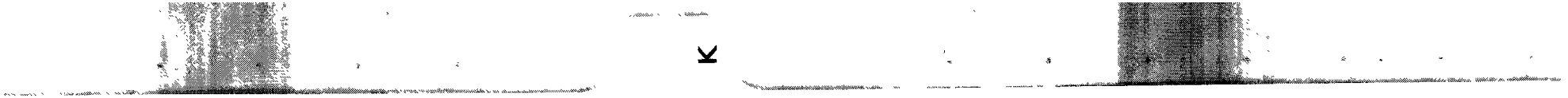

Isolation from Man of "Avian Infectious Bronchitis Virus-like" Viruses (Coronaviruses*) similar to 229E Virus, with Some Epidemiological Observations

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With standard tissue culture techniques it has been possible to determine the etiologic agent of only 20%–35% of upper respiratory illnesses in adults [1–4]. However, in 1965 Tyrrell and Bynoe reported successful attempts to increase the efficiency of virus recovery from patients with upper respiratory tract illness using human embryonic tracheal and nasal organ cultures. They found that organ cultures were more sensitive than conventional tissue cultures for isolation of some rhinoviruses; in addition, they isolated an ether-labile virus, strain B814, which could be grown only in organ culture and which produced common cold-like illnesses in volunteers [5]. In 1966, Hamre and Procknow described the recovery of a new virus, using standard tissue culture techniques, from 5 students, 4 of whom had an upper respiratory tract illness [6]. The prototype strain, 229E, was ether-labile, possessed an RNA core, measured approximately 89 mμ, and yet was apparently unrelated to any of the myxoviruses of man. In 1967, we described the isolation of 6 new ether-labile viruses (hereafter referred to as National Institutes of Health organ culture [NIH O.C.] viruses) from patients with upper respiratory illnesses; these agents could be isolated only in human embryonic tracheal organ cultures [7]. The B814 virus, the 229E virus, and the NIH O.C. viruses were all shown to possess a similar morphology which resembled that of the avian infectious bronchitis virus (IBV) but was distinct from that of the myxo- or paramyxoviruses [7–9].[1] Subsequently, it was found that mouse hepatitis virus (MHV) shared the common morphologic features of these viruses [10–13].

As a group, the "IBV-like" viruses of man are fastidious in their host-cell requirements: neither the B814 nor the 6 NIH O.C. viruses could be adapted to grow in a monolayer tissue culture system; 229E virus was isolated in such tissue culture, but only with difficulty. Virus 229E was isolated originally after a second blind passage in human embryonic kidney (HEK) cells; attempts to isolate this virus in human diploid cell strain (HDCS) WI38 cultures after 4 blind passages were unsuccessful although it was adapted subsequently to grow in these cells [6]. Virus 229E was capable of producing respiratory illness in volunteers after 1 passage in human embryonic tracheal organ cultures [11].

In an attempt to study the incidence of 229E virus infection in a population of civilian adults, complement fixation (CF) tests were performed on paired sera obtained during a previously reported cross-sectional study of upper respiratory illness which spanned 2 years (1962–1964) [2]. Five of 256 patients developed serologic (CF) evidence of 229E virus infection. At the same time, various experimental cell cultures were

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* Subsequent to the submission of this manuscript for publication the term "coronavirus" was proposed to include the avian infectious bronchitis virus (IBV) group, the mouse heaptitis virus (MHV) group, and the human "avian IBV-like" virus group (Coronaviruses, Nature 220:650, 1968).

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[1] D. A. J., Tyrrell, Presentation at Pan American Health Organization, First International Conference on Vaccines against Viral and Rickettsial Diseases of Man (November, [illegible]

under evaluation for their sensitivity to rhinoviruses and strain 229E. We therefore took advantage of this opportunity by attempting to isolate virus strains resembling 229E from the original specimens of these 5 patients in such cell cultures. In addition, specimens were obtained from patients with upper respiratory tract illnesses who participated in a more recent (1965–1967) cross-sectional study.[2] With the use of semi-continuous human embryonic intestine (HEI) cell cultures, a virus similar to 229E virus was isolated from 3 of the 5 patients with serologic evidence of infection and from 6 patients in the more recent study whose serologic response to 229E virus was not known at the time of the attempts at isolation. The recovery of these 9 virus strains represents the only reported isolation of viruses similar to 229E virus from natural infections since the original description of this agent. A description of these virus strains and certain observations concerning their epidemiology form the basis of this report.

Materials and Methods

Human embryonic intestine (HEI) tissue culture. HEI tissue culture tubes (MA- 177) were purchased from Microbiological Associates. The intestine was obtained from a $3^1/_2$-lb stillborn male infant with an estimated gestational age of 34 weeks. This fibroblast cell strain could not be grown consistently beyond the twentieth passage; passages 13–17 were used for isolation of the 229E related viruses. Growth medium consisted of Eagle's minimum essential medium (MEM) in Earle's balanced salt solution (BSS), supplemented with 0.1 mmole each of 7 "nonessential" amino acids, 1 mmole sodium pyruvate, 10% inactivated (56 C for 30 min) fetal calf serum, 100 units of penicillin, and 100 μg of streptomycin per milliliter; maintenance medium consisted of equal parts of Eagle's MEM in Earle's BSS and Medium 199 in Hanks' BSS supplemented with 2% inactivated calf serum and antibiotics as above [14].

Source of specimens and virus isolation procedures. Specimens were obtained on or before the fourth day of illness from employees of the NIH who had acute upper respiratory tract illnesses. In the 1962–1964 study, 0.2 ml of freshly collected nasopharyngeal wash fluid (veal infusion broth with 0.5% bovine albumin) was inoculated into 2 roller tubes each of Hep-2, rhesus monkey kidney (MK), and HDCS WI26 or WI38 [2]. In the 1965–1967 study of NIH employees, 0.85% NaCl was used as nasal wash fluid, and this fluid was immediately diluted approximately $^1/_2$ in veal infusion broth with 0.5% bovine albumin. These specimens were inoculated within 1 hr of collection into 2 roller tubes each of Hep-2, MK, WI26 and/or WI38, human aorta (AT-39), and HEK cultures [15]. The remaining nasal fluid was stored at −60 C. Tissue cultures were obtained from commercial sources, maintained as previously described, incubated at 33–34 C on drums rotating at 12 revolutions per hour, and observed for cytopathic effect (CPE) twice weekly [16]. MK cultures were tested for hemadsorption at 5–7-day intervals, and a single blind subpassage of the HEK culture harvests was made at 21 days. Acute phase sera were obtained at the time the washings were collected and convalescent sera about 3 weeks later. Five specimens from the 1962–1964 study and all specimens from the more recent study (1965–1967) were also inoculated as above into HEI tissue culture; although some specimens were inoculated into HEI cultures within 1 hr of collection, most had been stored at −60 C prior to inoculation.

Organ cultures. Human embryonic tracheal organ cultures were prepared and maintained by a modification of the method of Hoorn and Tyrrell as described previously [7, 17, 18].

Electron microscopy. One to 5 ml of tissue culture or O.C. fluid was clarified by low-speed centrifugation at 2,000 rpm for 10–15 min in the PR-2 International centrifuge, and then centrifuged at 111,000 × *g* for 60 or 90 min in the SW-39 rotor of a Spinco model L ultracentrifuge. Pellets were resuspended in 0.1–0.2 ml of 1% ammonium acetate, negatively stained with 2% phosphotungstic acid (PTA) at *p*H 5.0 or 7.0, and spread on formvar-coated copper grids [7]. All electron micrographs were taken with a Siemens Elmiskop 1A at magnifications of × 40,000–80,000.

Viruses and sera. Dr. Dorothy Hamre kindly supplied 229E virus and guinea pig antiserum. The virus had been purified by the terminal dilution technique in WI38 cultures.

Chloroform sensitivity, 5-iodo-2-deoxyuridine

[2] A. Z. Kapikian, H. D. James, Jr., S. J. Kelly, K. McIntosh, and R. M. Chanock, "Etiology of upper respiratory tract illnesses among civilian adults" (in preparation).

sensitivity, and acid lability tests. These tests were performed as previously described [16, 19–22]. The 229E-related viruses isolated in HEI cells were adapted to HDCS WI38, and tests were performed in tube cultures of HDCS WI38.

Infectivity titrations. HEI or WI38 cultures were used in infectivity titrations. Tenfold dilutions of virus were made in Hanks' BSS containing 0.5% gelatin, 100 units of penicillin per milliliter, and 100 μg of streptomycin per milliliter. Two-tenths milliliter of the appropriate virus dilution was inoculated into each of 2–4 tube cultures; the cultures were examined for the appearance of CPE 3 times weekly for approximately 2 weeks. Infectivity titers were based on CPE and calculated by the method of Reed and Muench [23].

Inoculation of suckling mice. Swiss mice of the CD-1 strain were obtained from Charles River Mouse Farms, Incorporated, Wilmington, Massachusetts. Retired breeders from this mouse colony were tested and found to be free of CF antibody to MHV, strain A59 [24]. One litter each of suckling mice, 0–3 days old, was inoculated intracerebrally with 0.01 ml of tissue culture harvests of viruses 489, 511, 515, and 844 and observed for 21 days. If a mouse developed illness, it was sacrificed and brain suspension was passaged to additional suckling mice intracerebrally and intraperitoneally; in addition, such suspensions were inoculated into HDCS WI38 cultures.

Complement fixation (CF) tests. 229E virus was inoculated into 32-oz bottles of WI38 cultures and allowed to adsorb for 1 hr. Fluids harvested after 2 freeze-thaw cycles, about 48 or 72 hr after inoculation, were employed as CF antigen. CF tests with appropriate controls were performed as previously described [7]. CF tests involving large numbers of paired sera could not for practical reasons be completed in a single day of testing. Therefore, such CF results reported in this study were from tests performed several days in succession.

Neutralization tests. Equal volumes of virus and fourfold dilutions of inactivated serum (56 C for 30 min) were incubated at room temperature for 2 hr. Two-tenths milliliter of the mixture was inoculated into each of 2 WI38 cultures. These were examined for CPE at a time when a simultaneous titration indicated that approximately 32–320 TCD_{50} of virus were present. The cultures were examined at 2–3-day intervals for approximately 2 weeks, since CPE with the 229E-related viruses did not appear before the fifth or sixth day. Serum neutralizing end points were calculated according to the method of Reed and Muench and were expressed as initial serum dilutions [23].

Results

Isolation and growth in tissue culture. The 3 nasal wash specimens which yielded 229E-related viruses from the 1962–1964 study were obtained within a 7-week period, March 2 to April 13, 1964, while the 6 virus-positive nasal wash specimens from the 1965–1967 study were obtained during the first 3 months of 1967 (table 1). The specimens were inoculated into roller tube cultures as stated in the Methods section. None of the inoculations into rhesus MK, Hep-2, HDCS WI26 or WI38, AT-39, or HEK cultures gave CPE. However, all 9 specimens produced CPE on initial passage in HEI tissue culture. The CPE was characterized by a gradual elongation of the cells throughout the monolayer beginning on the fifth or sixth day after inoculation; specific foci were not evident. Gradually, small granular round cells appeared throughout the monolayer. The cell sheet was rarely destroyed completely. At times uninoculated HEI tissue cultures appeared somewhat "stringy," and for this reason recognition of virus-specific CPE was occasionally difficult, especially at the beginning of the study.

Table 1. Source of "IBV-like" viruses recovered in human embryonic intestine cultures from nasal washings taken from patients with upper respiratory illnesses

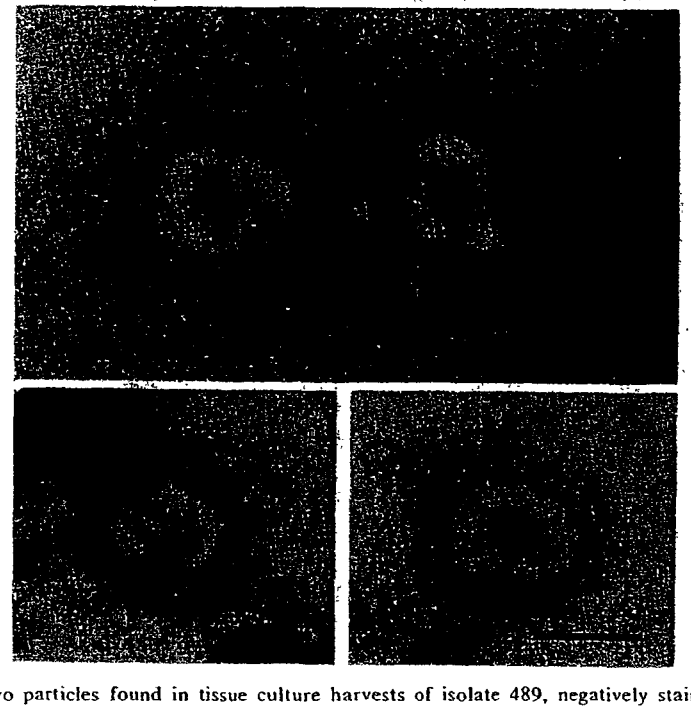
Patient number	Age	Sex	Date of specimen
489	21	female	3/2/64
511	33	female	4/9/64
515	24	female	4/13/64
840	39	male	1/23/67*
844	41	male	1/30/67
862	31	male	3/3/67
865	54	female	3/7/67
868	36	female	3/9/67
879	21	female	3/16/67

*CPE was visualized in HEI_1, but specimen was not available for further testing. Therefore WI_1, which did not show CPE, was passaged to HEI, which demonstrated typical CPE.

Electron microscopic and filtration studies. HEI culture harvests were clarified and concentrated by ultracentrifugation and examined by electron microscopy. Figure 1 shows representative particles seen in the harvests of isolate 489. Morphologically similar particles were seen in harvests of each of the other 8 isolates. A comparison of the isolates with strain 229E (figure 2), and avian IBV (figure 3), revealed their remarkable similarity. The particles usually appeared round or elliptical, but many were somewhat pleomorphic. A characteristic feature of the particles was the widely spaced club- or pear-shaped surface projections, which were narrow at the base, about 10 mμ wide at the outer edge, and approximately 20 mμ in length. The mean of the largest diameter (including projections) of 67 samples of the 9 isolates was 152 mμ ± 6 mμ (i.e., ±2 × standard error [SE] of mean) with a range of 104–250 mμ; the mean of the shortest diameter of these 67 samples was 107 mμ ± 4 mμ (i.e., ±2 × SE of mean) with a range of 76–160 mμ. Details of the morphology of this group of viruses have been described elsewhere [7–10, 25]. In filtration studies, in which Swinnex-25 filter units attached to 20-ml vacuum tubes were employed, virus strain 844 passed through a 450-mμ, 220-mμ, and 100-mμ but not a 50-mμ or 10-mμ Millipore filter [26, 27].

Attempts to grow agents in organ culture (O.C.). Attempts were made to cultivate these agents in human embryonic tracheal O.C. and to detect them by electron microscopy using methods described previously [7]. Three passages of the original specimens obtained from 5 of the patients with serologic evidence of 229E virus infection (in the 1962–1964 study) were made in human embryonic tracheal O.C. Virus particles could not be visualized in the third passage O.C. harvests by electron microscopy. HEI-positive harvests of viruses 489, 511, 515, and 844 were also passaged 3 times in O.C., and again virus particles could not be visualized by electron microscopy. Ciliary action of the organ cultures was unaffected by these 4 isolates during the 3 passages. Titrations in HEI or WI38 tissue cultures of harvests of first, second, and third O.C. passages revealed that growth in O.C. of low-titered inocula of strains 489, 511, and 515 could not be detected, while a higher-titered inoculum of strain 844 appeared to initiate replication at a low level (table 2).

Determination of nucleic acid type. Viruses 489, 511, 515, and 844 were tested for nucleic



Figures 1–3. Figure 1: two particles found in tissue culture harvests of isolate 489, negatively stained with PTA. The bar in all 3 figures represents 100 mμ. ×144,000. Figure 2: 229E virus, negatively stained with PTA. ×144,000. Figure 3: infectious bronchitis virus, Beaudette 42 strain, negatively staind with PTA. ×144,000.

Table 2. Attempts to grow 229E-related viruses in organ culture

Strain	Passage history of material inoculated into O.C.	Titer* in TCD_{50}/0.2 ml (log_{10})							Visualization of particles by electron microscopy of	
		Inoculum	Pooled harvests 1st passage in O.C.†	Pooled harvests 2d passage in O.C.†	Harvests from indicated day of 3d passage in O.C. (days)				Pooled 3d passage O.C. harvests	Tissue culture harvests (passage level) (titer* in TCD_{50}/0.2 ml [log_{10}])
					4	7	12	2–12		
489	HEI_3	2.5	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	No	Yes (HEI_2) (3.5)
511	HEI_3	3.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	No	Yes (HEI_2) (4.0)
515	HEI_2	2.5	1.5	<1.0	<1.0‡	<1.0	<1.0	<1.0	No	Yes (HEI_1) (2.0)
844	HEI_3WI38_1	5.0	2.0	3.0	N.T.	N.T.	N.T.	0.5	No	Yes (HEI_3WI38_1§) (5.0)

* Titrations of viruses 489, 511, and 515 were performed in HEI tube cultures, whereas titrations of virus 844 were performed in HDCS, WI38 tube cultures.

† Pools of fluids harvested at 2–3 day intervals for 12 or 13 days after inoculation.

‡ Day 5.

§ The same passage as that inoculated into O.C.

Table 3. Effect of 5-iodo-2-deoxyuridine (5-IUDR) and 5-IUDR plus thymidine on virus multiplication

Virus	Infectivity titer in indicated medium*		
	Maintenance medium (MM)†	MM + $10^{-4.3}$ M 5-IUDR	MM + $10^{-4.3}$ M 5-IUDR & $10^{-4.0}$ thymidine
489	3.5	3.5	4.0
511	4.0	4.0	4.0
515	4.0	3.5	3.5
844	3.5	4.0	4.0
Polio virus type 1(LSC-1)	≥6.5	≥6.5	≥6.5
Vaccinia	4.5	<0.8	4.5

* Infectivity titer expressed as log_{10} TCD_{50}/0.2 ml.

