

Virus-Like Particle, 35 to 40 nm, Associated with an Institutional Outbreak of Acute Gastroenteritis in Adults

KOKI TANIGUCHI,* SHOZO URASAWA, AND TOMOKO URASAWA

Department of Hygiene, Sapporo Medical College, Sapporo, 060 Japan

Received for publication 6 August 1979

In an outbreak of acute gastroenteritis in an institution for the mentally retarded at Otofuke, Hokkaido, Japan, virus-like particles were observed by electron microscopy in five of seven stool specimens from the patients. The particles had 10 rod-shaped and 10 round projections or capsomeres on the periphery, measured 35 to 40 nm in diameter, and had a buoyant density of 1.35 to 1.37 g/ml in cesium chloride. Attempts to culture these particles in tissue culture or in mouse brain were unsuccessful. Immune electron microscopy performed with the virus from the patients as antigen demonstrated significant serological responses in all eight patients examined. Antigenic similarity of the virus particles obtained from five patients was also confirmed by immune electron microscopy with the paired acute- and convalescent-phase sera of one of the patients. Furthermore, in immune electron microscopy these particles appeared to have no antigenic relationship to three candidate viruses for gastroenteritis so far reported: the Norwalk agent, the W agent, and the calicivirus-like particle. These results suggested the possibility that this agent, tentatively designated as the Otofuke agent, might be a new candidate virus for gastroenteritis.

Rotaviruses are probably the commonest cause of acute nonbacterial gastroenteritis in infancy and childhood (6, 9, 11). The possible association of enteric adenovirus (8) and enteric coronavirus (3, 4) with this disease has also been reported, although their etiological role should still be regarded as controversial since they have often been observed in control subjects.

In addition, small round virus particles have been detected by electron microscopy (EM) in stools from gastroenteritis patients. Of these particles, the etiological importance of the Norwalk and Hawaii agents—parvovirus-like agents—in gastroenteritis in both children and adults has been established in extensive volunteer studies (12, 18, 20). Other suggested causative agents include enteroviruses (7), calicivirus (15), astrovirus (14), "mini-reovirus" (17), the W agent (5), and the Ditchling agent (1). These are all 22 to 30 nm in diameter.

An outbreak of acute gastroenteritis occurred in an institution for the mentally retarded at Otofuke, Hokkaido, Japan. Direct EM examination revealed the existence of virus-like particles in stool specimens from the patients. In this paper we report the results of immune EM (IEM) studies with the particles detected and paired sera from the patients, together with a characterization of these particles. We also discuss the differences between these particles and some other candidate viruses for gastroenteritis.

MATERIALS AND METHODS

Purification of stool specimens. Stool specimens were processed by the method described by Bishop et al. (2) with slight modifications. Stool suspensions (25 to 100 ml; about 20%, wt/vol) were clarified by low-speed centrifugation and then treated twice with trifluorotrchloroethane. To the aqueous phase collected, polyethylene glycol 6000 was added (final concentration of 8%, wt/vol). After holding overnight at 4°C, the mixture was centrifuged at $8,500 \times g$ for 30 min. The resultant pellet was suspended in distilled water, and the suspension was layered onto 3 ml of 25% sucrose in 10 mM phosphate-buffered saline (pH 7.2) and centrifuged at $100,000 \times g$ for 3 h (Hitachi 65P). The final pellet was resuspended in 0.5 ml of 10 mM phosphate buffer (pH 7.4), and this suspension was further centrifuged at $600 \times g$ for 15 min. The supernatant fluid was used as partially purified antigen.

Attempted virus isolation. Filtrates of fecal suspensions were inoculated onto HeLa, LLC-MK2, and monkey kidney stable (MS) cells. Cells were examined for the development of cytopathic effect during two successive blind passages. The same filtrates were injected into 1-day-old mice by the intracerebral route. The mice were observed for 2 weeks for signs of paralysis.

EM. Negative staining was carried out in a routine manner as follows: 0.05 ml of partially purified virus suspension was mixed with an equal volume of 3% potassium phosphotungstate adjusted to pH 7.0. A drop of this mixture was then placed on a 400-mesh carbon-collodion-coated grid, and excess fluid was withdrawn with the edge of a filter paper disk. The grid was immediately placed in a Hitachi H500 elec-

tron microscope and examined. Micrographs were taken at an instrumental magnification of 30,000 or 48,000.

