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Proteins of Norwalk Virus

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The proteins of the Norwalk virus were studied by polyacrylamide gel electrophoresis. Highly purified specifically immunoprecipitated virions appeared to contain a single primary structural protein with a molecular weight of 59,000. In addition, a soluble Norwalk viral protein with a molecular weight of 30,000 was identified in fecal specimens containing Norwalk virus. The protein structure of the virion is similar to that of the Calciviridae family.

The Norwalk virus is the best-characterized member of a group of small noncultivatable viruses that cause acute epidemic gastroenteritis in humans (21). Infection with Norwalk virus is common; seroepidemiological studies have shown that antibodies to the Norwalk or antigenically related viruses are found in more than 50% of adults around the world (4, 7).

The virus is a frequent cause of gastroenteritis; it has been associated etiologically with 24 of 70 separate outbreaks of nonbacterial gastroenteritis which have occurred over the past decade (8, 9; H. B. Greenberg, R. G. Wyatt, A. R. Kalica, R. H. Yolken, R. Black, A. Z. Kapikian, and R. M. Chanock, *in* M. Pollard, ed., *Perspectives in Virology*, in press). Studies with volunteers have shown that the Norwalk virus can be serially transmitted and that in this experimental setting it causes a disease identical to the illness observed during natural outbreaks (6). By immune electron microscopy, Norwalk virus has been shown to be shed briefly, coincident with the peak of gastrointestinal illness (18). There are several other antigenically distinct small gastroenteritis viruses which resemble the Norwalk virus morphologically and epidemiologically (21), but these agents have been studied less thoroughly.

Despite the clinical importance of Norwalk virus and its antigenically distinct relatives, investigation of these agents has proven difficult. None of these small gastroenteritis viruses has been successfully cultivated in vitro, and only humans and chimpanzees have been shown to be susceptible to infection (20). Norwalk virus is shed in the feces in rather small quantities (at least 10- to 50-fold less than is hepatitis A virus when particle-rich fecal specimens are compared [unpublished data]), and the shedding period for Norwalk virus is brief (18).

Because the Norwalk virus has not been successfully cultivated in vitro and is shed only in limited amounts, it has been difficult to purify and hence classify. This is also true for the other antigenically distinct 27-nm gastroenteritis agents. In an effort to aid in the classification of the Norwalk virus, we have attempted to characterize its proteins.

MATERIALS AND METHODS

Virus. Norwalk virus and viral protein were isolated from a single diarrheal stool of a volunteer experimentally infected with the agent. This particle-positive stool was used because it contained the highest concentration of antigen, as assayed by radioimmunoassay (RIA), in a survey of sequential stools from more than 30 ill volunteers. Partially purified feline calicivirus (vaccine strain F-9) was kindly furnished by Frederick Schaffer, Naval Bioscience Laboratory, University of California, Berkeley. This calicivirus was iodinated and immunoprecipitated in a manner similar to that used for the Norwalk virus and Norwalk protein preparations.

Antisera. Preinoculation and 4-week convalescent sera from two volunteers experimentally infected with Norwalk virus, as well as acute-phase and convalescent sera from a child naturally infected with a gastroenteritis virus antigenically related to Norwalk virus (2), were used for immunoprecipitation. Paired anti-Norwalk sera were selected for having little or no measurable antibody in the preinfection or acute-phase specimen and a high titer in the convalescent specimen. Hyperimmune feline antiserum to feline calicivirus F-9 was kindly furnished by James Gillespie, Cornell University, Ithaca, N.Y.

RIA. RIA for Norwalk virus antigen or antibody was performed as described previously. The assay was shown to be both sensitive and specific (9).

