IgG increase remains arbitrary. While recognizing this limitation, we will continue to use IgG seroconversions in 10 or 50% of patients as working criteria for classifying outbreaks as class II or class III, respectively. The IgA response, in contrast, appears to be more "type specific," and individual fourfold increases were rarely associated with outbreaks previously classified as class I or class II (Fig. 7B). This subclass specificity has also been observed in the antibody responses of volunteers experimentally infected with Norwalk, Snow Mountain, or Hawaii viruses (24). On the basis of these observations, we will consider rNV IgA seroconversions in 50% or more of patients to indicate that an outbreak is associated with Norwalk virus infection.

The results obtained with the recombinant antigen-based EIAs suggest that two of the outbreaks were incorrectly classified by the blocking BAI. Two of four serum specimen pairs from patients involved in outbreak C, originally assigned to class I, showed detectable seroconversions in the rNV IgG assay (Fig. 7A). Thus, this outbreak would be assigned to class II on the basis of the results of the newer test. Only one of four serum specimen pairs from outbreak R showed a seroconversion by the rNV IgG test, and none of the four showed a seroconversion by the rNV IgA test (Fig. 7). This outbreak was originally assigned to class III on the basis of several weak seroconversions detected by the blocking assay but would be characterized as class II on the basis of the results of the rNV EIAs.

The availability of the genomic RNA sequence for Norwalk virus (8, 11, 20) has made possible the design of oligonucleotide primers for amplifying the Norwalk and Norwalk-like SRSVs present in stool samples from patients with gastroenteritis (1, 4, 9). This development will allow the genetic characterization of viruses associated with outbreaks of gastroenteritis and should begin to identify regions of the genome encoding major epitopes. This genetic information, together with serologic information from recombinant antigen-based assays, should allow a clearer understanding of the evolutionary and antigenic relatedness among the numerous viruses currently classified as SRSVs.

REFERENCES

1. **De Leon, R., S. M. Matsui, R. S. Baric, J. E. Herrmann, N. R. Blacklow, H. B. Greenberg, and M. D. Sobsey.** 1992. Detection of Norwalk virus in stool specimens by reverse transcriptase-polymerase chain reaction and nonradioactive oligoprobes. J. Clin. Microbiol. **30**:3151–3157.
2. **Gary, G. W., Jr., J. E. Kaplan, S. E. Stine, and L. J. Anderson.** 1985. Detection of Norwalk virus antibodies and antigen with a biotin-avidin immunoassay. J. Clin. Microbiol. **22**:274–278.
3. **Glass, R. I., S. S. Monroe, S. Stine, P. Madore, D. Lewis, D. Cubitt, G. Grohmann, and C. Ashley.** 1989. Small round structured viruses: the Norwalk family of agents, p. 87–90. *In* M. J. G. Farthing (ed.), Viruses and the gut. Swan Press, Ltd., London.
4. **Graham, D. Y., X. Jiang, T. Tanaka, A. R. Opekun, H. P. Madore, and M. K. Estes.** Norwalk virus infection of volunteers: new insights based on improved assays. Submitted for publication.
5. **Green, K. Y., J. F. Lew, X. Jiang, A. Z. Kapikian, and M. K. Estes.** 1993. 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. J. Clin. Microbiol. **31**:2185–2191.
6. **Greenberg, H. B., R. G. Wyatt, J. Valedsuso, A. R. Kalica, W. T. London, R. M. Chanock, and A. Z. Kapikian.** 1978. Solid-phase microtiter radioimmunoassay for detection of the Norwalk strain of acute nonbacterial, epidemic gastroenteritis virus and its antibodies. J. Med. Virol. **2**:97–108.
7. **Hayashi, Y., T. Ando, E. Utagawa, S. Sekine, S. Okada, K. Tabuchi, M. Takashi, and M. Ohashi.** 1989. Western blot (immunoblot) assay of small, round-structured virus associated with an acute gastroenteritis outbreak in Tokyo. J. Clin. Microbiol. **27**:1728–1733.
8. **Jiang, X., D. Y. Graham, K. Wang, and M. K. Estes.** 1990. Norwalk virus genome cloning and characterization. Science **250**:1580–1583.
9. **Jiang, X., J. Wang, D. Y. Graham, and M. K. Estes.** 1992. Detection of Norwalk virus in stool by polymerase chain reaction. J. Clin. Microbiol. **30**:2529–2534.
10. **Jiang, X., M. Wang, D. Y. Graham, and M. K. Estes.** 1992. Expression, self-assembly, and antigenicity of the Norwalk virus capsid protein. J. Virol. **66**:6527–6532.
11. **Jiang, X., M. Wang, K. Wang, and M. K. Estes.** 1993. Sequence and genomic organization of Norwalk virus. Virology **195**:51–61.
12. **Kapikian, A. Z., and R. M. Chanock.** 1990. Norwalk group of viruses, p. 671–693. *In* B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsch, J. L. Melnick, T. P. Monath, and B. Roizman (ed.), Virology, 2nd ed., vol. 1. Raven Press, New York.
13. **Kaplan, J. E., G. W. Gary, R. C. Baron, N. Singh, L. B. Schonberger, R. Feldman, and H. B. Greenberg.** 1982. Epidemiology of Norwalk gastroenteritis and the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis. Ann. Intern. Med. **96**:756–761.
14. **Keswick, B. H., T. K. Satterwhite, P. C. Johnson, H. L. DuPont,**