IEM. A 0.05-ml amount of partially purified virus suspension was mixed with 0.05 ml of a 1/25 dilution of serum. After the mixture was incubated at room temperature for 1 h, 4 ml of cold 10 mM phosphate buffer was slowly added to the mixture which was then centrifuged at $50,000 \times g$ for 1 h. The supernatant fluid was carefully removed and discarded, and the pellet or sediment was resuspended in one drop of distilled water and stained with 2% phosphotungstate.

Determination of the buoyant density in cesium chloride. A 0.5-ml amount of partially purified virus suspension was layered onto 4 ml of a continuous CsCl density gradient (1.1 to 1.6 g/ml) which was centrifuged at 35,000 rpm for 10 h at 4°C in an RPS50 rotor in a Hitachi 65P centrifuge. Nineteen fractions were collected from the gradient, and 0.05 ml of each fraction was examined by IEM by the method described by Kapikian et al. (10). The number of particles recovered in seven squares on a 400-mesh grid was counted. The density of each fraction was determined with a refractometer (Atago, abbe no. 302).

RESULTS

Outbreak. The institution where acute gastroenteritis occurred had 92 mentally handicapped inmates and 32 teaching and other staff. Thirty-two (34.8%) of the inmates and two (6.3%) of the staff contracted the disease; ages of those infected ranged from 22 to 51 years. Attack rates did not differ appreciably among different age groups. The main clinical features were nau-

sea, vomiting, and diarrhea. Recovery took 1 to 3 days. Figure 1 shows the number of patients by date of onset as well as frequencies of main clinical symptoms observed. Examinations of feces from all patients as well as some of the contacts, food, and vomit did not reveal *Salmonella*, *Shigella*, *Enterococcus*, or *Staphylococcus aureus*. Epidemiological inquiries failed to furnish clear evidence of the mode of spread.

Detection and characterization of virus-like particles. The stool specimens obtained from seven patients and one apparently healthy contact were examined for the virus particles by direct EM. Of these, specimens from five of seven patients contained virus-like particles such as those shown in Fig. 2. In the specimens of patients 4 and 5, the particles were present in large numbers. These particles, whose capsomeres or projections could be observed, stood out clearly from the surrounding matter and could be readily differentiated from other numerous spherical objects normally found in stool specimens. These virus-like particles generally occurred singly; spontaneous clumping was rare. The appearance and diameter of the negatively stained virus-like particles were identical in all specimens. For convenience, we tentatively called these particles the Otofuke agent.

We examined the distribution of particle size of the Otofuke agent. Poliovirus type 1 (Mahoney strain) particles were added to the Otofuke agent suspension as a reference. We could easily

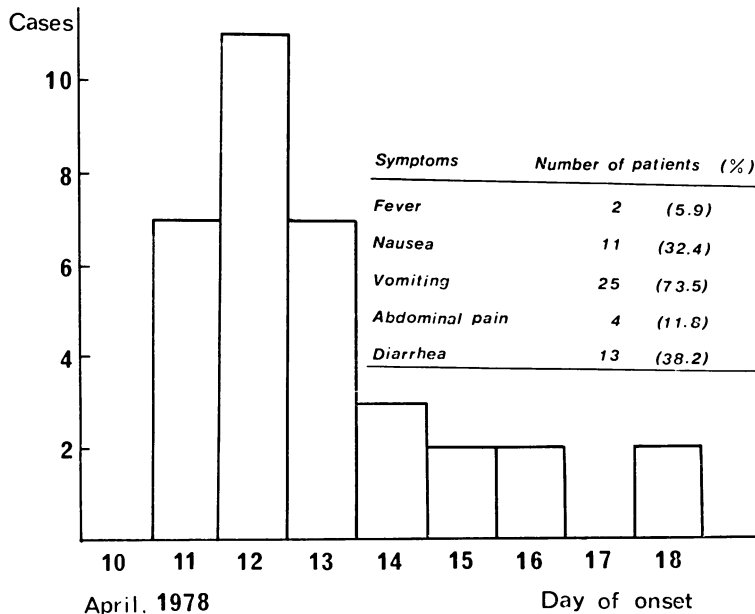


FIG. 1. Date of onset of acute gastroenteritis at an institution for the mentally retarded and main clinical symptoms.