† MM as described in Materials and Methods.

acid type. Multiplication of these 4 viruses was not inhibited by $10^{-4.3}$ M 5-iodo-2-deoxyuridine (5-IUDR), a concentration inhibitory for DNA viruses. As seen in table 3, vaccinia virus (a known DNA virus) was markedly inhibited by 5-IUDR while poliovirus type 1 (a known RNA virus) was unaffected. Vaccinia virus multiplication was not inhibited when 10^{-4} M thymidine was added to the maintenance medium containing 5-IUDR. These findings suggest that the nucleic acid core of these 4 viruses was RNA.

Chloroform sensitivity. Table 4 shows that all 9 virus strains were inactivated by chloroform. The table also shows that a control virus, rhinovirus 1A (a known chloroform-resistant virus) was resistant to inactivation by chloroform,

Table 4. Stability of 229E-related viruses

Virus	Chloroform sensitivity test Infectivity titer (TCD_{50}/0.2 ml [log_{10}])		Acid lability test Infectivity titer (TCD_{50}/0.2 ml [log_{10}])	
	Treated with chloroform	Untreated	*p*H 2.7*	*p*H 7.0*
489	<0.8	2.3	<0.8	3.0
511	<0.8	4.8	<0.8	4.5
515	<1.0	5.0	<0.8	4.5
840	<1.0	≥3.5	<1.0	3.0
844	<0.8	2.3	<0.8	4.5
862	<1.0	≥3.5	<1 0	2.5
865	<1.0	≥3.5	<1 0	2.5
868	<1.0	2.5	<1.0	2.5
879	<0.8	3.0	<1.0	3.0
Rhinovirus 1A	≥3.5	≥3.5	≤1.5	≥3.5
Herpes simplex	<1.0	3.5	N.T.	N.T.
Polio virus type 1 (LSC-1)	N.T.	N.T.	≥5.5	≥6.0

* 3 hr at 25 C.

while another control virus, herpes simplex (a known chloroform-sensitive virus) was inactivated by chloroform.

Acid lability. Each of the 9 virus isolates exhibited at least a 100-fold reduction in infectivity titer after exposure to *p*H 2.7 for 3 hr at 25 C (table 4). The table also shows that rhinovirus 1A (a known acid-labile virus) demonstrated at least a 100-fold reduction in infec-

tivity titer, while the infectivity of poliovirus type 1 (a known acid-resistant virus) was unaffected after exposure to *p*H 2.7 for 3 hr.

Pathogenicity in mice. Virus strains 489, 511, 515, and 844 were inoculated into suckling mice as described in Materials and Methods. No illness was observed in mice inoculated with strains 489, 515, and 844; 4 of 8 mice inoculated with virus 511 died on the fourth day after inoculation. However, brain suspensions passaged intracerebrally and intraperitoneally to additional suckling mice failed to produce illness; in addition, such suspensions inoculated into WI38 cultures failed to produce CPE.

Serologic studies. 10–32 TCD_{50} of each of the 9 isolates were neutralized by a 1:160–1:1280 dilution of 229E guinea pig antiserum which had a homologous titer of 1:320–1:1280 against 32–100 TCD_{50}. This would indicate that each of the 9 isolates was similar, if not identical, to 229E virus.

Table 5 shows that 8 of the 9 patients from whom the 229E-related viruses were isolated had a significant CF antibody response to 229E virus. In neutralization tests, 3 of the 9 virus-positive patients exhibited significant increases in antibody to both strain 229E and their own isolate; in addition, 2 patients had such rises in antibody to strain 229E alone, while 2 others had such rises only to the strain which they shed.

Efficiency of techniques for detection of virus infection. The CF test was a more sensitive index of 229E infection than virus isolation. Table 6 shows that, during the period of 229E prevalence in 1966–1967, 22 of 89 individuals exhibited evidence of infection; 21 of the 22 developed a fourfold or greater CF antibody rise, whereas only 9 of the 22 shed virus detectable in HEI cultures.

Clinical findings. Each of the 9 virus-positive patients was cultured either on the day of onset (1 of 9) or the day after onset of symptoms. Their average age was 33 years, with a range of 21–54 years. The most common symptoms recorded were coryza (in all 9), nasal congestion (in 8), sneezing (in 7), and sore throat (in 5). Less common were headache (in 4), cough (in 3), muscle or general aches (in 3), and chills and feverishness (in 2). The chief complaint in 8 of the 9 patients was coryza or nasal congestion; 1 patient's chief complaint was sneezing. None of the patients had an abnormal temperature elevation on the day of examination.

Epidemiologic observations. Sera obtained from pediatric patients with acute respiratory illnesses from several populations were tested by the CF technique for evidence of 229E infection. Only 1 of 892 paired sera obtained from October, 1962, through August, 1965, from infants and children admitted to Children's Hospital, Washington, D.C., for predominantly lower respiratory tract illness, exhibited serologic evidence of 229E infection. In a similar study of 222 infants and young children admitted to Children's Hospital from December, 1966, through April, 1967, none of these patients developed an antibody rise for 229E virus. In addi-

Table 5. Serologic response of individuals from whom 229E-related viruses were recovered

Patient number	Serum tested	Reciprocal of neutralizing antibody titer against 32 TCD_{50} of 229E virus	Reciprocal of neutralizing antibody titer against indicated TCD_{50} of patient's isolate	Reciprocal of CF antibody titer to 229E virus
489	Acute	<4	<4 (32)	<8
	Conv.	16	6 (32)	16
511	Acute	24	<4 (1,000)	2
	Conv.	64	12 (1,000)	16
515	Acute	8	6 (32)	<2
	Conv.	64	24 (32)	8
840	Acute	4	4 (32)	4
	Conv.	32	24 (32)	32
844	Acute	<4	<4 (32)	8
	Conv.	16	24 (32)	≥32
862	Acute	<4	24 (32)	<4
	Conv.	6	48 (32)	16
865	Acute	<4	6 (10)	<4
	Conv.	12	16 (10)	16
868	Acute	24	8 (32)	8
	Conv.	64	24 (32)	16
879	Acute	24	<4 (10)	<4
	Conv.	24	8 (10)	8

Table 6. Evidence of infection with 229E-related viruses during period of prevalence (December, 1966 through April, 1967)

	Number of individuals		Total
	Virus isolated	Virus not isolated	
CF antibody rise	8	13	21
No CF antibody rise	1	67	68
Total	9	80	89

Table 7. Serologic (CF) evidence of 229E virus infection by month and year in civilian adults with upper respiratory illnesses

Months	1962		1963		1964		1965		1966		1967	
	No. tested	No. with ≥4-fold rise	No. tested	No. with ≥4-fold rise	No. tested	No. with ≥4-fold rise	No. tested	No. with ≥4-fold rise	No. tested	No. with ≥4-fold rise	No. tested	No. with ≥4-fold rise
Jan.–Feb.	*	..	24	0	28	2 (7%)	..	.	38	1†	26	5 (9%)
March–April		..	54	0	22‡	3 (14%)	.	..	30	0	46	13 (9%)
May–June	..	.	27	0	1	0	..	.	18	0	8	0
July–Aug.	..	..	24	0		.		.	19	0	9	0
Sept.–Oct.	4	0	25	0	..	.	40	1	35	0	...	...
Nov.–Dec.	28	0	19	0	..		37	0†	21	3 (14%)	...	...

NOTE. For 1962–1964, 5 (2%) of 256 had rises; for 1965–1967, 23 (7%) of 317 had rises.

* Not studied.

† From December, 1965, through February, 1966, 18 (31%) of 59 patients developed ≥4-fold rises to NIH O.C. virus strains OC38 (664) and OC43 (690); 5 NIH O.C. viruses (663, OC38, OC43, 691, 703) were recovered in O.C. from 5 of these 18 patients [7].

‡ NIH O.C. virus 501 recovered in O.C from 1 patient who did not develop 229E CF antibody rise [7].

tion, none of 261 paired sera obtained from June, 1964, through June, 1965, from infants and young children with predominantly lower respiratory tract illnesses studied in Jamaica, Trinidad, Hong Kong, Cairo, Singapore, or New Delhi as part of a World Health Organization collaborative program, demonstrated evidence of 229E infection [28].

In the first period of the cross-sectional study of acute respiratory illness among NIH employees which extended from October, 1962, to May, 1964, infection with strain 229E was not detected for the first 15 months of the investigation (table 7). During the next 4 months, 5 of 50 patients studied developed a CF antibody rise to 229E virus. Over-all, in the first period, 5 (2%) of 256 patients exhibited serologic evidence of 229E virus infection. During the second period, which extended from September, 1965, through August, 1967, 229E virus infection was detected during 2 separate 4- and 5-month intervals. Infection was infrequent during the first interval, from October, 1965, through January, 1966; but during the second interval, from December, 1966 through April, 1967, 21 (24%) of 89 patients developed a rise in CF antibody to strain 229E. Over-all, during the 1965–1967 period, 23 (7%) of 317 patients exhibited serologic evidence of infection. During the interval in which 229E was prevalent, infections with rhinovirus and other cytopathic or hemadsorbing viruses were uncommon. Detailed data will be published in the future. As indicated previously, serologic evidence of infection with 229E virus was not detected among pediatric patients hospitalized with predominantly lower respiratory tract illness in the Washington, D.C., area during the interval when 229E virus was prevalent among adults with upper respiratory tract illness.

During 1965–1967, 152 (48%) of 317 adults studied had detectable CF antibody (1:4 or greater) for 229E virus, while 48 (15%) had serum antibody levels of 1:8 or greater. In contrast, only 4 (2%) of 222 infants and young children studied during the period December, 1966, through April, 1967, possessed serum CF antibody at a titer of 1:4 or greater.

Discussion

Viruses resembling avian IBV and MHV in morphology have recently emerged as possible important etiologic agents of acute upper respiratory illnesses in adults. Only 12 isolations from natural infections have been reported: B814, the first "IBV-like" virus isolated from man could be cultivated only in human embryonic tracheal O.C. [5]; 229E and 4 serologically identical strains were recovered in HEK cultures [6]; and 6 NIH O.C. strains (501, 663, OC38 [664], OC43 [690], 691, and 703) were isolated in human embryonic tracheal O.C. [7]. Two of the 6 NIH O.C. strains (OC38 and OC43) were subsequently adapted to suckling mouse brain, but none of the 6 strains grew in monolayer tissue culture [29]. The 9 strains recovered in this study

were found to be similar, if not identical, to strain 229E by one-way neutralization tests and, in addition, to possess similar morphologic and biophysical properties.

The 9 229E-related strains were recovered on initial passage in semi-continuous HEI tissue cultures; they could not be isolated on initial passage in conventional tissue cultures. In addition, since the technique of O.C. passage followed by electron microscopic examination of pooled, concentrated O.C. harvests had proved to be a sensitive system for the recovery of NIH O.C. viruses, this technique was applied both to several clinical specimens yielding agents resembling strain 229E and to several tissue-culture-adapted strains as well. In all cases, attempts to detect virus by electron microscopy in O.C. harvests failed, although titration of such harvests showed in 1 case that low-grade replication of virus probably occurred. It appears, therefore, that HEI tissue culture is preferable to O.C. for isolation of certain strains of "IBV-like" viruses such as these 229E-related agents. It should be noted, however, that the 6 "IBV-like" strains recovered in O.C. in this laboratory could not be adapted to grow in HEI tissue culture [K. McIntosh, unpublished studies]. Therefore, for isolation of "IBV-like" viruses, it would appear to be necessary to employ both HEI tissue culture and O.C. However, additional ways to facilitate the growth of "IBV-like" viruses such as 229E and others must be found since HEI cultures, although more sensitive than any conventional tissue culture or O.C. system, were much less sensitive than the measurement of CF antibody rises for detection of 229E infection.

The serologic survey of 229E virus infection in various population groups revealed that infection with this agent was rare in infants and children with lower respiratory tract illnesses. In the 1962–1964 study of upper respiratory tract illness among adults, 5 (2%) of 256 individuals had serologic evidence of 229E infection. It was noteworthy that these 5 infections occurred from January through April, 1964, when rhinovirus and other known respiratory virus infections were uncommon but upper respiratory tract illnesses were prevalent; during this period 229E virus infection was associated with 5 (10%) of the 50 illnesses sampled. In the 1965–1967 study of upper respiratory tract illness in adults, 23 (7%) of 317 patients developed serologic evidence of 229E infection. It was striking that 21 of the 23 infections occurred during a 5-month period (December, 1966–April, 1967) when infections with rhinoviruses and other cytopathic or hemadsorbing viruses were uncommon while upper respiratory tract illness remained prevalent. During this 5-month period, 21 (24%) of 89 patients studied developed serologic (CF) evidence of 229E infection. It was previously reported that 5 NIH O.C. viruses were recovered in O.C. from specimens obtained from 9 patients during a 3-month period of the previous year (December, 1965–February, 1966) [7]. In addition, in a serologic survey using as antigen 2 serologically identical NIH O.C. viruses (strains OC38 and OC43) originally recovered in O.C., and subsequently adapted to grow in the brain of suckling mice, 18 (31%) of 59 patients (including the 5 patients above who yielded NIH O.C. viruses) developed serologic evidence of infection with these viruses during this 3-month period (December, 1965–February, 1966) [29]; only 1 of the 59 patients developed a CF antibody rise to 229E virus during this period. It was of interest that, in these CF antibody surveys, patients with serologic evidence of 229E infection rarely developed concurrent CF antibody rises to the OC38 and OC43 mouse brain antigens [30].

These observations suggest that "IBV-like" viruses may be etiologic agents of a portion of respiratory illnesses which occur during the winter season, when the prevalence of rhinovirus and other known respiratory virus infections is often low, but that of upper respiratory tract illnesses quite high [2–4].

The occurrence of a typical common-cold-like illness in the 9 patients from whom 229E-like viruses were isolated is consistent with the clinical findings in volunteer studies with B814 and 229E viruses [5, 11].

Summary

Nine virus strains resembling "IBV-like" virus 229E of Hamre were recovered in human fetal intestine fibroblast cultures from nasopharyngeal washings of adults with acute upper respiratory tract disease. Eight of the 9 virus-positive individuals developed serologic (CF) evidence of 229E virus infection. The virus isolates exhibited properties typical of the "IBV-like" virus group: distinctive morphology, resistance to 5-IUDR, and chloroform sensitivity. Measurement of CF

antibody response was found to be approximately twice as sensitive as virus recovery for detection of infection. Standard monolayer tissue cultures as well as human fetal tracheal O.C. were ineffective for recovery and recognition of the 229E related isolates. A seroepidemiologic survey indicated that 229E virus infection was rare in children with lower respiratory tract disease. However, such infection occurred in 10%–24% of adults with upper respiratory tract illnesses during 2 of 4 winters—a season when rhinovirus infection was uncommon but respiratory disease morbidity was high.

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CHAPTER 49

Rotaviruses

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Rotaviruses are the single most important etiologic agents of severe diarrheal illness of infants and young children world-wide (24,124,171,288,336,342,348,355). Although diarrheal diseases are one of the most common illnesses of infants and young children throughout the world, they assume a special significance in less developed countries, where they constitute a major cause of mortality among the young.

The scope of the problem in the United States was highlighted during the Cleveland Family Study; in this study, infectious gastroenteritis (considered nonbacterial) was found to be the second most common disease experience, accounting for 16% of approximately 25,000 illnesses over a period of about 10 years (139). Although diarrheal diseases are not a prominent cause of mortality in infants and young children in the United States, a recent analysis revealed that between 1973 and 1983, 5539 children 1 month to 4 years of age died from diarrheal diseases (average 504 deaths per year); this comprised 2% of the total postneonatal deaths or 10% of the preventable portion (284).

The toll from diarrheal diseases in developing countries is staggering. An analysis of vital statistics by the World Health Organization indicates that diarrheal diseases are responsible for a large proportion of the total reported deaths in developing countries, accounting for as many as 15–34% of all deaths annually in certain countries (742). It is likely that most of these deaths occurred primarily during infancy and early childhood. In 1975 it was estimated that 450,000,000 diarrheal episodes occurred in infants and young children less than 5 years of age in Asia, Africa, and Latin America and that 1–4% of the episodes were estimated to be fatal (553). In this estimate, the deaths of 5–18 million infants and young children were attributed to diarrheal illness. More recently, it was estimated that in these regions there were 3–5 billion cases of diarrhea and 5–10 million deaths associated with diarrhea in a 1-year period (1977–1978), ranking diarrhea first among infectious diseases in the categories of both frequency and mortality (723). In another analysis of selected

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studies, it was estimated that 744 million to 1 billion diarrheal episodes and 4.6 million deaths from diarrhea occur every year in children less than 5 years of age in Africa, Latin America, and Asia (excluding China) (613).

HISTORY

Despite the magnitude of the problem of infantile diarrheal diseases, the search for important etiologic agents—bacterial, viral, or parasitic—was unrewarding until the 1970s. This was especially disappointing for virologists because with the advent of tissue culture technology, scores of enteric viruses were discovered in the 1950s and 1960s, but none was found to be an important etiologic agent of infectious diarrhea (116,122,333,460,774).

This frustration ended with the discovery in 1972 by Kapikian et al. (354) of the 27-nm Norwalk virus and its association with viral gastroenteritis in older children and adults, followed by the discovery in 1973 by Bishop et al. (48) of the 70-nm human rotavirus and its association with severe diarrhea in infants and young children. Both of these fastidious agents were discovered without the use of tissue culture technology; by necessity, their identification relied on direct visualization by electron microscopy. The Norwalk virus was identified in feces by immune electron microscopy, whereas the first human rotavirus was visualized by thin section electron microscopy of duodenal mucosa. Shortly afterwards, rotavirus was identified in feces by electron microscopy by Flewett et al., Bishop et al., and others (49,177,352,451). The Norwalk virus is the cause of 40% of community outbreaks of nonbacterial gastroenteritis in older children and adults, and although this virus is associated with mild gastroenteritis in infants and young children, it is not the cause of severe diarrhea in this age group (24,124,348).