Virus purification. A 10-g amount of diarrheal stool was suspended in 40 ml of TN buffer (0.01 M Tris, 0.15 M NaCl, 0.05% sodium azide, pH 8.0). An equal volume of trichlorotrifluoroethane (Genetron 113) was added to this suspension, and the mixture was homogenized for 1 min. Then the preparation was centrifuged (4,000 × *g* for 5 min) in a Sorvall GSA centrifuge rotor. The aqueous supernatant was decanted, and the Genetron layer and interface were reextracted twice more with TN buffer. The pooled

supernatants (approximately 120 ml each) were centrifuged (96,300 × *g* for 12 h) in an SW27 rotor. The high-speed supernatant was carefully decanted and saved (see purification procedure described below). The crude stool pellet (P1) was suspended in 10 ml of TN buffer and repelleted through 3 ml of 30% (wt/vol) sucrose at 150,000 × *g* for 6 h in an SW40 rotor. The supernatant was discarded, and the pellet (P2) was resuspended in 2 ml of TN buffer and layered on top of a 1.2- to 1.6-g/cm^3 discontinuous cesium chloride gradient and centrifuged at 150,000 × *g* for 18 h in an SW40 rotor. Fractions (1 ml each) of the gradient were collected and assayed for Norwalk virus antigen by RIA. The virion-associated peak fractions (fractions 4 and 5, Fig. 1) were pooled, diluted approximately sixfold, and pelleted (150,000 × *g* for 4 h in an SW40 rotor). The pellet (P3) was suspended in 0.4 ml of TN buffer and layered on a continuous 10 to 30% (wt/vol) sucrose gradient with a 1-ml, 1.6-g/cm^3 cesium chloride cushion and centrifuged at 100,000 × *g* for 90 min in an SW40 rotor. Fractions (5/10 ml each) were collected and assayed for Norwalk antigen activity by RIA (Fig. 2). The peak fraction (fraction 16, Fig. 2) was taken and layered on a continuous 30 to 60% (wt/vol) Renografin (Squibb) gradient in TN buffer and centrifuged for 15 h at 150,000 × *g* in an SW40 rotor. Fractions (0.6 ml each) were collected and assayed for antigen activity by RIA (Fig. 3). The peak fraction (fraction 7, Fig. 3) was diluted fivefold in TN buffer and pelleted at 200,000 × *g* for 5 h in an SW56 rotor. The virus pellet was suspended in 100 μl of 0.25 M phosphate buffer, pH 7.4.

Purification of the soluble Norwalk virus protein. The pooled high-speed supernatant (120 ml; see above), which remained strongly positive for Norwalk virus antigen activity in RIA, was recentrifuged for 18 h at 96,000 × *g* in an SW27 rotor. The supernatant was again carefully collected and concentrated approximately 10-fold by pressure dialysis, using an Amicon PM10 filter. A 5-ml amount of the 10× high-speed supernatant was layered onto a gel filtration column (2.6 by 100 cm; G-200 Sephacryl superfine; Pharmacia Fine Chemicals) which was run at a flow rate of 20 ml/h with TN buffer. The column elution profile was calibrated with Pharmacia low- and high-molecular-weight standards. Fractions (4 ml each) were collected, and each was assayed for Norwalk virus antigen by RIA. The peak eight fractions were pooled (fractions 50 to 57, Fig. 4), concentrated 10-fold, and dialyzed against 0.005 M phosphate buffer (pH 8.0) by using an Amicon PM10 filter. The concentrated peak fractions from the Sephacryl gel filtration were then layered onto a DEAE-cellulose (Whatman DE 52) column (1.5 by 30 cm). A stepwise discontinuous molar phosphate buffer (pH 8.0) gradient (0.007, 0.016, 0.031, 0.06, 0.12, 0.25, and 0.5 M) with 20-ml steps was used to elute the Norwalk virus antigen. Fractions (5 ml each) were collected and assayed for Norwalk virus antigen by RIA. The peak fractions were pooled and concentrated 10-fold as before and equilibrated back to TN buffer. By RIA, greater than