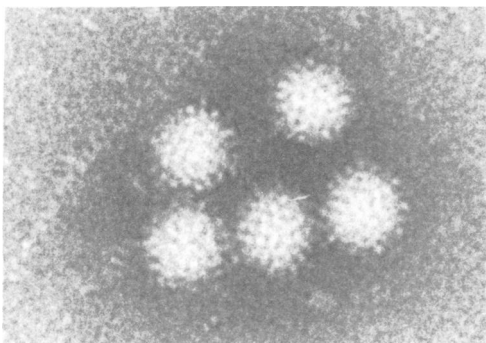


FIG. 2. Representative EM of virus-like particles (the Otofuke agent) detected in a stool specimen from patient 5. Magnification, 270,000 \times .

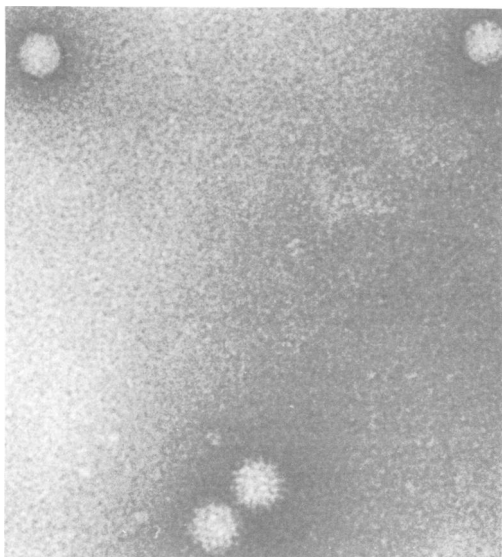


FIG. 3. Differences in size and morphology between the Otofuke agent and poliovirus type 1 (Mahoney strain) compared in the same field. Magnification, 190,000 \times .

distinguish the Otofuke agent from poliovirus particles on the basis of their size and morphological features. The size distribution of poliovirus was confirmed to be 28 nm, in agreement with observations of most workers. The majority of the Otofuke agent particles, in contrast, accumulated in the size range of 35 to 40 nm. Figure 3 shows the relative sizes of poliovirus and the Otofuke agent in the same field.

The negatively stained Otofuke agent appeared to be approximately spherical in shape with projections on its surface. When subjected to rotational enhancement (16), 20 capsomeres were clearly visible on the periphery of the particles on $n = 5$ rotation but not on $n = 6$ rotation in all subjects enhanced (Fig. 4). There were two

kinds of capsomeres; one was round, and the other was rod shaped. These peripheral capsomeres might not be arranged exactly along a meridian. These observations do not yet permit us to propose a detailed model of the arrangement of capsomeres of this particle. However, the presence of 20 capsomeres on the periphery of this particle is one of the items of information that any scheme of the structure of this particle must take into account.

Next, by the technique of IEM, we determined the buoyant density of the Otofuke agent (from a stool specimen from patient 4) in CsCl. After dividing the centrifuged gradient into 19 fractions, 0.05 ml of each fraction was mixed with 0.05 ml of a 1/25 dilution of acute-phase serum from patient 1 (see Table 1) and examined under EM. The number of the particles recovered in seven squares of a 400-mesh grid is shown in Fig. 5. The density of this agent was found to be 1.35 to 1.37 g/ml in CsCl. The density of the particles

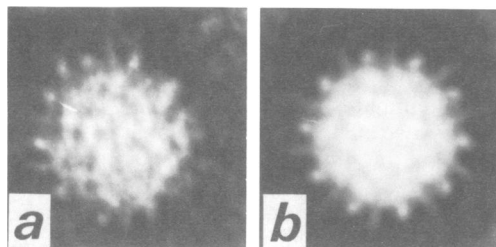


FIG. 4. High magnification of a single particle of the Otofuke agent. (a) Original electron micrograph. (b) Rotated $n = 5$ by the Markam rotation technique. Enhancement of peripheral capsomeres is evident in $n = 5$ rotation. Magnification, 560,000 \times .