It soon became apparent that the 70-nm particle (Fig. 1), subsequently designated *rotavirus*, was an impor-

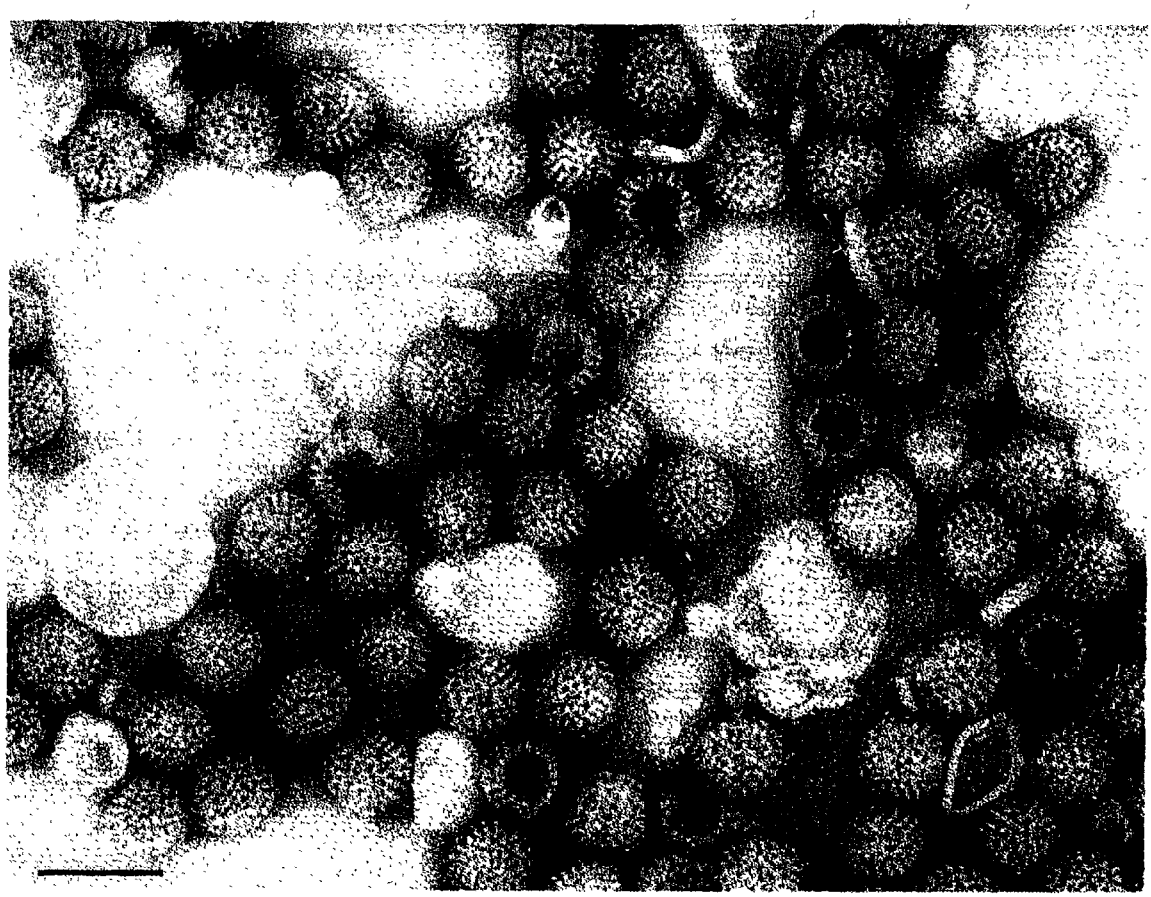

FIG. 1. Human rotavirus particles observed in a stool filtrate (prepared from a stool of an infant with gastroenteritis) after incubation with phosphate-buffered saline and further preparation for EM. The particles appear to have a double-shelled capsid. Occasional "empty" particles are seen. Scale bar: 100 nm. (From refs. 332 and 352 with permission.)

tant etiologic agent of infantile diarrhea, causing 35–50% of hospitalization for this condition during the first 2 years of life (171,288,342). In a relatively short period, investigators from many countries reported the detection of rotaviruses in feces of pediatric patients with diarrheal illness, and it was soon apparent that rotaviruses were the long-sought-after major viral etiologic agents of severe diarrhea of infants and young children.

Although the human rotaviruses were discovered in 1973, it should be noted that in 1963, Adams and Kraft, using thin-section electron microscopy, described virus-like particles in intestinal tissue of mice infected with the epizootic diarrhea of infant mice (EDIM) virus (1,22). These particles were similar to those observed by Bishop et al. (48). In 1963, Malherbe et al. (408) described the isolation of a 70-nm virus designated SA-11 (simian agent 11). This virus, derived from a rectal swab obtained from a healthy vervet monkey, was recovered in vervet monkey kidney cell culture. In 1967, Malherbe and Strickland-Cholmley (409) described another virus similar to SA-11, the O (Offal) agent. The O agent was isolated in vervet monkey kidney cell culture from the mixed washings of intestines of cattle and sheep. In addition, in 1969, Mebus et al. (444) demonstrated the presence of 70-nm virus particles in stools from calves with a diarrheal illness and showed that this agent could be passaged serially in calves with production of disease. In 1971, Mebus et al. (443) reported successful cultivation of the Nebraska calf diarrhea virus (NCDV) in primary fetal bovine cell cultures; in 1972, Fernelius et al. (173) reported that the NCDV resembled the reoviruses morphologically but was distinct antigenically. The murine, simian, O, and bovine agents were later found to exhibit characteristic rotavirus morphology and to share a group antigen with other rotaviruses (180,338,339,352,388,466).

In 1943, Light and Hodes (393) induced a diarrheal illness in calves by feeding a filtrate of stools from infants with diarrhea. About 30 years later, following the detection of human rotaviruses, rotavirus particles were visualized in a stool filtrate from a calf that developed illness during this study (285,286). Unfortunately, it could not be determined whether these particles represented human or bovine rotavirus.

CLASSIFICATION

Rotaviruses are classified as a genus in the family Reoviridae (430). This family contains six distinct genera: *Reovirus, Orbivirus, Rotavirus, Phytoreovirus, Fijivirus*, and a presently unnamed group of cytoplasmic polyhedrosis viruses. Antigenically, rotaviruses are distinct from the reoviruses by complement fixation (CF) and immune electron microscopy (IEM), from type 1 reovirus by neutralization, from reovirus types 2 and 3 by radioimmunoassay (RIA), from a large number of orbiviruses by CF, and from bluetongue virus by IEM (130,181,327,338,339,352). Rotaviruses have not been tested serologically against the other genera of the Reoviridae. Classification is described in detail in Chapters 2, 45, and 48.

INFECTIOUS AGENTS

Morphology and Morphogenesis

Rotaviruses have a distinctive morphologic appearance by negative-stain electron microscopy. Complete particles measure ~70 nm in diameter and have a double-layered icosahedral protein capsid that consists of an outer and an inner layer (Fig. 1). Within the inner capsid is the core that contains the 11 segments of double-strand RNA. The complete particles are also designated "smooth" particles because the outermost margin of the outer capsid layer has a well-defined, smooth circular appearance. Single-shelled particles measure ~55 nm in diameter and are designated "rough" particles because they lack the smooth outer layer and the capsomeres of the inner capsid project to the periphery, giving a circular "bristly" appearance. The core, which appears to be hexagonal in outline (not shown in the figure), measures ~37 nm in diameter (156,181,291,352,413,505,518,545).

The term *rotavirus* is derived from the Latin word "rota," which means wheel, and was suggested because the sharply defined circular outline of the outer capsid gives the appearance of the rim of a wheel placed on short spokes radiating from a wide hub (174,180). Rotaviruses resemble the reoviruses and orbiviruses morphologically; thus, morphology alone cannot distinguish these viruses (473,489,505,750). However, subtle morphological differences can sometimes be observed. The sharply defined circular outline of the outer capsid of rotaviruses differs from the comparatively indistinct outer capsid of orbiviruses; the distinct outer capsid of the reoviruses is often not as sharply defined as that of the rotaviruses. A small proportion of human rotavirus preparations may contain flattened tubular structures 54–100 nm (mean 70 nm) in width and of varying length; these structures have been shown by IEM to be related antigenically to the virion (162,181,291,367,368,504). In one study, both large (75–80 nm in diameter) and small (50 nm in diameter) tubular structures were observed. By IEM, both types of tubules reacted not only with homologous human rotavirus antiserum but also with heterologous bovine rotavirus antiserum, suggesting that these structures were related to the inner capsid (366). It has been proposed that they may be the result of aberrant assembly of viral capsid material (368). The

complete tubular forms may have a cap-like structure on either end and are ~1000 nm in length.

Recent studies of the three-dimensional structure of unstained, unfixed, double- and single-shelled rotavirus particles using cryoelectron microscopy and image processing techniques have yielded some important new information on the ultrastructure of rotaviruses (524). These and other findings relating to ultrastructure and morphogenesis are described in Chapter 48.

Physicochemical Properties

Double-shelled ("complete" or "smooth") rotavirus particles have a density of 1.36 g/cm^3 in cesium chloride (CsCl) and have a sedimentation coefficient of 520–530S. Single-shelled particles ("rough") have a density of 1.38 g/cm^3 and a sedimentation coefficient of 380–400S (77,156,244,339,350,519,545,546,645). "Empty" particles (penetrated by negative stain when viewed by electron microscopy) have a density of 1.29–1.30 g/cm^3 in CsCl (156,545,645). Infectivity is associated with the double-shelled particles (1.36 g/cm^3). The buoyant density of the core particle is 1.44 g/cm^3 in CsCl, and its sedimentation coefficient is 280S (38).

The infectivity of SA-11 virus as measured by plaque titration in tissue culture is stable or relatively stable following treatment with ether, genetron, chloroform, repeated freeze-thawing, sonication, or incubation at 37°C for 1 hr or at ambient temperature (25°C) for 24 hr (169). After 5 min incubation at 50°C, SA-11 loses 80% of its infectivity; after 30 min, 99% of infectivity is lost (169). Incubation with trypsin enhances infectivity of SA-11 and is necessary for infectivity of human rotaviruses for tissue culture (167,372,580,603). The enhancement of viral infectivity by proteolytic enzymes such as trypsin, pancreatin, or elastin is now recognized as essential for isolation and cultivation of human rotaviruses and other fastidious strains (169,206,531,580). At pH 3.5 or 10.0, SA-11 retains infectivity, although there is some loss of titer (505). Below pH 3.0 or above pH 10, the outer capsid of human rotavirus collapses, but the particles do not disintegrate. Following exposure to acid buffers at a pH of 2.0 or to stomach acid at a pH of 1.8 or 2.1, rotaviruses are rapidly inactivated (729). The inactivation of rotavirus at low pH observed in this and other studies is an important property that must be taken into consideration in vaccine development because major emphasis is being given to live oral vaccines (243, 707). Hemagglutination activity is reduced following treatment with chloroform, following repeated freezing and thawing, or incubation at low pH (pH 2.0) and is lost following treatment with ethanol or methanol. It is not reduced by treatment with alkaline solutions (pH 10.6) or diethylether (45).

SA-11 loses most of its infectivity following incubation in 2M $MgCl_2$, $CaCl_2$, or NaCl for 15 min at 50°C, whereas infectivity is stable in $MgSO_4$ (596). SA-11 also loses most of its infectivity after freezing in 2 M $MgCl_2$ or 2 M $CaCl_2$. Infectivity of human rotavirus is stabilized by low levels of $CaCl_2$ (1.5–15 mM) or strontium chloride (0.15–15 mM) but not by $MgCl_2$.

Human rotavirus and SA-11 lose infectivity following treatment with chelating agents such EDTA or EGTA (169). Chelating agents convert double capsid particles (density of 1.36 g/cm^3 in CsCl) to single capsid particles (density of 1.38 g/cm^3) by reducing the concentration of calcium; this cation is necessary for maintenance of intact virions (114,587). The loss of the outer shell apparently activates the endogenous RNA polymerase.

Chaotropic agents such as $CaCl_2$ or potassium thiocyanate degrade calf rotavirus single capsid particles to 37-nm core particles with a density of 1.44 g/cm^3 in $CsCl_2$ (38). This change is associated with loss of polymerase activity.

Hospital disinfectants such as ethanol, 0.95 by volume (95% v/v), and Biogram (a chlorinated phenolic compound), 5% v/v, are more effective than 4% or 10% w/v formaldehyde for inactivation of SA-11 (64,647, 648,704); 95% ethanol is the most effective of the disinfectants thus far tested (45,647). Sodium hypochlorite solution containing 11% available chlorine (chloros) used at a 3% solution is not effective in reducing infectivity of lamb rotavirus (607). An extensive survey of the effect of numerous commercially available disinfectant formulations on the infectivity of rotaviruses has demonstrated the relative resistance of human rotaviruses to a wide range of other chemical disinfectants in common use (397,620).

Genetics

The genetics of rotaviruses, including the gene organization and the coding assignments, are presented in detail in Chapter 48. Only key points that relate to the present chapter are presented here.

Genome

The rotavirus genome contains 11 segments of double-strand RNA which range in molecular weight (MW) from 2×10^5 to 2.2×10^6 daltons (171,288,324, 328,329,440,489,545,673). The estimated MW of the 11 segments is 11×10^6 to 14×10^6 daltons. The RNA segments fall into four size classes based on contour length measurements by electron microscopy and confirmed by nucleotide sequence analysis of nine of the 11 genes (see Chapter 48). The distribution of the 11 segments into these four size classes is evident using

polyacrylamide gel electrophoresis (PAGE) of RNA (Fig. 2) (165,205,328). Characteristically, these RNA segments are numbered in order of migration during PAGE, with the slowest RNA segment designated gene 1, etc. There are four large segments, two medium segments, three smaller segments, and the two smallest segments. This pattern contrasts with that of the reoviruses, which contain 10 RNA segments that are distributed into three size classes—large, medium, and small. These size designations are relative because the 10th (i.e., smallest) RNA segment of reovirus is larger than the five smallest segments of rotavirus (288).

Some rotaviruses do not display the characteristic RNA migration pattern described above. For example, murine rotavirus segment 10 migrates close to segment 11 (605); avian rotavirus segment 4 migrates close to segment 5, and segments 10 and 11 are difficult to resolve (674); segment 11 was missing from a bovine rotavirus and appeared to be transposed between segments 6 and 7 (522); and two lapine rotaviruses had patterns of 4,3,3,1 and 4,2,4,1 instead of the usual 4,2,3,2 distribution shown in Fig. 2 (672). In some human rotavirus strains, the 10th and 11th RNA segments migrate more slowly than usual, yielding a "short" pattern characteristic of almost all subgroup 1 human rotaviruses (which are, in almost every instance, serotype 2) (325,382,542). A human strain with a "long" RNA pattern was recently described which exhibits the unusual combination of subgroup 1 and serotype 3 antigenic specificities (371,476). Moreover, several "super-short" patterns of RNA migration in which the 10th gene segment migrated even more slowly than the "short" pattern rotaviruses have been observed for viruses recovered from humans and bovines (6,421,522). These strains also exhibit subgroup I antigenic specificity. In addition, several human, porcine, bovine, ovine, rodent, and avian rotavirus strains, which do not share the common group antigen of conventional (Group A) rotaviruses, have quite distinct RNA migration patterns (59,71,75,171,311,312, 440,511,543,676). Such strains have been designated non-group A rotaviruses (also referred to as "pararotaviruses").

Because human rotaviruses initially could not be propagated in tissue culture, comparison of the migration patterns of RNA during PAGE became an important laboratory and epidemiologic technique for characterization of strains (165,542). The term "electropherotyping" was applied to this method of differentiating strains (542). It soon became apparent that human rotaviruses exhibited a wide variety of electropherotypes and that these patterns differed from those observed with animal rotavirus strains. Furthermore, rotaviruses derived from animals exhibited a species-specific pattern (or set of patterns). However, it was not known whether differences or similarities in electrophoretic pattern had any relationship to antigenic specificity of the human strains as defined by neutralization (i.e., serotype). Subsequent studies indicated that viruses of the same serotype can exhibit different electropherotypes and that viruses of the same electropherotype can belong to different serotypes (29,193,218).

The extent of genetic relatedness among rotavirus strains has also been studied using labeled (+)ssRNAs transcribed from viral cores as hybridization probes as shown in Fig. 3 (186,187,189,193,196,252,428,453, 456,628). The (+)ssRNAs represent messenger RNAs that are transcribed from the negative strand of genomic RNA by the viral polymerase. These (+)mRNAs appear to be colinear and coterminal with genomic (−)RNA. None of the ^{32}P-labeled (+)ssRNAs transcribed from the Wa strain of human rotavirus hybridize with denatured dsRNAs of the bovine NCDV or simian rotavirus SA-11 to yield labeled dsRNAs with an electrophoretic mobility characteristic of genomic RNA. Similarly, (+)ssRNAs of NCDV do not hybridize with denatured dsRNA of Wa strain or SA-11 to yield labeled dsRNA with the electrophoretic mobility of genomic RNA. Also, (+)ssRNA probes transcribed from SA-11 exhibit specificity when tested against genomic RNA of Wa or NCDV.

Examination of rotaviruses obtained on a single day from children with gastroenteritis in Venezuela revealed numerous electropherotypes by PAGE (193). However, when these rotaviruses were tested with hybridization probes prepared from the human Wa (se-

FIG. 2. Polyacrylamide gel electrophoresis pattern of RNA from human rotavirus strain D (serotype I, subgroup II). The 11 segments of double-strand RNA comprising the rotavirus genome are identified. (From refs. 328 and 751 with permission.)