S. L. Secor, J. A. Bitsura, G. W. Gary, and J. C. Hoff. 1985. Inactivation of Norwalk virus in drinking water by chlorine. Appl. Environ. Microbiol. **50:**261–264.
15. **Lambden, P. R., E. O. Caul, C. R. Ashley, and I. N. Clarke.** 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science **259:**516–519.
16. **Lewis, D. C.** 1990. Three serotypes of Norwalk-like virus demonstrated by solid-phase immune electron microscopy. J. Med. Virol. **30:**78–81.
17. **Lewis, D. C.** Personal communication.
18. **Madore, H. P., J. J. Treanor, R. Buja, and R. Dolin.** 1990. Antigenic relatedness among the Norwalk-like agents by serum antibody rises. J. Med. Virol. **32:**96–101.
19. **Madore, H. P., J. J. Treanor, K. A. Pray, and R. Dolin.** 1986. Enzyme-linked immunosorbent assays for Snow Mountain and Norwalk agents of viral gastroenteritis. J. Clin. Microbiol. **24:**456–459.
20. **Matsui, S. M., J. P. Kim, H. B. Greenberg, S. Wanchuang, S. Qiming, P. C. Johnson, H. L. DuPont, L. S. Oshiro, and G. R. Reyes.** 1991. The isolation and characterization of a Norwalk virus-specific cDNA. J. Clin. Invest. **87:**1456–1461.
21. **Okaka, S., S. Sekine, T. Ando, Y. Hayashi, M. Murao, K. Yabuuchi, T. Miki, and M. Ohashi.** 1990. Antigenic characterization of small, round-structured viruses by immune electron microscopy. J. Clin. Microbiol. **28:**1244–1248.
22. **Plikaytis, B. D., S. H. Turner, L. L. Gheesling, and G. M. Carlone.** 1991. Comparisons of standard curve-fitting methods to quantitate *Neisseria meningitidis* group A polysaccharide antibody levels by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **29:**1439–1446.
23. **Tijssen, P.** 1985. Practice and theory of enzyme immunoassays. Elsevier Science Publishers, Amsterdam.
24. **Treanor, J. J., X. Jiang, H. P. Madore, and M. K. Estes.** 1993. Subclass-specific serum antibody responses to recombinant Norwalk virus capsid antigen (rNV) in adults infected with Norwalk, Snow Mountain, or Hawaii virus. J. Clin. Microbiol. **31:**1630–1634.
25. **Treanor, J. J., H. P. Madore, and R. Dolin.** 1988. Development of an enzyme immunoassay for the Hawaii agent of viral gastroenteritis. J. Virol. Methods **22:**207–214.