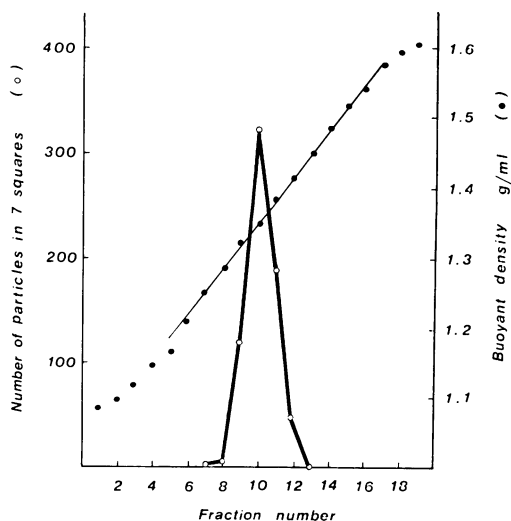


FIG. 5. Isopycnic banding of the Otofuke agent in a CsCl density gradient.

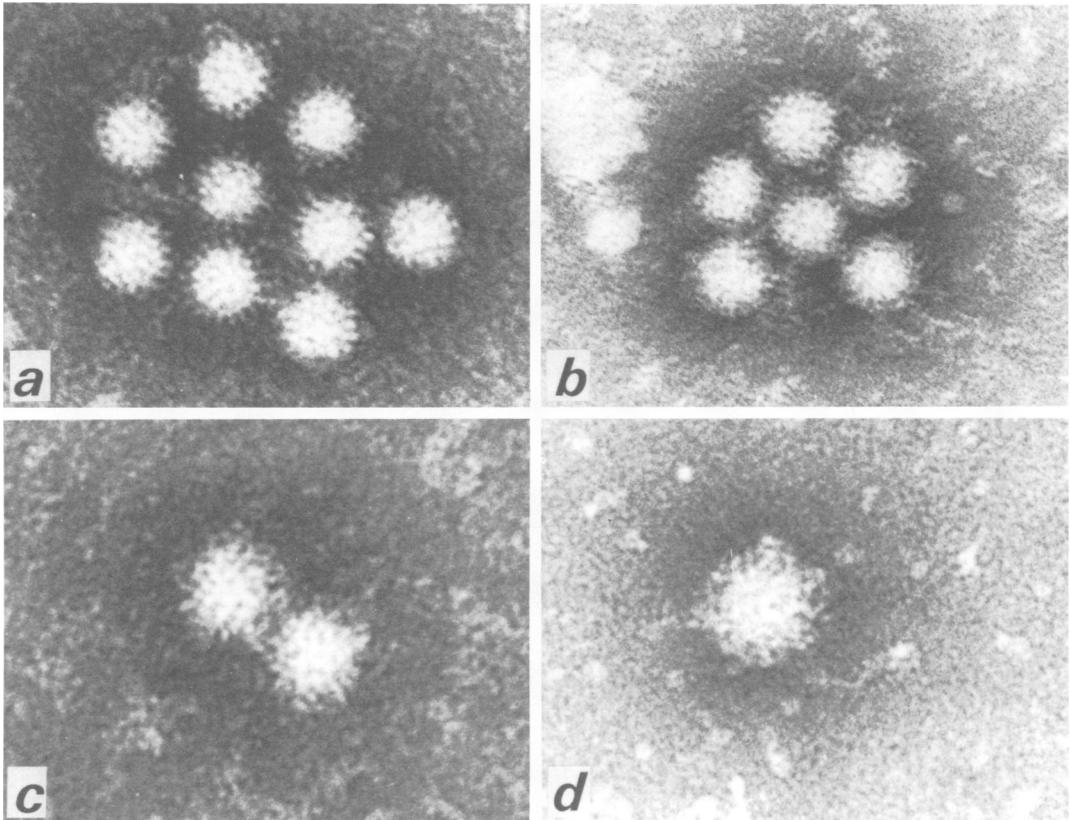


FIG. 6. Aggregates observed in IEM tests. Each of the aggregates was scored as 1+ (a), 2+ (b), 3+ (c), and 4+ (d), respectively. Magnification, 270,000 \times .

derived from patient 5 was the same as that of patient 4.

In attempted virus isolation, no cytopathic effects were found in the cells and no paralytic signs were observed in the suckling mice inoculated with stool specimens.