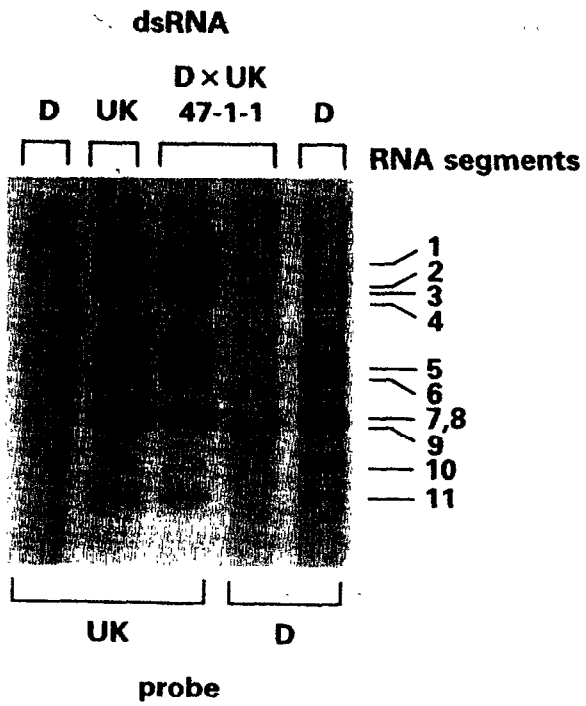

FIG. 3. RNA–RNA hybridization as used for (a) study of genetic relatedness among rotaviruses and (b) analysis of genotype of rotavirus reassortants. Single-strand (ss) ^{32}P-labeled RNA transcripts (probe) of the bovine UK rotavirus or the human D rotavirus were hybridized to denatured, genomic double-strand (ds) RNAs of the UK or D rotavirus (reaction of D transcripts with UK genomic RNAs not shown). After hybridization at 65°C for 14 hr, the hybridized RNAs were pelleted and subjected to polyacrylamide gel (10%) electrophoresis. The resulting pattern was visualized by autoradiography. Note that incubation of denatured human D rotavirus dsRNAs with the bovine UK rotavirus ss probe did not result in formation of genomic length hybrids visualizable by autoradiography (*first lane*). However, the homologous reaction of ss-labeled bovine UK rotavirus probe and bovine UK rotavirus dsRNAs yielded hybrids that exhibited the electrophoretic mobility of bovine rotavirus genomic RNAs (*second lane*). Similarly, in a homologous reaction the ss human D rotavirus probe produced hybrids with the electrophoretic mobility of human D rotavirus genomic RNAs (*fifth lane*). Analysis of reassortant 47-1-1 indicated that only the ninth gene was derived from its human D rotavirus parent, whereas the remaining 10 genes were derived from its bovine UK rotavirus parent. (Courtesy of Dr. Karen Midthun; after ref. 252 with permission.)

rotype 1) or DS-1 (serotype 2) virus, the RNA segments of each rotavirus hybridized significantly to either the Wa probe or the DS-1 probe but not to both. This observation led to the suggestion that there are at least two distinct families of human rotavirus (191,193). Additional studies revealed that gene segments with similar migration patterns by PAGE did not necessarily exhibit homology by hybridization; conversely, some gene segments that exhibited homology by hybridization varied in their electrophoretic migration (193).

Gene Coding Assignments

The polypeptides comprising the capsids of human and animal rotaviruses appear to be similar. There has been some disagreement concerning (a) their number, (b) their precise location in the inner or outer capsid, (c) their molecular weight, (d) whether they are structural or nonstructural, and (e) whether they are glycosylated or nonglycosylated (171,288). Also, coding assignments for some genes are still in dispute. However, it appears that there is general agreement on the coding assignments for most genes (see Chapter 48).

The gene coding assignments were established in various ways, including: (a) correlation of phenotype and genotype following the reassortment of genes during coinfection by two distinct rotaviruses; (b) analysis of the reaction of monoclonal antibodies with various gene products; (c) isolation of individual dsRNA segments followed by *in vitro* translation of the fractionated denatured dsRNAs and analysis of the polypeptide products; and (d) translation of isolated (+)ssRNA transcripts produced by the viral polymerase, followed by identification of the products. The peptides of SA-11 have been studied more thoroughly than those of other rotaviruses, in part because this virus was among the first of this group of agents to be propagated efficiently in cell culture (408). As shown in Fig. 4, RNA segments 1, 2, 3, and 6 code for the inner capsid polypeptides VP1, VP2, VP3, and VP6, respectively; segments 4 and 9 code for the major outer capsid polypeptides VP4 (formerly designated VP3 but renamed VP4 when RNA segment 3 was conclusively found to encode a structural protein) and VP7, respectively (396). Segment 11 codes for a polypeptide of uncertain location which may be the precursor to the minor outer capsid polypeptide VP9 or may be a nonstructural protein (171,184,419). Segments 5, 7, 8, and 10 code for nonstructural proteins.

VP4 is cleaved by proteolytic enzymes such as trypsin to form cleavage products designated VP5* (MW 60,000) and VP8* (MW 28,000). Proteolytic cleavage of VP4 protein potentiates infectivity by enhancing penetration of virus into cells. VP4 is a multifunctional protein because, in addition, it has the following functions: (a) It is responsible for hemagglutination activity of certain rotaviruses; (b) it plays an important role in virulence of heterologous rotaviruses for mice; and (c) it induces neutralizing antibodies that can protect against experimental rotavirus illness (297,299,323, 373,419,491,497). The 6th gene product encodes VP6,

which contains the major subgroup antigen as well as the antigen that acts as the main determinant of group reactivity. The 9th gene (or 7th or 8th gene in certain viruses) encodes the neutralization protein VP7, which is largely responsible for serotype specificity defined by neutralization with hyperimmune antiserum (419).

Propagation and Assay in Cell Culture

Although various animal rotaviruses, such as SA-11 and the O agent, grow readily in cell culture, the human rotaviruses are rather fastidious agents; until recently, these viruses had not been grown efficiently in any tissue culture system (4,9,11,19,25,142,166,409,425, 437,439,441,443,463,582,662,663,680,690,745,747, 756). Initially, human rotaviruses were observed to induce an abortive infection of cells in culture; viral antigens were produced, but infectious virus could not be passaged serially. The first human rotavirus that grew well and could be passaged serially in cell culture was a mutant of the Wa human rotavirus (serotype 1) that emerged during 11 serial passages of this strain in gnotobiotic piglets (748). Efficient growth of the mutant required pretreatment of virus with trypsin.

Because attempts to propagate the noncultivatable human rotavirus DS-1 (serotype 2) in piglets were not successful, another strategy to cultivate this virus was adopted (250). This strategy took advantage of the well-known property of the Reoviridae to undergo genetic reassortment with high efficiency during coinfection in cell culture. Cell cultures were coinfected with a "noncultivatable" human rotavirus (such as DS-1) and a cultivatable bovine rotavirus *ts* mutant. Subsequently, the growth yield from the coinfected cultures was analyzed for cultivatable reassortants (i.e., viruses with a mixed genotype) which were phenotypically similar to the human rotavirus parent. Rescue of noncultivatable human rotavirus was achieved using 2 types of selective pressure which favored cultivatable reassortants with the neutralization specificity of the human rotavirus parent: (a) The bovine rotavirus *ts* mutant parent and reassortant bearing *ts* genes were selected against by incubating cultures inoculated with the growth yield at restrictive temperature; and (b) viruses with bovine rotavirus neutralization specificity were selected against by incubating the growth yield with bovine rotavirus antiserum. The reassortants that were selected in this manner grew efficiently in cell culture, produced plaques at the restrictive temperature, had the neutralization specificity of the noncultivatable human rotavirus parent, and were of mixed genotype. With this technique, 33 of 52 human rotaviruses were successfully rescued, and the serotypic diversity of these viruses was demonstrated by conventional neutralization assays (255). In addition, as noted before, these studies yielded information concerning the genes that code for certain rotavirus proteins, such as (a) proteins that stimulate neutralizing antibodies (i.e, VP4 and VP7), (b) protein bearing the subgroup antigen (i.e., VP6), and (c) the protein responsible for restriction of growth of human rotaviruses in cell culture (i.e., VP4). It should also be noted that the generation of rotavirus reassortants may have an important application in development of vaccines, as will be described.

Recently, efficient techniques were developed for the direct cultivation of most human rotaviruses in cell culture (580,695). This was achieved by pretreatment of virus with trypsin (10 μg/ml), incorporation of tryp-

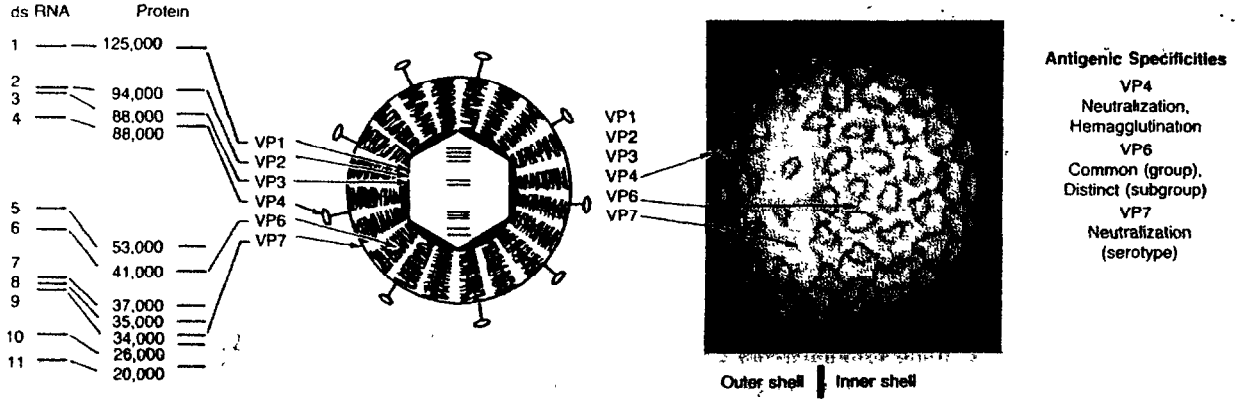

FIG. 4. The figure on the left is a schematic representation of the rotavirus double-shelled particle. The figure on the right (from ref. 524) shows surface representations of the three-dimensional structures of a double-shelled particle (on the left half) and a particle (on the right half) in which most, if not all, of the outer shell and a small portion of the inner shell mass have been removed.

sin (1 μg/ml) in the maintenance medium, the use of roller tube cultures of simian MA104 cells, and incubation of inoculated cultures at 37°C. Following growth in roller tube cultures, most human rotavirus strains also produced plaques under an overlay medium containing 0.6% purified agar, 3 μg/ml acetylated trypsin, and 50 μg/ml DEAE dextran. Virus in over 75% of fecal samples known by other tests to be rotavirus-positive can be cultivated successfully in roller tube cultures using the above procedures. However, this method has not proved to be efficient for the cultivation of rotaviruses from rectal swab specimens. Primary AGMK or cynomolgus MK cell cultures may be more efficient than MA104 cultures for isolation of rotaviruses from clinical specimens (273,726). The non-group A rotaviruses are also fastidious viruses, since only one non-group A rotavirus (a strain belonging to group C) has been successfully grown in cell culture (566,661).

ANTIGENIC COMPOSITION

Designation of Serotypes and Subgroups

Human rotaviruses were initially studied for antigenic relationships by IEM, enzyme-linked immunosorbent assay (ELISA), specific CF, and neutralization of foci of abortive infection in cell culture (32,182,199,550,670,773,777). These foci were detected by immunofluorescence, and hence the technique was designated neutralization of immunofluorescent foci (NIFF) (32,182,670). Initially, strains derived from cross-sectional studies were classified by specific CF or ELISA into two distinct groups designated types 1 and 2 (199,773,777). In addition, rotaviruses were classified by the NIFF technique into three distinct groups (32). It was generally assumed that the various techniques just described measured the same antigenic specificity and that once the fastidious human rotaviruses were cultivated in cell culture, conventional neutralization assays would confirm the relationships established in the earlier studies.

The situation became more complex when analysis of rotaviruses from humans and a variety of animal species was performed by the immune adherence hemagglutination assay (IAHA) (335,337). Calf rotaviruses (NCDV or UK), the O agent, rhesus rotavirus, and the DS-1 strain of human rotavirus reacted with type 1 antiserum that had been defined previously by CF and ELISA. Thus, certain animal rotaviruses appeared to belong to the same antigenic group as a human rotavirus. Up to that time, animal rotaviruses, some of which grew in cell culture, had been considered to be antigenically distinct from one another and also from human rotaviruses.

These discordant observations were resolved when examination of reassortants of human rotavirus Wa and bovine rotavirus UK revealed that different genes coded for (a) the major protein involved in neutralization of infectivity and (b) the antigen detected by IAHA (326). In view of these findings, it was suggested that the term "serotype" be reserved to identify neutralization specificity and that the term "subgroup" be used to identify the specificity that had previously been defined by specific CF, IEM, ELISA, and IAHA (337). It was shown that gene 9 of the human Wa virus coded for the outer capsid protein VP7, which is primarily responsible for neutralization by hyperimmune antiserum, and that gene 6 coded for the subgroup antigen which is located on the major inner capsid protein, VP6 (326). Thus, in designating the antigenic composition of a rotavirus, these two specificities must be considered. For example, the human Wa virus is serotype 1 and subgroup II, whereas the human DS-1 virus is serotype 2 and subgroup I. The arabic numbers are reserved for serotype, and the roman numerals designate subgroup (20).

Thus far, with few exceptions, all subgroup I human rotaviruses possess slower-moving RNA segments 10 and 11 ("short pattern") or an even-slower-moving RNA segment 10 ("super-short" pattern of serotype 8) than do subgroup II human rotaviruses ("long pattern") (3,6,7,272,325,421,476,621). This makes it possible to identify subgroup I viruses by PAGE, but this technique is not of value in determining the serotype of a "long" RNA pattern human rotavirus because four serotypes (1,3,4,9) exhibit this pattern (31,112,302,749). A single human subgroup II strain with the "short" pattern has been described (621). However, with this exception, human viruses with the short pattern form a homogeneous group that is serotype 2 by neutralization. The mobility of the 10th–11th gene segments of rotaviruses is not helpful in classification of viruses from other species because almost all animal strains tested thus far exhibit the "long pattern", although most strains belong to subgroup I (302). A "super-short" RNA pattern of two bovine rotavirus strains (subgroup I) were recently described (664). Studies with reassortants derived from a human subgroup I and a human subgroup II rotavirus demonstrated that the 10th and 11th gene segments were not involved in subgroup specificity (211,697). It should be noted that several human and animal rotaviruses have been detected which cannot be classified into either subgroup I or II (296,637).

The development of techniques for cultivation of human rotaviruses in cell culture made it possible to identify virus serotype by conventional assays which include (a) neutralization of cytopathic effect (CPE), (b) reduction of virus yield in tube cultures, (c) plaque

reduction, and (d) viral interference (219,302,304,383, 579,636,654,696,746). The criterion for establishing a distinct serotype is a reciprocal 20-fold or greater difference in serum antibody titer when a candidate serotype is tested against prototype viruses representing established serotypes (302,746). With this standard, six distinct human rotavirus serotypes have been identified (Table 1). The uniformity of serotypes initially characterized (i.e., serotypes 1–4) in different locations is striking (32,182,698,746,749). The four U.S. serotypes appear to be similar, if not identical, to the four Japanese serotypes. In addition, the three serotypes established in early English studies by NIFF are similar to three of the serotypes established by standard plaque reduction neutralization (32,182). For this reason, the numbering system assigned in the early English studies has been adopted (20).

TABLE 1. *Group A rotavirus serotypes determined by neutralization*[a]

Serotype	Human rotavirus reference strains (subgroup)	Animal rotavirus reference strains (subgroup)
1	Wa, K8, KU, D, M37, DB, RV4(II)[b]	None
2	DS-1, S2, KUN, 390, HN126, RV5, 1076 (I)	None
3	P, M, Walk, 57/14 Mo, Ito, Nemoto, YO, McN, RV1, RV3 (II), AU-1 (I)	Simian SA-11, Rhesus monkey MMU18006, canine CU-1, feline (TAKA) (I); equine H-2 (not I or II), FI-14 (both I and II), E1 (II); lapine C11, ALA (I), R2 (II); murine EW(EDIM), EB (not I or II); porcine MDR-13, CRW-18 (I)
4	St. Thomas No. 3 and 4, Hosokawa, Hochi, VA70 (II)	Porcine SB-2 (I); porcine Gottfried, SB-1A (II)
5	None	Porcine OSU, EE, equine H-1 (I)
6	None	Bovine NCDV, UK, WC3 (I)
7	None	Chicken Ch.2, turkey Ty.1 (not I or II)
8	69M, B37, B38, 57M (I)	
9	WI-61, F45 (II)	

[a] According to VP7 specificity when known. Data from refs. 1,3,4,6,7,20,32,40,60,112,118,221,245,251,254,255, 272,296,298,302,304,313,383,421,439,456,474,486,513, 579,580,649,668,688,695,696,698,746, and 749.

[b] Subgroup antigen as determined by ELISA or IAHA is shown in parentheses.

Although the sharing of subgroup specificity by certain animal and human rotaviruses was surprising, the sharing of neutralization specificity by certain human and animal viruses was unexpected (302,304,746,749) (Table 2). Initially, rhesus rotavirus and strain SA-11 were shown by plaque reduction to be similar, if not identical, to human rotavirus serotype 3. Further studies that involved extensive reciprocal cross-neutralization tests among human and animal rotaviruses revealed that: (a) serotype 1, 2, 8, and 9 strains are restricted to humans; (b) serotype 4 strains have a broader host range which includes humans and pigs; (c) serotype 3 viruses have the broadest host range which includes humans, monkeys, dogs, cats, horses, pigs, mice, and rabbits; (d) serotype 5 strains are restricted to horses and pigs, whereas serotype 6 and 7 strains are restricted to calves or birds, respectively. It is of interest that the porcine rotavirus strains Gottfried and SB-2, which share serotype specificity, belong to different subgroups: The former is a subgroup II virus, whereas the latter belongs to subgroup I (302). This is the first example of strains that share neutralization but not subgroup specificity. It is surprising that this dissociation has not been observed more often, since serotype and subgroup specificities are encoded by different genes.