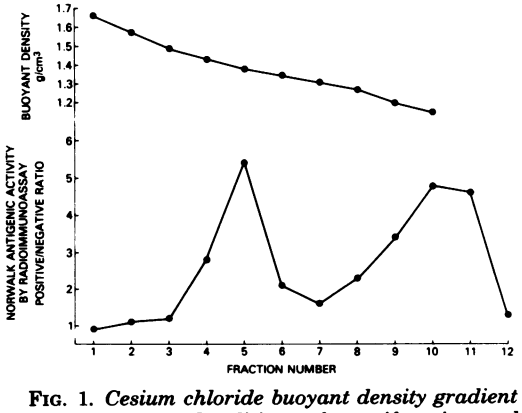

FIG. 1. *Cesium chloride buoyant density gradient of Norwalk virus. Conditions of centrifugation and preparation of Norwalk virus were as described in the text. Fractions 4 and 5 were pooled and used for further virion purification.*

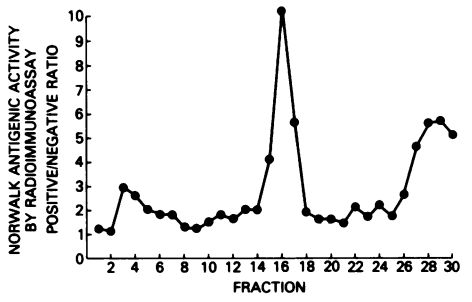

FIG. 2. *Rate zonal sedimentation of Norwalk virions in 10 to 30% sucrose gradient. Conditions of centrifugation and viral preparation were as described in the text. Fraction 16 was used for further virion purification.*

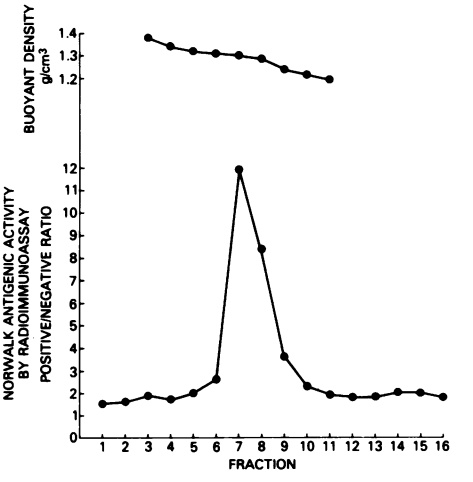

FIG. 3. *Renografin buoyant density gradient of Norwalk virion. Conditions of centrifugation and viral preparation were as described in the text. Fraction 7 was used for iodination and immunoprecipitation studies.*

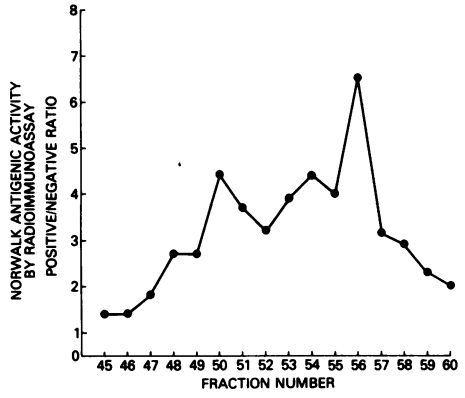

FIG. 4. *Sephacryl G-200 fine column chromatography of Norwalk virus-soluble viral protein. Conditions of elution were as described in the text. Fractions 50 to 57 were used for further purification (molecular weight range, 61,000 to 35,000).*

70% of the eluted Norwalk virus antigenic activity was contained in the 0.06 and 0.12 M fractions.