Serological evidence of infection confirmed by IEM. We used the partially purified virus suspension derived from a stool specimen (from patient 4 or 5) obtained during the acute phase to examine paired sera for antibody response to this virus-like antigen by IEM. The tests were carried out by one of us (K. T.), who used coded serum samples to eliminate the possibility of biased interpretation. The relative concentration of antibody in each serum specimen was estimated by scoring the amount of antibody coating the particle on a 0 to 4+ scale. Figure 6a to d shows representative samples rated as 1+ to 4+. A change of 1+ in antibody rating between paired sera was considered to be significant. All acute-phase sera had preexisting antibody to this agent in moderate quantities. However, all patients examined demonstrated distinct increases in antibody (Table 1). The

individual shown in the bottom line of Table 1 is a research worker who contracted gastroenteritis accidentally in our laboratory after working with the stool specimen from patient 4. His convalescent-phase serum had a rating of 4+, whereas the acute-phase serum was scored as 1+. So, possibly, he was infected with the agent derived from this institutional outbreak of gastroenteritis.

To confirm whether the virus particles from the different patients were antigenically similar, we carried out IEM with the paired sera of one of the patients (patient 1). Although the quantity of virus particles used as antigen in IEM tests differed, we confirmed a significant antibody response against all stool specimens examined (Table 1).

Serological comparison with some other agents associated with acute nonbacterial gastroenteritis. As shown in Table 2, the antigen observed during this institutional outbreak of gastroenteritis (the Otofuke agent) was tested by IEM against the paired sera from a chimpanzee infected with the Norwalk agent and against those from a volunteer infected with the W

TABLE 1. Detection of virus-like particles in feces by EM and serological evidence of infection confirmed by IEM

Subject	Age	Symptoms ^a	Virus-like particles in feces	IEM with paired sera of patient 1 ^b		IEM with particles of patient 4 or 5 ^c	
				Acute	Convalescent	Acute	Convalescent
1	30	V	+	1+	4+	1+	3+
2	29	V	+	2+	4+	2+	4+
3	22	D	+	2+	4+	— ^d	—
4	36	V, D	+	1+	3+	—	4+
5	30	N, EF	+	1+	4+	—	4+
6	29	V, D	—	—	—	2+	4+
7	43	V	—	—	—	—	—
8	36	V	NS ^e	—	—	2+	4+
9	23	V	NS	—	—	2+	4+
10	23	V	NS	—	—	2+	4+
11	31	N, D	NS	—	—	2+	4+
12	44	N, V	NS	—	—	2+	4+
13	26	—	—	—	—	—	—
14 ^f	26	N, D	NS	—	—	1+	4+

^a V, Vomiting; D, diarrhea; N, nausea; EF, epileptic fit; —, no symptoms.

^b IEM with the paired sera from patient 1 and the stool specimens from individual patients.

^c IEM with the paired sera from individual patients and the stool specimen from patient 4 or patient 5 (in the cases of 11, 12, and 14 the stool specimen from patient 5 was used as antigen).

^d No serum sample obtained.

^e NS, No stool specimen obtained.

^f The man who contracted gastroenteritis accidentally in our laboratory after working with the stool specimen from patient 4.

TABLE 2. Serological comparison of the Otofuke agent with some other agents by IEM

Paired sera against	Preillness or acute	Convalescent
W agent (volunteer) ^a	1+	1+
Norwalk agent (chimpanzee) ^b	0	0
Norwalk agent (patient) ^c	0-1+	0-1+
Calicivirus (patient) ^d	1+	1+
Human rotavirus (patient)	0	0

^a Provided by S. K. R. Clarke, Public Health Laboratory, Bristol, England.

^b Provided by A. Z. Kapikian, National Institutes of Health, Bethesda, Md.

^c Provided by T. Konno, Tohoku University, Sendai, Japan.

^d Provided by S. Chiba, Sapporo Medical College, Sapporo, Japan.

agent. The reaction of the Otofuke agent with the paired sera from a schoolboy infected with the Norwalk agent and with those of a 9-month-old baby infected with calicivirus was also examined. Both of the paired sera were obtained in outbreaks of gastroenteritis in Japan (13; S. Chiba, Y. Sakuma, M. Akihara, R. Kogasaki, K. Horino, T. Nakao, and S. Fukui, *J. Med. Virol.*, in press). Neither pair of sera, which had shown

significant seroresponses in a homologous system by IEM test or radioimmunoassay blocking tests, showed antibody responses against the Otofuke agent. Although the number of tests was small, it appeared that the W agent, the Norwalk agent, and calicivirus, whose reported sizes were smaller than that of the Otofuke agent, were also serologically different from the Otofuke agent.