Antigenic Specificities of Various Proteins

The VP6 Protein

VP6, which is located on the inner capsid of the virus, has a molecular weight of 41,000 and constitutes 51% of virion protein (396). Murine monoclonal antibodies to the VP6 of human Wa (serotype 1, subgroup II) or rhesus rotavirus (serotype 3, subgroup I) react with almost all mammalian group A rotaviruses in various immunoassays (249,251,253,601). Although other structural proteins may show some group reactivity under selected conditions, VP6 specifies the major group reactivity measured by such assays. However, two monoclonal antibodies that immunoprecipitate VP6 had a more restricted activity (251). One of these antibodies induced by rhesus rotavirus reacted only with subgroup I viruses; the other antibody, induced by Wa rotavirus, reacted only with subgroup II viruses. When reassortant viruses of known genotype derived from coinfection of cells with Wa (a subgroup II rotavirus) and UK (a subgroup I rotavirus) were studied, it was observed that the monoclonal antibody with subgroup II specificity reacted only with reassortants whose sixth gene was derived from Wa virus, whereas the subgroup I specific monoclonal antibody reacted only with reassortants whose sixth gene was

TABLE 2. *Host range of Group A rotavirus serotypes*

Serotype	Human	Simian	Equine	Porcine	Canine	Feline	Lapine	Murine	Bovine	Avian
1	+									
2	+									
3	+	+	+	+	+	+	+	+		
4	+			+						
5			+	+						
6									+	
7										+
8	+									
9	+									

derived from the UK rotavirus. None of the other rotavirus genes segregated with monoclonal antibody reactivity. Thus, the sixth rotavirus gene codes for the major inner capsid protein VP6 that specifies subgroup reactivity (251,601). It is of interest that a non-neutralizing monoclonal antibody directed to VP2 (a component within the inner capsid) had a subgroup reactivity pattern similar (but not identical) to a reference VP6 subgroup II monoclonal antibody (657). The VP6 protein also contains a separate domain that is the major determinant of group A rotavirus antigenic reactivity (162,214,601). The site of attachment of VP6 subgroup monclonal antibodies on rhesus rotavirus (RRV) has been visualized clearly by IEM (Fig. 5). These antibodies attach to the inner but not the outer capsid of the rhesus rotavirus.

Most group A rotaviruses are classified into either subgroup I or subgroup II. Human strains belong to either subgroup I or II, whereas animal rotaviruses (with few exceptions) belong to subgroup I. A few animal strains cannot be subgrouped, and some share both subgroup I and II specificities (296,302). The majority of human rotaviruses recovered during epidemiological studies belong to subgroup II, although subgroup I strains may occasionally predominate.

As noted in Chapter 48, the amino acid composition of the VP6 protein of several rotavirus strains has been determined, but the epitopes corresponding to group and subgroup specificities remain to be elucidated (63,115,170,238,287).

It was assumed until recently that all human and animal rotaviruses share common antigenic determinants present predominantly on VP6. However, several human and animal rotavirus strains do not share these determinants. Rotaviruses that share the group antigen are now classified into group A, whereas rotaviruses that lack this antigen are classified as non-group A viruses (75,310,511). The latter viruses also do not share subgroup antigens with group A viruses. The non-group A viruses have been given various names such as pararotaviruses, rotavirus-like viruses, novel rotaviruses, antigenically distinct rotaviruses,

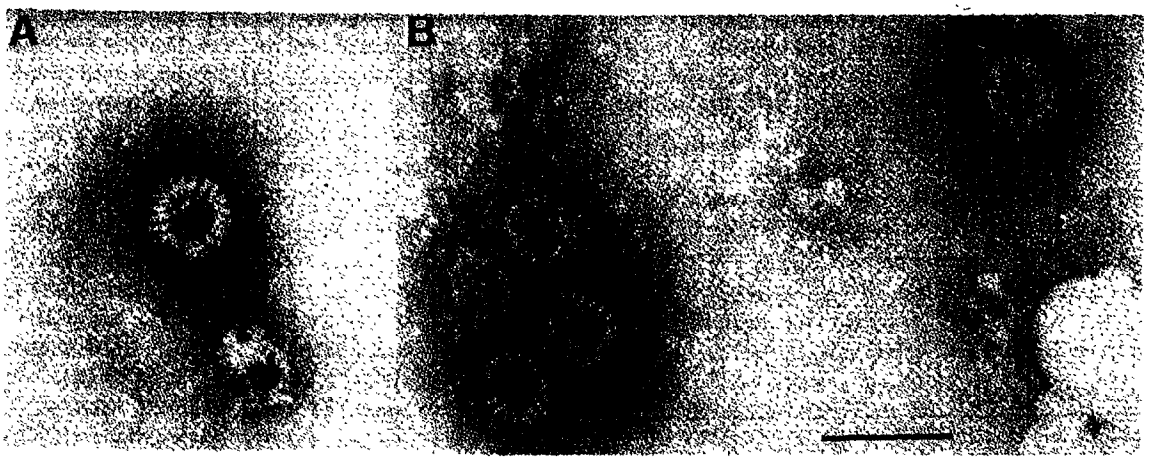

FIG. 5. Direct IEM of RRV after incubation with monoclonal antibody (mouse ascites fluid) to subgroup domain of major inner structural protein (VP6) of RRV. Antibody is not seen on the outer margin of the double-capsid rotavirus particle in **A**, whereas antibody is clearly visible on the particles in **B**, which lack the outer capsid. (From Kapikian et al., *unpublished observations.*)

and adult diarrhea rotaviruses (75,310). They currently are classified into groups B, C, D, E, F, and, tentatively, G. Group B viruses have been recovered from humans, pigs, cattle, sheep, and rats; group C viruses have been recovered from humans, pigs, and ferrets; group D viruses have been recovered from chickens; group E viruses have been recovered from pigs; group F viruses have been recovered from chickens; and viruses tentatively designated group G have been recovered from chickens (75,676). Recently, a group B rotavirus was implicated as the cause of (a) several large outbreaks of severe gastroenteritis in China which predominantly involved adults, and (b) a mild outbreak of diarrhea in neonates (101,127,310–312, 598,631,724). In contrast, relatively few human strains of a group C rotavirus have been detected, but these have been distributed in various countries (61a,74,78a, 100,311). The importance of non-group A rotaviruses outside of China has not been defined (78,138,155,543).

The VP7 Protein

The VP7 glycoprotein is the major neutralization antigen of rotaviruses detected by hyperimmune antiserum and serves as the basis for determination of serotype (28,363,387,419,422,465,563,589,591,617). VP7 is located on the outer capsid, has a molecular weight of 34,000, and constitutes 30% of the virion protein, thus making it (a) the second most abundant rotavirus protein and (b) the major constituent of the outer capsid (396). The VP7 of various serotypes contains one to three potential sites for N-linked glycosylation, but carbohydrate side chains are not required for infectivity because a fully infectious mutant of simian rotavirus SA-11 which lacks a glycosylation site has been described (168).

Early studies in which reassortant rotaviruses were analyzed for genotype and antigenic specificities indicated that most neutralizing antibodies in hyperimmune serum were directed against the VP7 protein, which is encoded by the 7th, 8th, or 9th gene, depending on the strain (248,250,255,326,419). Also, many rhesus rotavirus monoclonal antibodies which immunoprecipitate the 38,000-dalton outer capsid VP7 glycoprotein neutralize rhesus rotavirus to high titer (253). The site of attachment of these VP7 monoclonal antibodies has been identified by IEM as the outer capsid of the virus (Fig. 6). Thus far, group A rotaviruses have been classified into nine distinct serotypes in reciprocal neutralization tests with hyperimmune antisera (Table 1). Serotype identification of rotaviruses has been facilitated recently by the isolation of VP7 monoclonal antibodies which are specific for human strains of serotype 1, 2, 3, or 4 (653). Strains within a

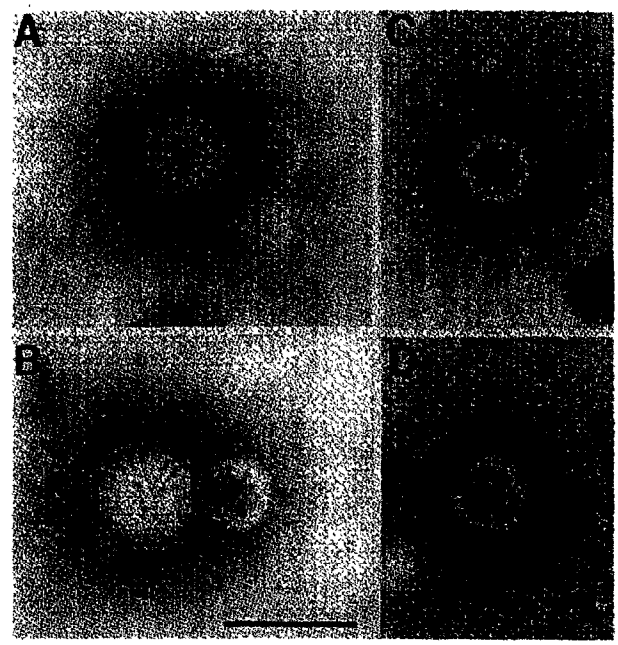

FIG. 6. Indirect IEM of RRV after incubation with monoclonal antibody (mouse ascites fluid) to major neutralizing outer capsid glycoprotein (VP7) of RRV and ferritin conjugated antimouse globulin. Antibody is clearly visible on the outer margins of the double-capsided particles in **A** and **B**, whereas antibody is not observed on the particles in **C** and **D**, which lack the outer capsid. (From Kapikian et al., *unpublished observations.*)

serotype which vary in their capacity to be neutralized by serotype-specific monoclonal antibodies have been termed "monotypes," analogous to "subtypes" defined with polyclonal hyperimmune antisera (117,119). VP7 has also recently been shown to be responsible for attachment of virus to host cells as described in Chapter 48 (207,208,330,633,634).

The amino acid sequence of the VP7 of strains belonging to each of the serotypes, except serotype 7, has been deduced from the nucleotide sequence of the VP7 gene (Fig. 7) (16,62,99,143,158,226,234,236,239, 245–247,260,414,537,558). Representative relationships of the VP7 amino acid sequence among homotypic and heterotypic strains of the first six serotypes are shown in Table 3. Within each serotype, amino acid homology was high (91–100%); however, between strains of different serotype, conservation of sequence was significantly less (245–247). Thus, there was complete concordance between relationships among rotaviruses as determined by neutralization or sequence analysis of VP7. Conservation of VP7 amino acid sequence has been examined in more detail within serotype 3; this serotype has the broadest host range, encompassing eight different species. Comparison of the deduced VP7 amino acid sequence of 27 strains

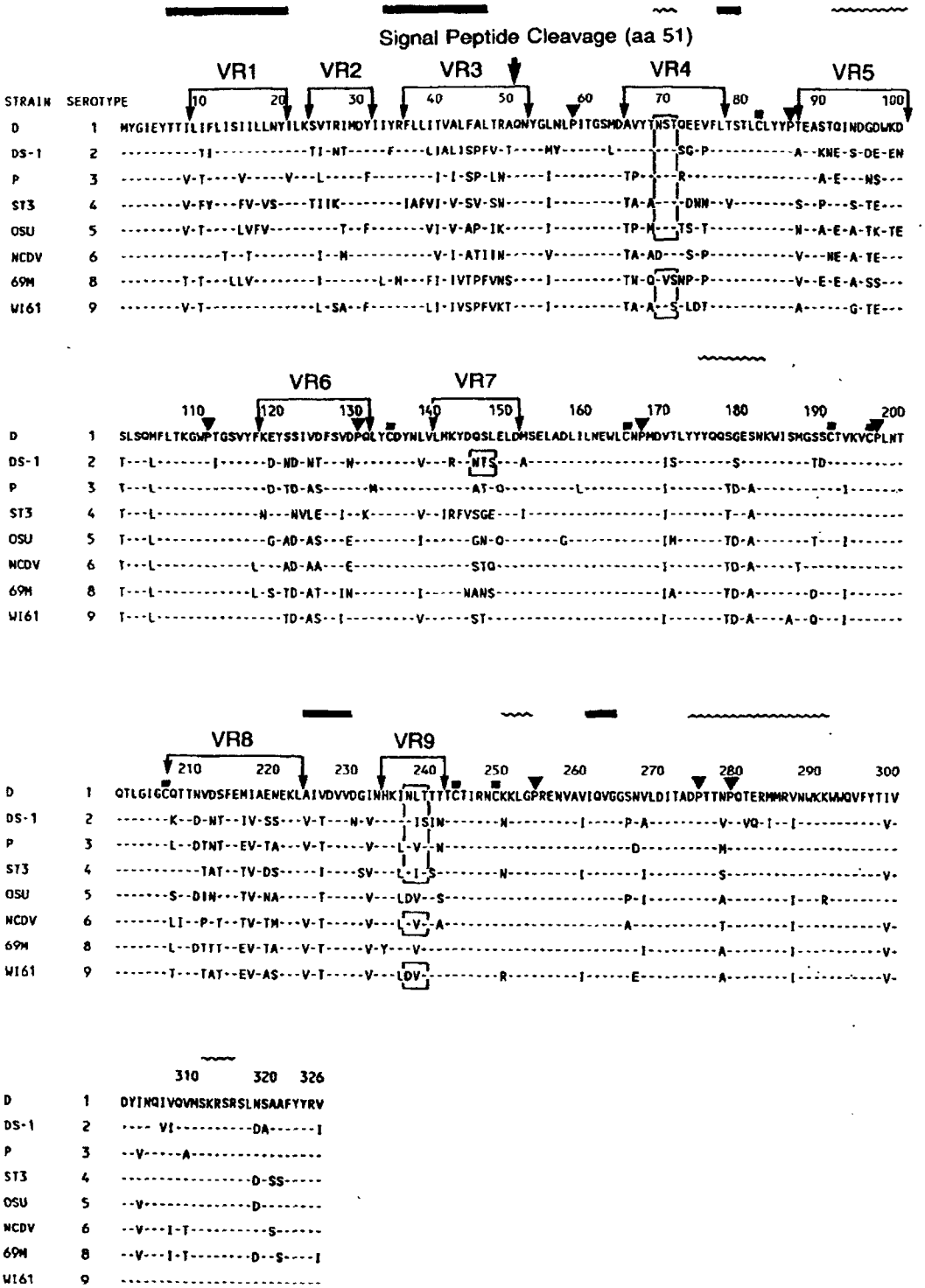

FIG. 7. Comparison of the deduced amino acid sequences of the VP7 protein from rotavirus strains of eight different serotypes. The hydrophobic regions are denoted by ■; the hydrophilic regions are denoted by ∿; variable regions are denoted by arrows and bracket; potential glycosylation sites are enclosed in rectangles; conserved prolines are indicated by ▼; and conserved cysteines are indicated by ■. (Adapted from ref. 245.)

TABLE 3. *Amino acid homology among the VP7 amino acid sequences from 17 human and animal rotaviruses*[a]

D																
100[b]	Mo															
95	95	M37														
99	99	95	Wa[c]													
75	75	74	75	DS1												
75	75	74	75	99	HN126											
75	75	74	75	99	99	HU5[c]										
75	75	74	75	96	96	96	S2[c]									
83	83	83	83	75	75	75	75	P								
81	81	81	81	75	75	75	75	93	RRV							
82	82	81	82	75	76	75	75	91	96	SA11[c]						
77	77	78	77	72	72	71	73	76	78	78	ST3					
78	78	79	78	71	71	71	71	76	78	79	94	VA70				
78	78	79	78	74	74	74	74	85	85	85	77	76	OSU[c]			
81	81	81	81	75	75	76	75	83	85	85	77	76	81	NCDV[c]		
82	82	82	82	76	76	76	76	84	86	86	79	77	82	97	UK[c]	
81	81	81	81	75	75	74	75	83	85	85	77	76	86	98	97	RF[c]
Serotype:	1			2				3			4		5	6		

[a] From ref. 246.
[b] Percentage amino acid homology (identity).
[c] The VP7 from these strains were previously published: Wa (ref. 537); HU5 (ref. 143); S2 (ref. 260); SA11 (refs. 16 and 62); OSU (ref. 234); NCDV (ref. 226); UK (ref. 158); and RF (ref. 99).

recovered from these eight species indicated an overall homology of 85% or greater (486). With the exception of two feline strains, conservation of VP7 sequence was greatest among strains recovered from the same species, with homology being 95% or greater. For example, among 13 serotype 3 human rotavirus strains, there was 95.3% or greater identity of VP7 sequence.

Comparison of the VP7 of strains belonging to eight serotypes indicated that there are nine regions of the linear amino acid sequence of VP7 which are highly divergent (Fig. 7) (245). Each of these regions is highly conserved in human rotavirus strains within the same serotype (Table 3) (245–247). There is sufficient conservation of sequence of human rotavirus strains within a serotype that it is possible to predict the serotype of an isolate by direct sequence analysis of two of these variable regions (VR5 and VR8) of VP7 (245–247).

The major neutralization epitopes on VP7 have been identified by analysis of the deduced amino acid sequence of mutants which are resistant to neutralization by the VP7 monoclonal antibody used for their selection (Fig. 8) (144,405,650). Each of the "neutralization escape mutants" analyzed sustained a single amino acid substitution in VP7, and these mutations occurred exclusively in variable regions VR5, VR7, or VR8 (Fig. 8). The observation that a single monoclonal antibody can select for a mutation in either VR5 or VR8 suggests that these regions occupy a close spatial relationship on the mature VP7 molecule (144,405,650).

Homotypic monoclonal antibodies select single amino acid substitution mutations in VR5, VR7, or VR8, whereas monoclonal antibodies that have only heterotypic activity select a mutation in VR5. Homotypic and heterotypic epitopes appear to be close together within VR5 because both homotypic and heterotypic monoclonal antibodies select mutations within a region of six amino acids (i.e., positions 94–99) (144,405,650).

The importance of glycosylation in the antigenicity of VP7 was shown by the effect of a single amino acid substitution at position 211 of simian virus SA11 (92,144). This mutation created a new glycosylation site, and the resulting mutant was rendered significantly resistant to neutralization by hyperimmune antiserum. Based upon these observations, it was suggested that VR8, which contains amino acid 211 (or a region contiguous to it in the folded protein) constitutes an immunodominant site that is rendered inaccessible to its corresponding neutralizing antibodies by the addition of a new carbohydrate side chain. This region appears to be the dominant antigenic site on VP7, but a generalization of its dominance to the whole virus is probably not valid because the role of VP4 in neutralization is not taken into consideration. Consistent with this view is the observation that the 211 mutant which bears a new glycosylation site is fully susceptible to neutralization by mouse postinfection sera even though it is resistant to neutralization by hyperimmune antisera. This difference in susceptibility to neutralization probably reflects (a) the presence of sufficient VP4 neutralization antibodies in the former sera and (b) the deficiency of these antibodies in the latter sera.