Affinity chromatography. An affinity column was prepared using immunoglobulin G from a volunteer known to possess a high level of antibody to Norwalk antigen as measured by RIA. Serum from this individual was not used in the immunoprecipitation experiments. A 5-mg amount of immunoglobulin G was coupled with 1 g of activated Sepharose 4B (Pharmacia Fine Chemicals) in $NaHCO_3$ buffer (0.1 M, pH 8.0) at 4°C overnight. The coupled Sepharose was then blocked with 1.0 M glycine and equilibrated with TN buffer in a Pasteur pipette column. The peak, pooled, concentrated fractions from the DEAE-cellulose purification of the Norwalk virus-soluble protein were bound to the affinity column in TN buffer. The column was then washed with 30 ml of TN buffer with 0.01% Nonidet P-40. The Norwalk virus protein was eluted from the affinity column with glycine hydrochloride buffer (pH 2.8), and the eluant fractions were immediately neutralized (to pH 7.0) with 1.0 M Tris buffer, pH 8. The affinity column-purified Norwalk virus antigen was again concentrated approximately 10-fold and exchange dialyzed with 0.25 M phosphate buffer (pH 7.4).

Iodination of Norwalk virion and soluble protein. A total of 4 μl of ^{125}I (Amersham; 100 μCi/μl; carrier-free NaI) and 15 μl of chloramine T (3.5 μg/μl) in 0.25 M phosphate buffer were added to 20 μl of purified virion, soluble protein, or feline calicivirus in 0.25 M phosphate buffer (pH 7.4). After 40 s, 20 μl of sodium metabisulfite (4.8 μg/μl) was added to the reaction, and the free iodine was separated from the labeled protein by gel filtration (Sephadex G-50 medium) with phosphate-buffered saline. The labeled protein was made 1% with fetal calf serum and kept at 4°C until immunoprecipitation.

Immunoprecipitation. Serum (8 μl), either preinfection, acute phase, convalescent, or, in the case of feline calicivirus F-9, hyperimmune antiserum, was added to 400 μl of labeled Norwalk virion, soluble Norwalk virus protein, or labeled feline calicivirus. After an overnight incubation at 4°C, 150 μl of *Staphylococcus aureus* A whole cells (Pansorbin, Calbiochem.-Behring Corp.) was added to the reaction, and the mixture was incubated at 37°C for 0.5 h. The mixture was then centrifuged (10,000 × *g* for 2 min), and the supernatant was discarded; the *S. aureus* A-immunoglobulin-^{125}I-labeled protein complex was washed twice with 1 ml of phosphate-buffered saline, and the final pellet was suspended in 200 μl of sample buffer (0.062 M Tris [pH 6.8], 5% mercaptoethanol, 2% sodium dodecyl sulfate, 10% glycerol, 0.0175% bromophenol blue) and boiled for 5 min before electrophoresis.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was performed at 25 to 30 mA on 12% gels by the method of Laemmli (12). Molecular-weight markers from Amersham (200,000 to 14,300 daltons) were used to estimate the size of the Norwalk virus polypeptides. Autoradiographs were made by using Kodak no-screen X-ray film.

RESULTS

Virion purification. The three-step purification procedure used for the Norwalk virion (Fig. 1 through 3) was found to be necessary to separate this virus from contaminating fecal material. Even after this purification scheme, a final specific immunoprecipitation was necessary to clearly separate the Norwalk virus protein from contaminants. As had been previously reported (10), Norwalk virus had a buoyant density of between 1.40 and 1.38 g/cm^3 in cesium chloride (Fig. 1). The buoyant density of Norwalk virus in renographin was 1.30 g/cm^3 as calculated from weighed fractions. A broad second peak of Norwalk virus antigenic activity was detected in fractions 9 to 11 (Fig. 1). When these or comparable fractions were examined by immune electron microscopy, no Norwalk virions were seen. This antigenic peak probably represents residual soluble Norwalk virus protein that had been trapped in the second fecal pellet (P2).