We checked the relationship of this outbreak to human rotavirus infection by complement fixation tests with bovine rotavirus, which cross-reacts to human rotavirus, as the antigen and found no antibody response in all the paired sera included in Table 1. Furthermore, we showed that there was no antigenic relationship of this agent to human rotavirus by the fact that neither of the paired sera from the patients infected with human rotavirus showed antibody against the Otofuke agent in IEM tests.

DISCUSSION

The results of IEM tests described above imply that this institutional outbreak of gastroenteritis in adults might be caused by the same agent, measuring 35 to 40 nm in diameter.

Various small round virus-like particles, measuring 22 to 30 nm in diameter, associated with acute nonbacterial gastroenteritis have been reported to date (1, 5, 7, 12, 14, 15, 17). Serological comparison among the Ditchling, W, Norwalk,

and Hawaii agents was carried out by Appleton et al. (1). According to those results, it would appear that the Ditchling and W agents are antigenically similar and that they both differ from the Norwalk agent. It was also suggested that the Ditchling and Hawaii agents differed antigenically in IEM tests. The particles observed in the Otofuke outbreak had a larger diameter and seemed to be antigenically different from both the Norwalk and the W agents. Morphologically, well-delineated capsomeres were observed on the surface of the Otofuke agent, and rotational enhancement revealed an alternating arrangement of 10 each of two kinds (round and rod shaped) of capsomeres on the periphery of the Otofuke agent. These morphological features of the Otofuke agent are quite different from those of astrovirus and calicivirus, whose surfaces are patterned by dark spots due to deposits of negative stain filling depressions (14, 15, 19). Since clear pictures of the Norwalk, W, and Ditchling agents are not yet available, we were unable to compare their morphology to that of the Otofuke agent. Furthermore, the density of the Otofuke agent in CsCl (1.35 to 1.37 g/ml) was lower than that of the Norwalk and Ditchling agents (1.38 to 1.39 g/ml and 1.38 to 1.40 g/ml), respectively (1, 10). These results suggest that the Otofuke agent might be a new agent differing from any candidate virus so far reported to be associated with acute nonbacterial gastroenteritis.

In addition to the IEM tests shown in Table 1, we examined 30 serum samples from patients of various ages in other outbreaks of gastroenteritis in Hokkaido and from the staff in our laboratory for antibodies against the Otofuke agent. We found that most preillness or acute-phase sera examined contained a weak or moderate antibody (1+ to 2+) against the Otofuke agent, and the antibody content of preillness or acute-phase sera seemed to increase with age (unpublished data). In addition, we recently found particles whose morphology and size are almost the same as those of the Otofuke agent in other institutional outbreaks of acute gastroenteritis. These findings suggest that infection caused by the Otofuke agent or serologically cross-reacting agent(s) is common in Hokkaido and perhaps elsewhere in Japan. To clarify this, we must await further epidemiological studies as well as additional characterization of this agent.

Proving a causal role of the Otofuke agent in gastroenteritis would involve volunteer studies; however, the case of the man who accidentally contracted gastroenteritis after working with a particle-containing stool specimen and showed

a significant antibody response against this agent seems to indicate the pathogenicity of the Otofuke agent.

ACKNOWLEDGMENTS

We are grateful to A. Z. Kapikian, National Institutes of Health, Bethesda, Md.; S. K. R. Clarke, Public Health Laboratory, Kingsdown, Bristol, England; T. Konno, Department of Pediatrics, Tohoku University; and S. Chiba, Department of Pediatrics, Sapporo Medical College for supplying the paired sera against the Norwalk agent (chimpanzee), the W agent (volunteer), the Norwalk agent (patient), and calicivirus (patient), respectively. We thank S. Kawamura and his colleagues at the Obihiro Health Center for the field survey and for collecting the specimens. We also thank N. Sakurada, Hokkaido Institute of Public Health, for his attempts to culture the particles detected in this outbreak.