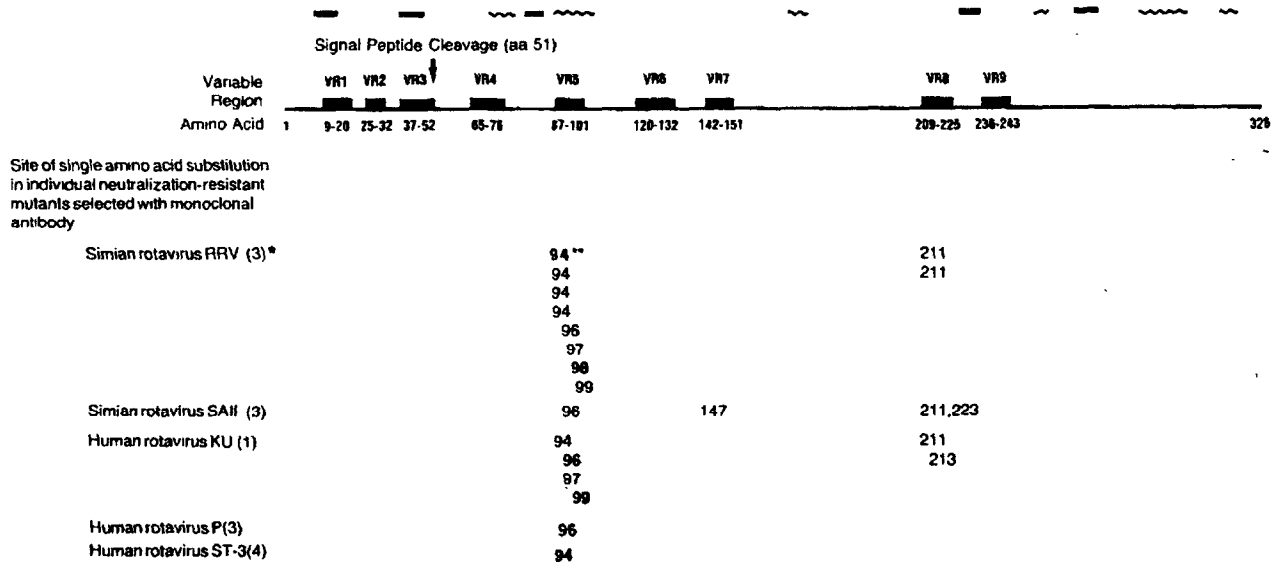

FIG. 8. Linear structure and antigenic organization of rotavirus outer capsid VP7. *Serotype, as determined by VP7. **Boldface numbers indicate amino acid substitutions in mutants selected with monoclonal antibody exhibiting cross-reactive (i.e., heterotypic) neutralizing activity; other numbers indicate amino acid substitutions selected with monoclonal antibody with homotypic neutralizing activity. At the top of the figure, hydrophobic regions are denoted by ▬, whereas hydrophilic regions are denoted by ∿. (Data were taken from refs. 144, 405, and 650.)

The VP4 Protein

Study of rotaviruses by cryoelectron microscopy indicates that VP4 is present on the outer capsid from which it protrudes as a series of short spikes (Fig. 4) (524). VP4 has a molecular weight of 88,000 and constitutes 1.5% of virion protein (396). Although it is a minor constituent of the outer capsid, VP4 performs a number of functions that have been defined by genetic and immunologic analyses (88,419). Analysis of rotavirus reassortants indicated that VP4 is encoded by the 4th gene (i.e., RNA segment 4) of all strains studied thus far. Restriction of growth of human rotaviruses in tissue culture was assigned to VP4 during a study of reassortants produced during dual infection of cell cultures by a cultivatable bovine rotavirus and by a poorly cultivatable human rotavirus (248). The only gene that segregated with efficient growth of virus in cell culture was the gene that encodes VP4. Study of reassortants produced during dual infection of a rotavirus that has hemagglutination activity (rhesus rotavirus) and a rotavirus that lacks this property (bovine UK rotavirus) identified VP4 as the viral hemagglutinin (323). This assignment was confirmed when it was observed that monoclonal antibodies to rhesus rotavirus which inhibited hemagglutination immunoprecipitated VP4 (253).

In addition, some rhesus rotavirus monoclonal antibodies to VP4 were found to neutralize infectivity (253). This was unexpected because in earlier studies, VP4 had not been shown to play a role in neutralization, and it was thought that this function was mediated exclusively by VP7 (248). In these early studies, the bovine rotavirus antiserum that was used to measure neutralization of reassortants bearing genes of a cultivatable bovine rotavirus and a poorly cultivatable human rotavirus contained VP7-neutralizing antibodies but not antibodies that neutralized this bovine rotavirus via its VP4. Hence, the role of VP4 in neutralization was missed because all of the cultivatable reassortants recovered in this study bore the VP4 of the bovine rotavirus.

The ability of two outer capsid proteins (VP4 and VP7) to separately induce neutralizing antibodies has necessitated a reevaluation of serotype classification which heretofore was based upon neutralization mediated primarily by VP7. The need for a reevaluation also became apparent when various strains were discovered which shared neutralization specificities with two distinct serotypes. The first such "intertypic" virus recognized was the M37 strain, which was recovered from an asymptomatic infant in a newborn nursery (298,299,458). Hyperimmune antiserum to strain M37 neutralized both serotype 1 and serotype 4 reference rotaviruses to high titer; similarly, antiserum to serotype 1 rotavirus neutralized M37 to high titer, whereas antiserum to serotype 4 virus neutralized M37 to low titer (299). Analysis of (a) M37 reassortants in

which the genes coding for VP7 and VP4 had segregated independently and (b) antisera to these reassortants, demonstrated that the neutralization of serotype 1 virus by M37 antiserum was mediated by VP7, the ninth gene product, whereas the neutralization of serotype 4 virus by M37 antiserum was mediated by VP4, the fourth gene product. The serotype 4 reference strain ST-3 is also an asymptomatic nursery strain with a VP4 sequence which is highly conserved among other nursery strains (235). The need for a binary system of classification of rotaviruses has been suggested in order to identify the independent role of VP7 and VP4 neutralization specificities (299,490). However, since reagents are not yet generally available to define VP4 neutralization antigens, only VP7 specificity can be used at the present time.

Although VP4 antigenic specificity cannot be assigned for all rotavirus strains, it is known from sequence analysis that there are at least three distinct human VP4s. One is the highly conserved VP4 present on serotype 1, 3, and 4 "virulent" strains, a second is present on serotype 2 "virulent" strains, and the third is a highly conserved VP4 present on the asymptomatic neonatal strains M37, 1076, McN, and ST-3, which have been assigned to serotypes 1, 2, 3, and 4, respectively, based on neutralization mediated by VP7 (Table 1) (188,235,237,301). Independent segregation of VP7 and VP4 neutralization antigens, along with the conservation of VP4 among asymptomatic nursery strains, has led to the confusing situation in which these strains contain a VP7 with serotype 1, 2, 3, or 4 specificity while sharing a highly conserved VP4 sequence (Table 4). Further clarification of this situation awaits the development of appropriate serological reagents. At present, it appears that animal rotavirus VP4 specificities are distinct from those of human rotaviruses (187,237). Also, at least two different VP4 antigenic specificities may be present among rotaviruses derived from the same animal species. For example, the bovine UK and NCDV strains possess antigenically distinct VP4 antigens, although these viruses contain highly related VP7 antigens (299). A similar situation has been identified among porcine rotaviruses (298).

The significant role played by VP4 in protective immunity was observed during a study in which infection of newborn piglets with a reassortant bearing the VP4 of one of its virulent porcine rotavirus parents and the VP7 of the other virulent porcine rotavirus parent was shown to induce resistance to disease caused by either virulent parental virus (297). This indicated that VP4 and VP7 function as independent protective antigens *in vivo*. In this study, VP4 was observed to stimulate the same high level of neutralizing antibodies as VP7. Also, passively acquired neutralizing antibodies to VP4, as well as to VP7, independently conferred resistance to disease in infant mice in an experimental model of rotavirus illness (494,497). A monoclonal antibody directed against VP4 of the porcine OSU strain which neutralized OSU, as well as heterotypic simian RRV and bovine UK strains, passively protected suckling mice against challenge by either homotypic or heterotypic strains, indicating that cross-reactive antibodies to VP4 can also be protective (497).

Sera from animals convalescent from rotavirus infection usually contain significantly higher levels of VP4-neutralizing antibodies than do hyperimmune antisera raised to the same virus. Thus, serotype dif-

TABLE 4. *Amino acid homology among the VP4 amino acid sequences of five symptomatic and four asymptomatic human rotavirus strains*[a]

	Symptomatic strains					Asymptomatic strains			
	Wa								
	89.7[b]	DS-1							
	89.2	98.5	RV-5						
	94.1	90.5	90.2	P					
	96.8	89.4	89.2	93.2	VA70				
	77.8	77.8	76.4	76.9	77.3	M37			
	77.0	75.7	75.4	76.4	76.4	95.1	1076		
	77.0	75.7	75.4	76.4	76.5	96.6	96.8	McN13	
	76.8	75.7	75.4	76.4	76.3	96.3	95.7	97.4	ST3
Serotype[c]:	1	2	2	3	4	1	2	3	4

[a] Adapted from Ref. 235.
[b] Percent identity.
[c] As defined by VP7.

ferences established with hyperimmune sera tend to be less clear when infection sera are employed in neutralization assays (740). It should be noted that infants vaccinated orally with a live attenuated rhesus rotavirus vaccine developed antibodies to epitopes on both VP7 and VP4 (588).

As noted in Chapter 48, the fourth genes of a wide variety of rotavirus strains have been sequenced (235,331,398,399,406,487,523). A comparison of the deduced amino acid sequences of these proteins revealed several areas of divergence, with the most prominent being the region including amino acids 92–192 (Fig. 9). In addition, analysis of the deduced amino acid sequence of single amino acid substitution escape mutants selected with neutralizing monoclonal antibodies led to the identification of eight regions on the linear sequence of VP4 which are involved in neutralization (406,651) (Fig. 9). With one exception, serotype-specific VP4 monoclonal antibodies selected mutations in the VP8 subunit of VP4, whereas cross-reactive monoclonal antibodies selected only mutations in the VP5 subunit (Fig. 9). Although the sites of mutation in VP8 ranged from amino acids 87 through 188, some of these sites, such as amino acids 89, 100, and 150, may be in close proximity on the folded protein because some mutants selected with a monoclonal antibody directed to one of these regions were also resistant to neutralization by a monoclonal antibody that selected a mutation at one of the other sites. One of the major sites at which a mutation is selected by cross-reactive antibodies is of special interest because it is within a hydrophobic region of 18 amino acids (388–405) which exhibits 45%-identity with the putative fusion site of the E1 glycoprotein of Sindbis virus (406).

In addition to its other functions, VP4 appears to play a major role in virulence of rotaviruses. When reassortant viruses derived from parental strains that varied in virulence for newborn mice were studied, it was observed that virulence segregated with the 4th gene (491). Evidence for a role of VP4 in virulence has also been provided by RNA–RNA hybridization and by sequence analysis of the VP4 gene of rotaviruses recovered from (a) infants and children with moderately severe diarrhea or (b) neonates infected in a nursery in which virus persisted for long periods of time and in which most affected newborns failed to develop significant symptoms (Table 4) (188,235,237). Virulence is not serotype-specific (VP7), since each of the four major human rotavirus serotypes (1, 2, 3, and 4) has been associated with both symptomatic and asymptomatic infections (301). In contrast, the VP4 gene of the asymptomatic rotavirus strains is highly conserved (95.5–97.5%) and is distinct from the VP4 gene of the virulent strains (25.4–27.1% divergence) (235). In addition, the VP4 gene of symptomatic strains exhibits significant conservation (87.4–97%) Thus far, long-term persistence of a uniform strain of rotavirus which does not cause significant symptoms has been documented in seven different nurseries in Africa, Europe, South America, and Australia (301). Four of the nursery strains are type 4, while the remaining three

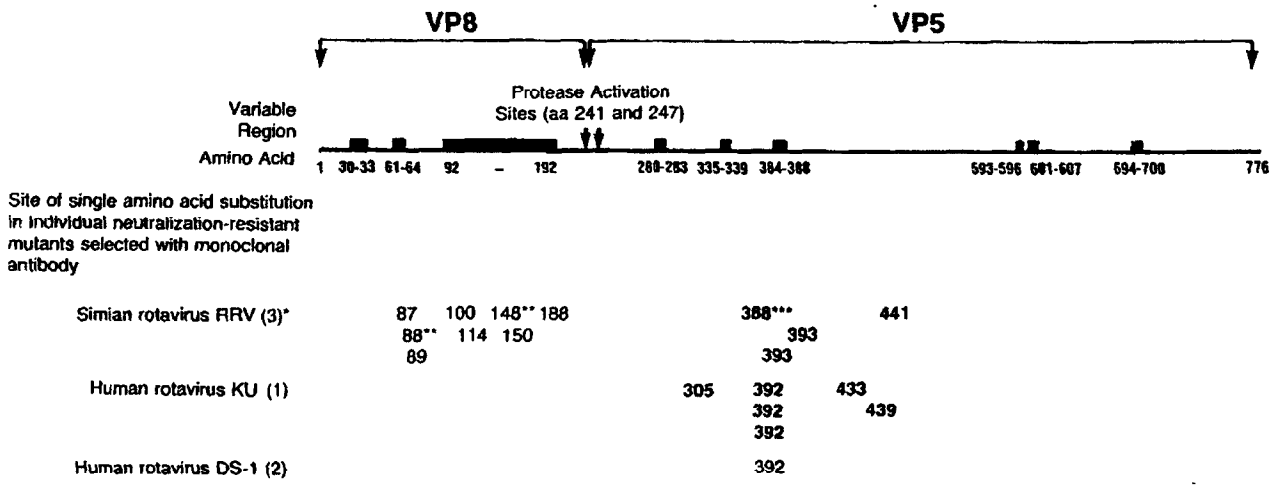

FIG. 9. Linear structure and antigenic organization of rotavirus outer capsid VP4. *Serotype, as determined by VP7. **Limited heterotypic activity. ***Boldface numbers indicate amino acid substitutions in mutants selected with monoclonal antibody exhibiting cross-reactive (i.e., heterotypic) neutralizing activity; other numbers indicate amino acid substitutions in mutants selected with monoclonal antibody with homotypic or limited heterotypic neutralizing activity. (Data were taken from refs. 406 and 651.)

strains are type 1, type 2, or type 3. However, each of these seven nursery strains possess the conserved VP4 gene, suggesting that this gene is associated with reduced virulence (235). Furthermore, none of the other 10 rotavirus genes appear to be conserved among the nursery strains.

INFECTION OF EXPERIMENTAL ANIMALS AND HOST RANGE

Rotaviruses have a wide host range, as indicated by their recovery from the newborn of many animal species (18,26,85,121,160,172,173,178,254,295,303–306, 322,409,438,444,466,469,470,510,517,533,544,554,555, 585,606,610,629,630,671,672,690,691,732,737). Most of these rotaviruses were detected in newborn animals with diarrhea. Rotaviruses have also been associated with respiratory illness in some species (172). Most animal rotaviruses are fastidious in their requirements for cultivation *in vitro*, but there are exceptions (e.g., certain strains of simian, porcine, or bovine origin).

Under experimental conditions, some human rotavirus strains induce a diarrheal illness or subclinical infection in newborn animals such as gnotobiotic calves, gnotobiotic or conventional piglets, rhesus monkeys, gnotobiotic lambs, puppy dogs, and newborn mice (33,241,289,390,447,450,458,608,677,678, 689,758). In China, a human rotavirus was shown to induce a severe diarrheal illness in a nonhuman primate, *Tupaia belangeri yunalis*, following administration by the alimentary route (506). Although rotaviruses are widely distributed in animals and have been identified in almost every species when appropriate techniques for virus detection have been employed, there is no evidence that in nature animal rotaviruses infect humans or that human rotaviruses infect animals. For example, an epidemiologic study in Panama of farm workers with frequent contact with cattle indicated that rotaviruses were transmitted within family units but not to or from animals (561).

PATHOGENESIS AND PATHOLOGY

Study of biopsies of the jejunal mucosa of infants and young children hospitalized with rotavirus disease has identified the following lesions: shortening and atrophy of the villi, mononuclear cell infiltration in the lamina propria, distended cisternae of the endoplasmic reticulum, mitochondrial swelling, and sparse, irregular microvilli (291,635). Denudation of microvilli was also noted. Virus particles were visualized in dilated cisternae of endoplasmic reticulum and in lysosomes of columnar epithelial cells. Virus particles were also detected in goblet cells and phagocytes in the lamina propria. Occasionally, tubular structures were visualized in dilated cisternae of the endoplasmic reticulum. Impaired *d*-xylose absorption has also been observed (431). In addition, some patients have depressed levels of disaccharidases (maltase, sucrase, and lactase) (48).

Information relating to the pathogenesis of rotavirus infection has also been obtained from experimentally infected animals that developed diarrheal illness following administration of human rotavirus. In one study, the pathogenesis of human rotavirus strain D (serotype 1) was studied in newborn, gnotobiotic, colostrum-deprived calves that developed illness following intraduodenal administration of virus (446). Morphological changes proceeded in a cephalocaudad direction in the small intestine. Within a half hour of experimentally induced diarrhea, morphological changes such as denuding of villi and flattening of epithelial cells were observed in the upper small intestine, but rotaviral antigens were not detected by immunofluorescence (IF). At this time, the lower small intestine was intact, but rotavirus antigens were abundant in swollen epithelial cells. Seven hours after onset of diarrhea, the lower small intestine showed morphological changes similar to those seen earlier in the upper small intestine; however, rotavirus antigens were not detected by IF at this time. Forty hours after onset of diarrhea, the intestine appeared relatively normal. Examples of some of these changes are shown in scanning electron micrographs in Fig. 10.

Piglets that developed rotavirus diarrhea following administration of human rotavirus demonstrated functional alterations in small intestinal villous epithelial cells (129,210). Glucose-coupled sodium transport was impaired, sucrase activity was decreased, and thymidine kinase activity was increased. However, adenylate cyclase and cyclic AMP were not stimulated.