Purification of the soluble Norwalk virus protein. During initial purification studies of the Norwalk virion, we noticed that appreciable antigenic activity, as measured by RIA, remained in the supernatant of fecal suspensions that were centrifuged at sufficient force to pellet all Norwalk virus particles. This soluble antigenic activity was present in every stool in which we detected Norwalk virus specific antigen by RIA and was independent of suspension buffer (phosphate- or Tris-buffered saline), fecal concentration, or fluorocarbon treatment. In preliminary filtration studies of crude 10% fecal suspensions in TN buffer we used Amicon Ultra filters (PM 10, 30, and XM 100 A) with molecular weight exclusion sizes of 10,000, 30,000, and 100,000 to show that the soluble antigenic activity had an apparent molecular weight of more

than 10,000 but less than 100,000. Since in most stools studied more than 50% of the total Norwalk virus antigenic activity appeared to be associated with the soluble fraction, we attempted to further characterize this activity. The same stool was used for purification of virion and soluble protein because it contained large amounts of both materials.

Gel filtration of the concentrated high-speed supernatant (Fig. 4) showed a heterogenous elution pattern with antigen detected over a broad molecular weight range (35,000 to 61,000) with peak activity found in the 37,000-dalton range (fraction 56). The concentrated gel filtration peak of the Norwalk virus-soluble protein was eluted from the ion exchange column at phosphate buffer molarities of 0.06 and 0.125 M. Preliminary affinity chromatography studies disclosed that the soluble protein was rendered nonantigenic by 4 M guanidine, 6 M urea, 4 M KSCN, 4 M $MgCl_2$, and glycine hydrochloride buffer (pH 2.4 and 2.6). However, 0.3 M glycine hydrochloride, (pH 2.8) removed Norwalk virus antigen from the affinity column without destroying its antigenicity.

Polyacrylamide gel analysis of the virion and soluble protein. Preinfection serum from a volunteer infected with the Norwalk virus (RIA anti-Norwalk virus titer, 1:10) failed to specifically precipitate labeled protein from the virion preparation (Fig. 5). The convalescent serum (antibody titer, 1:3,200) precipitated a single major protein with a molecular weight of 59,000 from the virion preparation (Fig. 5). In another gel (not shown) the immunoprecipitated single structural protein of feline calicivirus was found to have a molecular weight of 65,000, whereas in the same gel, the Norwalk virion protein was again found to have a molecular weight of 59,000. Occasionally (not visible in Fig. 5 or 6), when gels were exposed for 3 to 4 weeks, two additionally faintly visible bands (molecular weights 40,000 and 34,000) were seen in the specifically immunoprecipitated virion preparation. The single 59,000-dalton protein was again specifically precipitated by convalescent serum from a second infected volunteer and by convalescent infection serum from a child infected during a naturally occurring epidemic (preinfection or acute-phase titer, 1:10; convalescent titer, 1:3,200 for both pairs) (Fig. 6).

A single protein band with a molecular weight of 30,000 was specifically precipitated from the soluble protein preparation with convalescent anti-Norwalk virus serum from two volunteers (Figs. 5 and 6). The two preinfection serum specimens failed to precipitate detectable iodinated protein from the soluble protein preparation (Fig. 5 and 6). The soluble viral protein was also specifically immunoprecipitated (data not shown) by convalescent serum obtained from a patient with naturally acquired epidemic gastroenteritis caused by the Norwalk virus.