LITERATURE CITED

1. Appleton, H., M. Buckley, B. T. Thom, J. L. Cotton, and S. Henderson. 1977. Virus-like particles in winter vomiting disease. *Lancet* i:409-411.
2. Bishop, R. F., G. P. Davidson, I. H. Holmes, and B. J. Ruck. 1974. Detection of a new virus by electron microscopy of fecal extracts from children with acute gastroenteritis. *Lancet* i:149-151.
3. Caul, E. O., and S. K. R. Clarke. 1975. Coronavirus propagated from patient with non-bacterial gastroenteritis. *Lancet* ii:953-954.
4. Caul, E. O., W. K. Paver, and S. K. R. Clarke. 1975. Coronavirus particles in feces from patients with gastroenteritis. *Lancet* i:1192.
5. Clarke, S. K. R., G. T. Cook, S. I. Egglestone, T. S. Hall, D. L. Miller, S. E. Reed, D. Rubenstein, A. J. Smith, and D. A. J. Tyrrell. 1972. A virus from epidemic vomiting disease. *Br. Med. J.* 3:86-89.
6. Davidson, G. P., R. F. Bishop, R. F. Townley, I. H. Holmes, and R. J. Ruck. 1975. Importance of a new virus in acute sporadic enteritis in children. *Lancet* i: 242-246.
7. Eichenwald, H. F., A. Ababio, A. M. Arky, and A. P. Hartman. 1958. Epidemic diarrhea in premature and older infants caused by echo virus type 18. *J. Am. Med. Assoc.* 166:1563-1566.
8. Flewett, T. H., A. S. Bryden, H. Davies, and C. A. Morris. 1975. Epidemic viral enteritis in a long-stay children's ward. *Lancet* i:4-5.
9. Hamilton, J. R., D. G. Gall, and B. Kerzner. 1975. Recent developments in viral gastroenteritis. *Pediatr. Clin. North Am.* 22:747-755.
10. Kapikian, A. Z., J. L. Gerin, R. G. Wyatt, T. S. Thornhill, and R. M. Chanock. 1973. Density in cesium chloride of the 27 nm "8FIIa" particle associated with acute infectious nonbacterial gastroenteritis: determination by ultracentrifugation and immune electron microscopy (37135). *Proc. Soc. Exp. Biol. Med.* 142:874-877.
11. Kapikian, A. Z., H. W. Kim, R. G. Wyatt, W. L. Cline, J. O. Arrobio, C. D. Brandt, W. J. Rodriguez, D. A. Sack, R. M. Chanock, and R. H. Parrott. 1976. Human reovirus-like agent as the major pathogen associated with "winter" gastroenteritis in hospitalized infants and young children. *N. Engl. J. Med.* 294:965-972.
12. Kapikian, A. Z., R. G. Wyatt, R. Dolin, T. S. Thornhill, A. R. Kalica, and R. M. Chanock. 1972. Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis. *J. Virol.* 10:1075-1081.
13. Konno, T., H. Suzuki, A. Imai, T. Kutzuzawa, H. Yamamoto, K. Umezu, J. Konno, and N. Ishida.

1978. Acute gastroenteritis caused by Norwalk agent in Japan. *Igaku No Ayumi* **106**:604-605. (In Japanese.)
14. **Madeley, C. R., and B. P. Cosgrove.** 1975. Viruses in infantile gastroenteritis. *Lancet* **ii**:124.
 15. **Madeley, C. R., and B. P. Cosgrove.** 1976. Calicivirus in man. *Lancet* **i**:199-200.
 16. **Markham, R., S. Frey, and G. J. Hills.** 1963. Methods for the enhancement of image detail and accentuation of structure in electron microscopy. *Virology* **20**:88-102.
 17. **Middleton, P. J., M. T. Szymanski, and M. Petric.** 1977. Viruses associated with acute gastroenteritis in young children. *Am. J. Dis. Child.* **131**:733-737.
 18. **Parrino, T. A., D. S. Schreiber, J. S. Trier, A. Z. Kapikian, and N. R. Blacklow.** 1977. Clinical immunity in acute gastroenteritis caused by Norwalk agent. *N. Engl. J. Med.* **297**:86-89.
 19. **Studdert, M. J.** 1978. Caliciviruses. *Arch. Virol.* **58**:157-191.
 20. **Thornhill, T. S., R. G. Wyatt, A. R. Kalica, R. Dolin, R. M. Chanock, and A. Z. Kapikian.** 1977. Detection by immune electron microscopy of 26- to 27-nm virus-like particles associated with two family outbreaks of gastroenteritis. *J. Infect. Dis.* **135**:20-27.