EPIDEMIOLOGY

Morbidity and Mortality

Until the discovery of the rotaviruses, the majority of severe diarrheal illnesses of infants and young children could not be linked to an etiologic agent (116,122,333,460,774). However, as data from epidemiologic studies have emerged from both the developed and developing countries, it has become clear that rotaviruses are the major etiologic agents of serious diarrheal illness in infants and young children under 2 years of age throughout the world.

Although rotavirus diarrhea occurs with high frequency in the developed countries, the disease has a

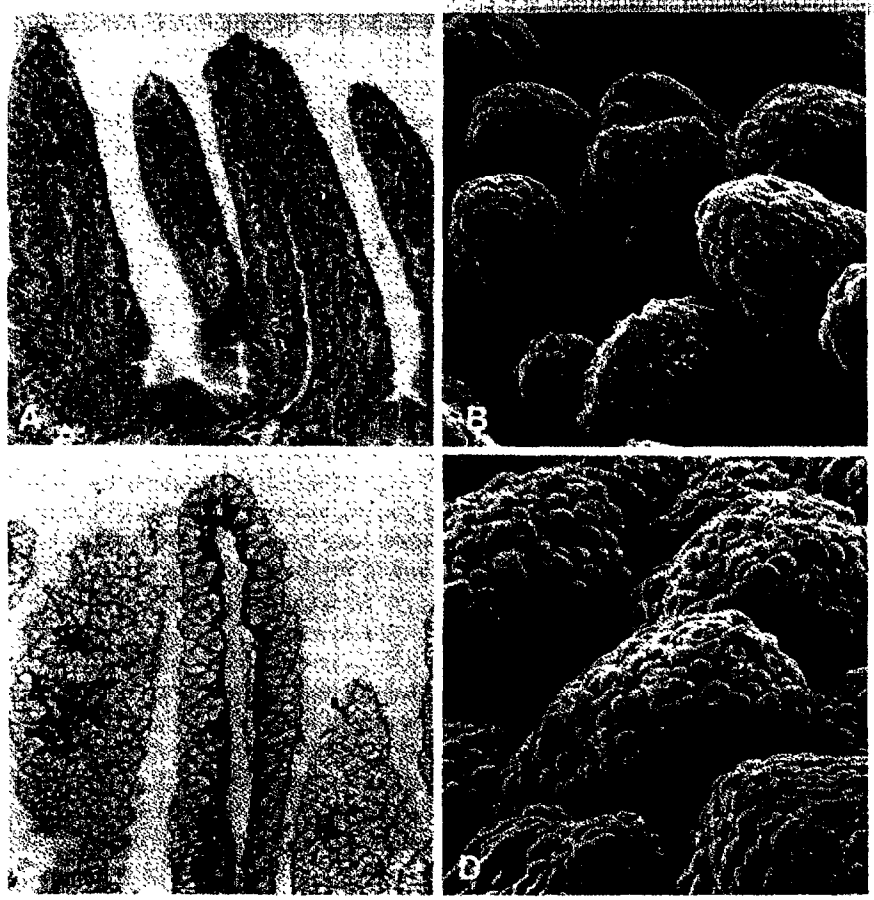

FIG. 10. Scanning electron and light micrographs of intestinal tissues from a gnotobiotic calf sacrificed 0.5 hr after onset of diarrhea induced by human rotavirus. **A**: Proximal small intestine with shortened villi and a denuded villus tip (second from right). Hematoxylin and eosin, ×120. **B**: Appearance of same level of intestine as in **A**, depicting denuded villi by scanning electron microscopy, ×180. **C**: Distal small intestine with normal vacuolated epithelial cells and normal villi. Hematoxylin and eosin, ×75. **D**: Same area as in **C**, seen by scanning electron microscopy. Epithelial cells appear round and protruding. ×210. (From refs. 446 and 751 with permission.)

low mortality in these locations. The burden of rotavirus diarrhea annually in the United States in the 1- to 4-year age group is estimated to include over one million cases of severe diarrhea and up to 150 deaths (283,284,316). A high morbidity but low mortality rate can be attributed to (a) the development during the 1940s of effective means for replacement of fluid and electrolytes lost during disease and (b) their routine use in developed countries. This improvement in therapy resulted in a marked decrease in mortality from all forms of infantile diarrhea. In contrast, in developing countries, rotaviruses are usually the leading cause of life-threatening diarrhea in infants and young children. In a recent analysis, the burden of rotavirus diarrheal disease in infants and young children under 5 years of age in developing countries was estimated to be over 125 million cases; over 18 million of these were considered moderately severe or severe (317). In addition, it was estimated that 873,000 infants and young children 1–4 years of age die from rotavirus diarrheal illness each year.

In developed countries the widespread distribution of rotaviruses in the community is indicated by the universal acquisition of serum antibody to these viruses at an early age. For example, in the Washington, D.C. area, over 90% of infants and young children acquire rotavirus antibody by the end of the third year of life, a pattern similar to that observed for respiratory syncytial and parainfluenza type 3 viruses (347,364,509). The high prevalence of rotavirus antibody is maintained into adult life, suggesting that subclinical rotavirus reinfection occurs. A similar pattern of acquisition and maintenance of rotavirus antibody has also been observed in most other parts of the world (44,55,58,83,148,157,263,321,339). The acquisition of serum antibody to specific serotypes may vary. In Germany and Japan, serum antibodies to serotypes 1 and 3 were most prevalent (83,698).

Cross-sectional studies of hospitalized infants and young children with diarrheal illnesses have yielded the most compelling evidence for the importance of rotaviruses as etiological agents of severe diarrhea of early life. For example, during a period of over 8 years, 34.5% of 1537 infants and young children admitted with diarrhea to a Washington, D.C. hospital shed rotavirus in their feces (Fig. 11) (66). Moreover, in a similar

study in Japan of over 6 years duration, 45% of 1910 pediatric patients with diarrhea were rotavirus-positive (379). In two similar shorter-term studies (1 year), one in Australia and the other in the United Kingdom, 52% of the infants and young children admitted with diarrhea were rotavirus-positive (128,392).

Prospective community-based studies of individuals enrolled in a health maintenance organization, a group of families, or infants and children followed in a medical practice have also yielded significant information concerning the incidence of rotavirus infection and disease. An estimate of the incidence of rotavirus disease requiring hospitalization in the Washington, D.C. area was derived from a defined population of about 29,000 infants and children less than 15 years of age whose primary health care was given by a health maintenance organization (548). One in 272 (3.7 per thousand per epidemic year) infants less than 1 year of age and one in 451 (2.2 per thousand per epidemic year) children 12–24 months of age were hospitalized for rotavirus gastroenteritis, with a marked decrease in the 25- to 60-month age group to one in 5519. After 5 years of age, the incidence of such hospitalization was nil. Moreover, rotavirus infection was associated with 63% of the diarrheal illnesses that were severe enough to require hospitalization.

In a family study extending over a 6-year period in Tecumsheh, Michigan, the overall incidence of enteric illnesses was 1.2 per person per year, with the highest rate occurring in the youngest age groups (461,462). Rotavirus was detected in stools of 3.8% of individuals with gastrointestinal symptoms; however, the annual rate was 10.4% for children under 2 years of age. Higher incidence rates were observed in a study in Virginia which included families served by a pediatric practice. Rotavirus infection was identified in 51% of the families during the 29-month surveillance period (552). Evidence of infection was detected in 28% of the children and in 13% of adults. The incidence of rotavirus gastroenteritis was highest in the 12- to 23-month age group (40 per 100 person-years), lower in the 6- to 11-month and 24- to 35-month age groups (12 per 100 and 13 per 100 person-years, respectively), and lowest in adults (5 per 100 person-years). Extrapolation of the nation-wide incidence figures from this study suggests the following annual number of rotavirus gastroenteritis cases in various age groups: 0–11 months, 410,000; 12–23 months, 1,474,000; 24–35 months, 475,000; and 36–60 months, 573,000. In addition, it was of interest that in the 36-month-and-under age group, 88% of rotavirus infections were symptomatic. Also, one of nine symptomatic children under

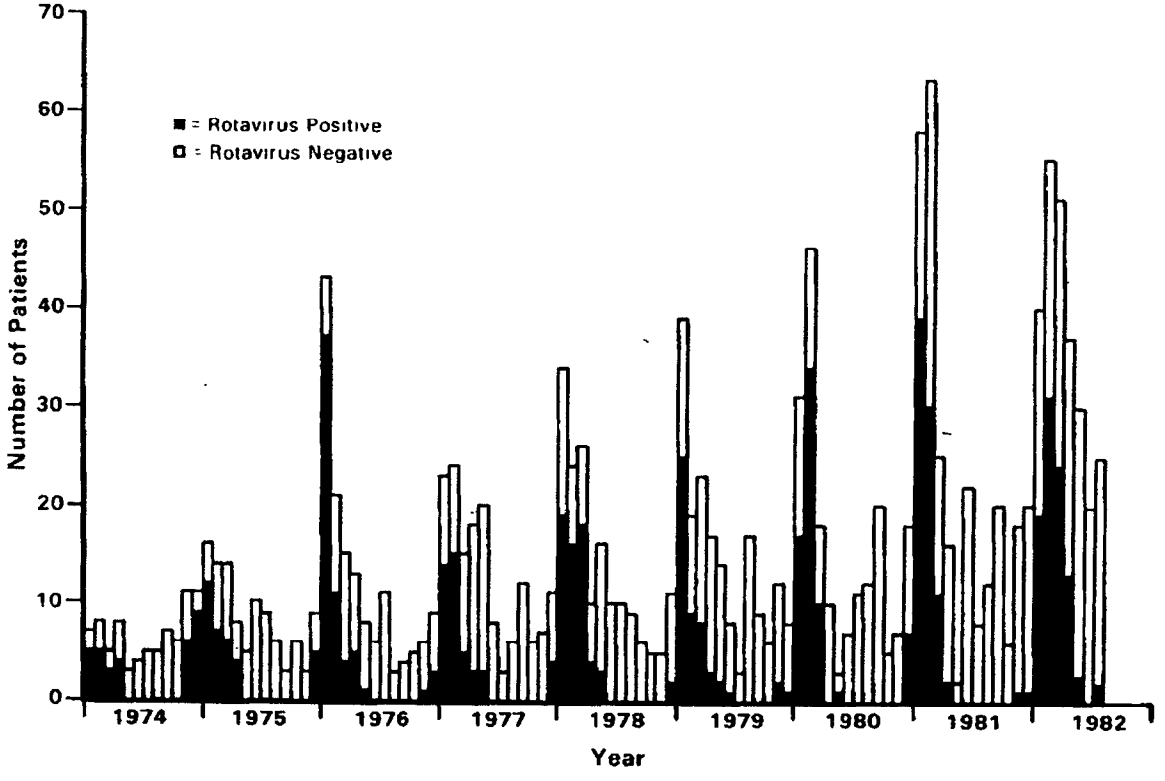

FIG. 11. Rotavirus infections in inpatients with gastroenteritis, January 1974 to July 1982, as demonstrated by EM, IEM, and rotavirus confirmatory ELISA. (From ref. 68 with permission.)

2 years of age who sought medical attention required hospitalization. Rotaviruses were detected only rarely (0.2%) in individuals who were not ill. This is in marked contrast to (a) a study in France in which asymptomatic rotavirus infection was described as a common occurrence in infants and young children at the time of admission to hospital and (b) a study of children from an area of Costa Rica where asymptomatic or mild rotavirus infections prevailed (93,94,600). The latter observations do not reflect the experience of most other investigators (except with regard to neonates). Thus, the general rule is that the rate of rotavirus infection is higher in ill infants compared to age-matched controls who are free of gastrointestinal disease (37,69).

Another perspective on the natural history of rotavirus infections was gained by studying the patterns and etiology of diarrhea in a group pediatric practice, an individual practice, and a hospitalized population in the same locality in Michigan (381). In both practices, 16% of the children with diarrhea not requiring hospitalization shed rotavirus, whereas 32% of children hospitalized for diarrhea were rotavirus-positive. During the rotavirus peak season of January, February, and March (1979–1980), when the practices and the hospital were studied simultaneously, the frequency of rotavirus diarrhea was 30% for the solo practice, 36% for the group practice, and 48% for the hospital study. Bacterial pathogens were detected infrequently throughout the study. With respect to incidence data in the solo practice, infants under 1 year of age experienced an average of 0.15 episodes of rotavirus diarrhea per year, whereas 1- to 2-year-old children experienced 0.05 such episodes per year. The marked difference in incidence of rotavirus diarrhea in the 1- to 2-year age group between this study and the longitudinal family study described above probably reflects differences in surveillance methods. The family study detected illnesses that were too mild to warrant an office visit. Eleven percent of patients under 2 years of age who had rotavirus diarrhea were hospitalized, a figure identical to that in the family study described above, whereas the hospitalization rate for nonrotavirus diarrhea was 4%. Overall, 38% of the diarrhea patients hospitalized from these practices were infected with rotavirus. The estimate of the annual number of hospitalizations in the United States for rotavirus diarrhea from this study was 80,000 (316).

In developing countries, the widespread distribution of rotaviruses is also shown by the universal acquisition of serum antibody to these viruses at an early age. By the end of the third year of life, over 90% of infants and young children acquire rotavirus antibodies, a pattern similar to that in developed countries (44,55,58, 83,148,157,263,321,339).

Cross-sectional studies have consistently demonstrated that rotaviruses are the major etiological agents of diarrhea in infants and young children in most developing countries. For example, in a comprehensive study of the etiology of diarrheal illnesses that included 6352 patients treated at the Matlab Treatment Center in Bangladesh over a 1-year period, rotaviruses were the most frequently detected pathogen in children less than 2 years of age, with 46% of them being rotavirus-positive (57). The next most important group of agents was toxigenic *Escherichia coli* (ETEC), detected in 28%. In the over-2-year age group, bacterial agents were recovered more frequently than rotavirus. The importance of rotaviruses in severe dehydrating diarrheal illnesses was demonstrated in another cross-sectional analysis in a hospital in Cairo, Egypt in which 145 infants and young children under 18 months of age with fatal or potentially fatal diarrheal illness were studied (597). Rotaviruses were the most frequently detected pathogen, accounting for 34% of the cases, with the enterotoxigenic *E. coli* ranking second (27% for LT and ST combined).

Prospective studies in developing areas of the world have demonstrated the magnitude of the problem of diarrheal diseases in general and also highlighted the unique capacity of rotaviruses to induce dehydrating illness with greater frequency than other agents. In a 30-month village-based study in northeastern Brazil, early childhood mortality exceeded 14% during the first 5 years of life; furthermore, in over 52% of recorded deaths, diarrhea was listed as either the primary or an associated cause of death (259). A sample of specimens from diarrheal episodes was tested for enteric pathogens, and 53% yielded a recognized etiologic agent. The most commonly detected pathogens were enterotoxigenic *E. coli* and rotaviruses, 21% and 19%, respectively. The poorest families had the highest diarrhea attack rates, which reached almost 10 per child per year in the 6- to 11-month age group.

When a cohort of 45 children were studied prospectively over a 3-year period in Santa Maria Cauque, Guatemala, the incidence of diarrhea was 7.9 per child per year; rotavirus was associated with 10% of such episodes (417,418). The incidence of rotavirus infection was 1.2 per child per year, whereas the incidence of rotavirus diarrhea was 0.8 per person per year. Each of the 45 children shed rotavirus at least once during the 3 years of the study. Reinfections occurred frequently, and the number of reinfections per child ranged from two to six (417). Significantly, dehydration was 14 times more frequent among children with rotavirus diarrhea than among those with diarrhea of bacterial, acute parasitic, or unknown etiology (759).

Two prospective longitudinal studies of diarrheal illnesses in Bangladesh deserve special mention because

they demonstrate the overall impact of diarrheal illness in a developing country. These studies also help to clarify the relative role of various microbial agents in the etiology of mild and severe diarrheal illnesses. In a longitudinal study in two contiguous villages, 197 children 2–60 months of age were studied prospectively for 1 year (52,53). Diarrhea, which was the second most common illness, occurred on 12.8% of all days. Infants 2–11 months of age had the highest prevalence of diarrhea (17% of all days); this decreased to 10.3% of all days in the 36- to 60-month age group. Diarrheal illness did not display a marked seasonal distribution, although it tended to occur more often in the hot, rainy months. The most frequent cause of admission to the treatment center was diarrhea. Twenty-three of the 197 children were admitted because of mild to moderate dehydration associated with diarrhea and vomiting; 10 others were admitted with bloody dysentery in which shigella was associated in nine instances. Ten additional children received treatment for diarrhea in the outpatient clinic. Diarrheal illness was responsible for 52% of all hospitalizations. A bacterial, viral, or parasitic agent was detected in 51% of the 920 diarrheal episodes. The agents most frequently identified were the enterotoxigenic *E. coli* (ETEC); these bacteria were detected in 27% of episodes as the only pathogen and in 4% with another pathogen. The second most commonly detected agents were the shigellae—12.8% as sole pathogen and 3% with another pathogen. The next most common pathogens were the rotaviruses—3.8% as sole pathogens and 0.9% in combination with another agent. Children 2–11 months of age had the highest incidence of diarrheal episodes (over 7 per child per year); incidence of diarrhea declined gradually to 4 per child per year in the 36- to 60-month age group. The incidence of ETEC diarrhea decreased from more than two episodes per child per year in the 2- to 11-month age group to one episode per child per year in the 36- to 60-month age group. The incidence of rotavirus diarrhea was 0.5 episodes per child per year up to the second year of life and decreased to a low level in the third year.

Although the incidence of rotavirus diarrhea was lower than that of diarrhea caused by the other pathogens, dehydration occurred significantly more often during rotavirus disease than during illness associated with ETEC, shigella, or other agents (52). Thus, in diarrheal episodes in which only a single pathogen was detected, nine of 35 rotavirus diarrheal episodes resulted in dehydration, whereas only seven of 248 episodes associated with ETEC, as well as four of 455 not associated with a known pathogen, were accompanied by dehydration. In addition, one of 10 episodes associated with non-O group 1 *Vibrio* and one of three associated with *Vibrio cholerae* O group 1 resulted in dehydration. None of 118 episodes of shigella diarrhea was accompanied by dehydration. Although implicated in less than 4% of all diarrheal episodes, rotaviruses were associated with 39% of illnesses that resulted in significant dehydration. It was the clinical impression of the investigators that untreated rotavirus diarrhea was more likely to be fatal than diarrhea caused by other pathogens. Although nearly all children in this study had serum ELISA antibody to rotavirus, those with the lowest levels had the greatest risk of developing rotavirus diarrhea.