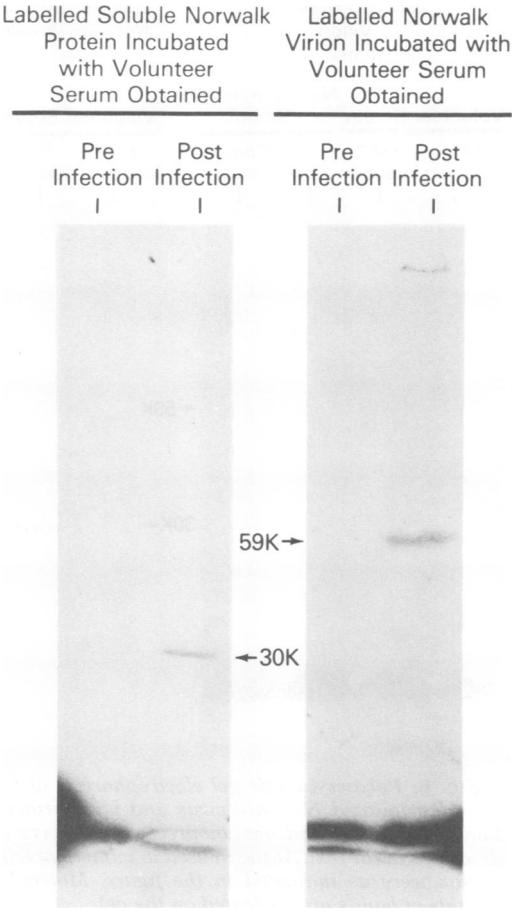

FIG. 5. *Polyacrylamide gel electrophoresis of immunoprecipitated Norwalk virus and viral protein. Conditions of gel and immunoprecipitation were as described in the text. Antisera used in immunoprecipitation were as indicated in the figure. Molecular weights of bands are indicated on the gel.*

DISCUSSION

The Norwalk virus can be considered as the prototype of a family of 27- to 30-nm gastroenteritis viruses (10, 21). These agents have been extremely difficult to study, primarily because they have a narrow host range, they are present in low titer in clinical specimens, and they have not been successfully adapted to tissue culture. Nevertheless, epidemiological studies of gastroenteritis indicate that infection with the Norwalk virus is common and is frequently a cause of epidemic gastroenteritis.

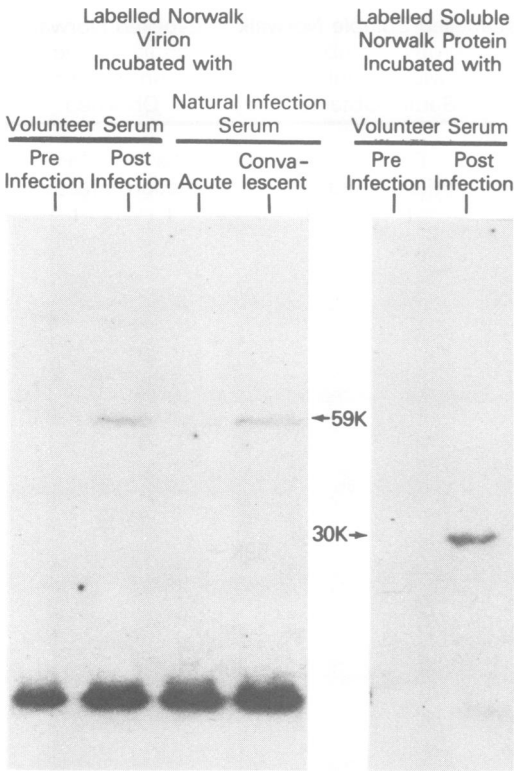

FIG. 6. *Polyacrylamide gel electrophoresis of immunoprecipitated Norwalk virus and viral protein. Conditions of gel and immunoprecipitation were as described in the text. Antisera used in immunoprecipitation were as indicated in the figure. Molecular weights of bands are indicated on the gel.*

In earlier work, the Norwalk virus was tentatively classified as "parvovirus-like" (5, 10). This preliminary classification was made because of the buoyant density of the particles, relative size, and stability in acid and ether. In this work we attempted to carry classification further by investigating the size of Norwalk viral proteins. The finding of one major structural protein with a molecular weight of 59,000 is at odds with a classification of parvovirus (16). In unpublished experiments, we used the chloramine T iodination procedure to label purified preparations of two parvoviruses, adeno-associated virus 3 and rat parvovirus (kindly supplied by David Hoggan, National Institutes of Health). As expected, three structural proteins with molecular weights of between 60,000 and 85,000 were visualized. This pattern is distinctly different from the pattern of a single major structural protein observed with Norwalk virus or feline calicivirus.

Caliciviruses are a newly proposed family of single-stranded RNA viruses that have been found to possess only one structural protein of approximately 65,000 daltons with a reported range of between 60,000 and 71,000 daltons (17). A feline calicivirus iodinated and immunoprecipitated in the same manner as the Norwalk virus was found to have one structural protein with a molecular weight of 65,000. The size and buoyant density of caliciviruses (35 to 40 nm and 1.36 to 1.39 g/cm^3) are similar although not identical to those of the Norwalk virus. In unpublished studies in which negative-stain electron microscopy was used, we found that feline calicivirus strain F-9 and Norwalk virus were comparable in size. The single Norwalk virion structural protein, when coelectrophoresed with feline calicivirus F-9, was found to be slightly smaller (59,000 versus 65,000). Caliciviruses are not known to possess minor smaller structural proteins analogous to the two faint bands occasionally seen in the Norwalk virus preparation; however, a minor protein with a molecular weight of 15,000 has been detected in some calicivirus preparations (17). The significance of these two very faintly labeled bands is unclear at present. The specific precipitation of the 59,000-dalton Norwalk virion protein by two convalescent sera from ill volunteers and one convalescent serum from a naturally infected patient, but not by the appropriate preinfection or acute-phase sera, is strong evidence for the specific relationship of this single protein to the Norwalk virion.

The finding of a single 30,000-molecular-weight soluble protein in Norwalk virus antigen containing fecal specimens was unexpected. This protein does not appear to represent an artifact of our procedure for preparing stool suspensions, since untreated fecal suspensions also appeared to contain a soluble Norwalk virus protein in large amounts. As with the virion protein, specific immunoprecipitation by several appropriate immune sera provided evidence for the specific relationship of this protein to the Norwalk virus. Whether this soluble protein represents a stable nonstructural viral protein made in great excess or whether the 30,000-molecular-weight protein is a subunit or cleavage product of the 59,000-molecular-weight virion protein is not clear at present. A nonstructural virus-associated protein with a molecular weight of 29,000 has been described in calicivirus-infected cells (17).

Caliciviruses have a unique ultrastructure characterized by cup-shaped depressions arranged symmetrically on their surfaces (17). The fine ultrastructure of the Norwalk virus has not been well visualized because of the difficulty in detecting the virion without using precipitating antibody. However, in some electron micrographs in which Norwalk virus has been seen with little or no coating antibody, a morphological similarity to calicivirus is apparent (Greenberg et al., in press). It is of interest that several

workers have recently identified morphologically characteristic calicivirus-like particles in fecal specimens from individuals involved in epidemics of nonbacterial gastroenteritis (3, 13; H. Suzuki, T. Konno, T. Kutsuzawa, A. Imai, F. Tazawa, and N. Ishida, J. Med. Virol., in press). These isolates have not been found to be serologically related to the Norwalk virus or to known animal caliciviruses.

The serologically distinct but morphologically similar small gastroenteritis viruses such as Hawaii agent (19), Ditchling agent (1), and Colorado agent (15) have not been purified or cultivated, so their taxonomic relationship to the Norwalk virus is not clear. This is also true for the morphologically distinct "astroviruses" (11, 14). Hopefully, more detailed biochemical characterization of other gastroenteritis agents will enable us to determine whether the various isolates represent several serotypes of a single viral family or several different types of viruses, all of which are relatively similar in size and biologic behavior.

The current findings are most compatible with the Norwalk virus being related to the calicivirus family. Certainly, nucleic acid studies would have been useful in clarifying this issue, but the minute amounts of Norwalk virus available and the lack of a tissue culture system have restricted us to protein analysis. In any case, from the available data we would recommend that Norwalk virus not be referred to as parvovirus-like. Hopefully, when workers identify other small gastroenteritis viruses that are present in larger quantity, nucleic acid studies can be carried out. Attempts are now under way to raise antibody to the purified 30,000-dalton protein to study its relationship with the virion.

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