The special capacity to induce severe diarrhea was underscored in another study of diarrheal disease in a rural village in Bangladesh from December 1977 to November 1978 (56). Again, the pathogens detected most frequently in all age groups were the ETEC (predominantly stable toxin producers), accounting for 23% of the diarrheal episodes. Shigella was the second most frequently detected pathogen, accounting for 11% of all episodes of diarrhea. Rotaviruses were associated with 5% of diarrheal episodes and were detected only in the under-2-year age group, where they accounted for 11% of such episodes. Although the incidence of rotavirus diarrhea was almost half that of ETEC diarrhea in the under-2-year age group, its impact was greater because one in six of the rotavirus diarrheas necessitated a visit to the treatment center, whereas only one in 15 ETEC diarrheas required treatment. A comparison of the etiologies of dehydrating diarrheal illness in 41 children less than 2 years of age indicated that 46% of episodes were associated with rotavirus, 24% were associated with ETEC, and 7% were associated with shigella; in 21%, a pathogen was not detected. An estimate was made of the number of children who would have died if fluid replacement therapy were not available, if dehydration reached a level of 7.5% or more, and if 50% of children failed to survive with this level of dehydration. The resulting estimate was 6.5 deaths per 1000 children less than 2 years of age per year. The estimate for rotavirus diarrhea was 2.9 deaths per 1000 persons less than 2 years of age per year (56). The latter estimate is remarkably similar to the observed incidence of rotavirus diarrhea severe enough to require hospitalization in Washington, D.C. (548).

Rotavirus Subgroups and Serotypes

The epidemiology of individual serotypes is poorly understood because techniques for routine identification of serotype have only recently become available. Thus, subgrouping has been used as a surrogate for serotyping because among human rotaviruses, subgroup I includes only serotype 2 strains, whereas

subgroup II includes strains of serotypes 1, 3, and 4. In a 1-year Swedish study, rotavirus isolates from children with gastroenteritis were analyzed by PAGE, which permits prediction of subgroup specificity by the mobility of RNA segments 10 and 11. Approximately one-third of the viruses recovered were subgroup I, while the remainder were subgroup II (693). While fever and a temperature exceeding 39°C developed significantly more often in children who shed subgroup I virus, the three dominant electropherotypes of subgroup II rotavirus appeared to be more virulent than other strains. The subgroup II viruses were associated with a higher rate of hospitalization, more severe symptoms, and a significantly higher occurrence of respiratory symptoms. In contrast, an analysis of the electropherotype of rotavirus strains derived from children less than 42 months of age treated in the clinic or in the hospital in Australia revealed that the etiologic agents were similar in both the clinic and hospital groups, suggesting that the severity of symptoms was associated with inherent host susceptibility rather than subgroup of the infecting rotaviruses (521). Overall, in various parts of the world, subgroup II rotaviruses are detected more frequently than subgroup I isolates (17,294,475,477,621,685,733,773).

Until recently, rotavirus serotype could only be identified by techniques not adaptable to large-scale epidemiologic surveys, such as neutralization of immunofluorescent foci, plaque reduction neutralization, roller tube tissue culture neutralization, or solid-phase immune electron microscopy. Recently, VP7 monoclonal antibodies with specificity for serotype 1, 2, 3, or 4 have been developed and are now being utilized extensively in epidemiological studies (39,121,275, 590,653). It is now possible to identify the serotype of a rotavirus present in stool by ELISA using these monoclonal antibodies. Thus far, there has been complete concordance between (a) serotype determined by ELISA and (b) by neutralization with hyperimmune antiserum. Several groups have retrospectively serotyped viruses present in stored specimens. In an Australian study in which specimens from neonates infected asymptomatically in a newborn nursery or from infants and young children hospitalized with acute gastroenteritis were analyzed, serotype 1 strains were detected most frequently during the period 1975–1986 (Fig. 12) (39). A similar distribution of serotypes, with type 1 predominating, has been observed in other locations such as Venezuela, Italy, and the Central African Republic (17,197,216,222).

Routine identification of serotype by ELISA has proven to be essential to the interpretation of clinical vaccine trials. However, typing of rotaviruses with serotype-specific monoclonal antibodies is limited, at

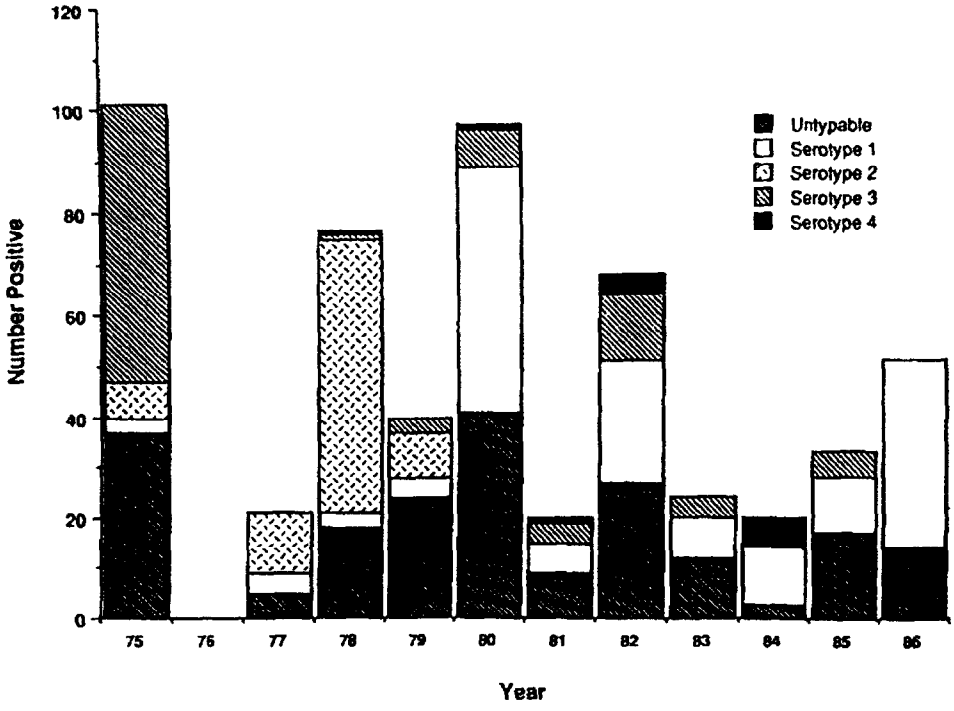

FIG. 12. Distribution of rotavirus serotypes in an Australian hospital among asymptomatically infected neonates and among infants and young children with acute gastroenteritis. (From ref. 39.)

present, to VP7. Because hyperimmune sera used for serotyping usually have low anti-VP4 neutralizing antibody titers, little is known about the distribution and epidemiology of different VP4 antigens.

Rotavirus Infections in Adults

Rotavirus gastroenteritis in adults has been described in army recruits in Finland as well as in patients (some geriatic) and staff in hospitals in England, Sweden, Canada, Finland, South America, Polynesia, and China (123,146,201,209,232,266,274,278,293,307,310, 351, 365, 394, 404, 411, 448, 449, 500, 551, 643, 686, 721, 730,779). Several outbreaks in geriatric groups had a high attack rate, and there were some fatalities (123,266,307,411). Nonetheless, these outbreaks are unusual because of the level of rotavirus immunity that most adults have acquired from previous infections. Subclinical rotavirus infection appears to be the rule in adults. The extent of subclinical infection in adults was documented in a study at the Children's Hospital National Medical Center (Washington, D.C.), in which 22 (55%) of 40 adult household contacts of children hospitalized with rotavirus gastroenteritis developed serological evidence of rotavirus infection at or about the time of their child's admission to the hospital (365). In contrast, only four (17%) of 24 other adults whose children were admitted for nonrotavirus gastroenteritis developed serological evidence of rotavirus infection. Only three of 26 adults with rotavirus infection developed a gastroenteric illness. Additional observations concerning adult infection were made during a prospective family study of diarrhea in families with newborn children in Winnipeg, Manitoba, Canada (730). Rotavirus infections were observed in adults on 43 occasions, for an incidence of 0.17 per adult per year. The incidence in children was 0.32 per child per year. Seventeen adults had gastrointestinal symptoms such as diarrhea (14) or abdominal cramps (11). Forty percent of adult rotavirus infections were symptomatic, whereas 70% of infections in their children were associated with gastrointestinal symptoms ($p < 0.001$). A pattern of intrafamilial spread of rotavirus infection and illness from infected infants and young children to adults was demonstrated in this study as well as in other family studies in the United States and New Zealand (256,552).

High rates of rotavirus illness in adults have also been reported in other situations. An unusual outbreak of rotavirus gastroenteritis occurred in adults associated with children in a play group (551). Nine children 15 months to 5 years of age, three of five mothers who participated in play-group activities, four of five fathers, and each of two grandparents developed gastroenteric illness which could be traced to the play group. Overall, 18 of 21 individuals developed gastroenteritis, and 10 of 11 persons tested demonstrated evidence of rotavirus infection. Other sharp outbreaks of rotavirus-associated acute diarrheal illness have been described in which individuals of all ages were affected (201,394). During an extensive outbreak in an isolated South American Indian community in northern Brazil, the attack rate in adults over 41 years of age was 80% (394).

Rotavirus infections have also been associated with travelers diarrhea in certain settings (61,150,361,560, 593,719). However, these viruses are not considered to be major etiological agents of this disease (51,147).

Group B rotaviruses have been implicated in several large outbreaks of severe gastroenteritis in adults in various parts of China involving 12,000 to 20,000 individuals. Affected individuals developed cholera-like, severe, watery diarrhea, and a few elderly patients died (310–312,479,631,724).

Nosocomial Infections

Nosocomial infections with rotavirus occur frequently (176,452,549,559). For example, in one study, 10 (17%) of 60 children hospitalized for nondiarrheal disease during a period of rotavirus prevalence developed a diarrheal illness associated with a rotavirus infection (559). In a 1-year period in another hospital study, about one of every five rotavirus infections appeared to be hospital-acquired (452).

Transmission

Rotaviruses appear to be transmitted by the fecal–oral route. Oral administration of rotavirus-positive stool material induces diarrheal illness in volunteers (356,357,725). Nevertheless, speculation continues as to whether rotaviruses are transmitted by the respiratory route (233,262,615). Evidence for this is circumstantial and rests on the following: (a) the rapid acquisition of rotavirus antibodies in the first few years of life in all settings, regardless of hygienic standards; (b) a few large outbreaks in which a fecal–oral spread could not be documented (201); and (c) the occurrence of respiratory symptoms in a proportion of patients with rotavirus gastroenteritis (262). However, there is no experimental evidence to support the respiratory mode of transmission. With the exception of two studies, neither rotavirus nor rotavirus antigen has been detected in respiratory secretions (90,202,229,357, 643,718).

The source of infection for the young infant who is normally not in contact with other infants and young

children with gastroenteritis is not well documented. One likely possibility is an older sibling or parent with subclinical infection (161,256). Several hospital-based studies have shown that a significant proportion of parents of infants and young children with rotavirus disease are infected with rotavirus at or about the time of their child's illness (351,365,643,686). In addition, in a day-care center study, rotavirus shedding prior to (as well as after) cessation of diarrhea was found to be quite common (520).

Resistance to physical inactivation may contribute to the efficient transmission of the human rotaviruses. This inference is drawn from the observed stability of simian rotavirus SA-11 and human rotavirus at ambient temperature (169,362,459). Also, it has been shown that calf rotavirus present in feces retains infectivity when kept at room temperature for 7 months (179). Other observations that also suggest environmental contamination as a source of infection include (a) the persistence of rotavirus infection in certain newborn nurseries and (b) the high frequency of nosocomial rotavirus infection in hospitals (21,46,104,176,442, 472,559). Effective disinfection of contaminated material and careful hand-washing may constitute important measures to contain rotavirus infection, especially in a hospital or institutional setting (54,380,571,623). Although rotaviruses have been detected in raw or treated sewage, it is unlikely that contaminated water plays an important role in transmission of group A rotaviruses (604).

There has been speculation on the role of animals as a source of rotavirus infection of humans (411). This speculation has intensified following the observation that certain animal rotaviruses, such as the canine and feline strains, share a neutralization antigen with the third human rotavirus serotype (Table 1). However, it should be noted that most of the corresponding genes of animal and human rotaviruses differ when studied by RNA–RNA hybridization (187,353). Thus, there is no evidence that animal rotaviruses spread to humans (or vice versa) under natural conditions (213,561).

Incubation Period

The incubation period of rotavirus diarrheal illness has been estimated to be less than 48 hr (128). The onset of experimentally induced rotavirus diarrhea in adult volunteers occurred 2–4 days after challenge (356,357). One of the volunteers who developed diarrhea 3 days after virus administration developed a fever and vomited on the first day after challenge, indicating that the incubation period under experimental conditions was 1–4 days.

Geographic Distribution

Rotaviruses have been detected throughout the world wherever they have been sought (2,171,342,410, 507,569,775). Furthermore, these viruses constitute major etiologic agents of infantile diarrhea in every country where this disease has been studied by appropriate techniques.

Temporal Distribution

Rotaviruses display a repetitive pattern of infection in developed countries, with peaks occurring in the cooler months of each year (8,42,66,67,69,84,128,351, 375,379,451). This recurring pattern was clearly documented in the Children's Hospital National Medical Center (Washington, D.C.) study of infants and young children admitted with diarrheal diseases over an 8-year period (Fig. 11) (66). The absence of rotavirus infections from July through October (1974 to 1982) was a consistent finding; none of the 256 patients sampled during the July–October intervals shed rotavirus in their feces. In contrast, during the month of January, 67% of 250 hospitalized diarrhea patients shed rotavirus; during the months of February, 58% of 219 patients were rotavirus-positive. Outpatients with diarrhea exhibited a similar pattern. In a Japanese study extending from December 1974 through June 1981, rotaviruses were detected in the feces of 66% of 785 patients hospitalized with diarrhea during the cooler months of December, January, and February and in the feces of 56% of 549 children admitted in the spring (March, April, and May) (379). In contrast, only 5.6% of 576 pediatric patients admitted during the summer and autumn months were rotavirus-positive. Although the cause for this striking seasonal pattern is not known, the influence of low relative humidity in the home has been suggested as a factor facilitating the survival of rotaviruses on surfaces (65). However, a correlation of relative humidity with temporal pattern of infection has not been observed in every epidemiologic setting (379,459). A recent analysis of hospitalizations for diarrhea in the postneonatal period and of the temporal distribution of rotavirus isolations from various centers suggests that rotavirus diarrhea spreads from the west to the east in the United States, appearing in the fall in the southwest and spreading progressively across the country so that by late winter and spring it has reached the northeast (283).

Since reagents for serotyping human rotaviruses from long-term studies have only recently become available, observations concerning the temporal distribution of specific serotypes are limited. However,

some information is available concerning pattern of virus subgroups (69,384,685,773). During the first 4½ years of the Children's Hospital National Medical Center study, 75% of the 238 rotavirus strains belonged to subgroup II while 25% belonged to subgroup I (69). This distribution was not consistent throughout the study, since subgroup I strains constituted 14% of the total number from November 1974 to July 1976, whereas from December 1977 to June 1978 they accounted for 46% of the strains studied. The distribution of subgroups in outpatients was comparable to that observed for inpatients. A survey of the distribution of subgroups among rotaviruses detected in patients from Sweden, Ethiopia, Guatemala, Costa Rica, Venezuela, Belgium, England, Australia, Asia, and other parts of Africa revealed that, as in Washington, D.C., subgroup II strains also predominated, ranging from 62% in Guatemala to 100% in Costa Rica and England (685,773). The predominance of subgroup II strains is consistent with emerging data on serotypes as described earlier. It was of interest that in the Venezuelan study, illnesses of less than 3 days duration occurred significantly more often among hospitalized infants and young children in the subgroup I infected group than in the subgroup II infected group (733). A more definitive pattern of temporal distribution of rotaviruses should emerge in the near future as strains are characterized in terms of both subgroup and serotype.

The usual seasonal pattern of rotavirus infection observed in the temperate climates does not occur uniformly in other settings. For example, a significant number of rotavirus infections has been documented throughout the year in South Africa, during the summer in Taiwan, during the "small rains" in Ethiopia, during most months in tropical climates (but with peak periods during the slightly cooler or dry months), during the summer in a newborn nursery in England, throughout the year in a newborn nursery in Australia or Venezuela, and during the autumn on a U.S. Indian reservation (21,149,161,217,277,407,416,472,508,583, 584,614,624,680,716). However, a marked seasonal pattern of rotavirus infection has been observed in Gambia over four consecutive years (epidemics occur during the cool, dry season, with a peak in January) and in Gabon, in equatorial Africa during the dry season months over a 1-year period (270,602).

Age, Sex, Race, Socioeconomic Status

Rotavirus gastroenteritis severe enough to require hospitalization occurs most frequently in infants and young children from 6 months to 2 years of age (69, 84,128,376). Infants under 6 months of age experience the next highest frequency of such illness; in certain studies, however, this age group had the highest frequency (161,467,626,694). However, in the Children's Hospital National Medical Center (Washington, D.C.) study, the age distribution of patients admitted to the hospital with gastroenteritis of any etiology was different for black and non-black patients; 59% of all black patients admitted for gastroenteritis were younger than 6 months of age (69). This difference was also reflected in admissions for rotavirus diarrhea. The median age of admission for black children with subgroup I infection was 5 months, whereas that for non-black children was 11.5 months. For subgroup II infections it was 8 months and 14 months for black and non-black children, respectively. Since the black children were predominantly from less affluent inner-city areas, these differences probably reflect the effects of crowded living conditions which may have allowed earlier and more efficient transmission of the virus.

The low frequency of clinical illness in most (but not all) normal neonates who shed rotavirus remains an intriguing paradox which is being actively investigated because of its implications for immunoprophylaxis (21,47,104,105,320,435,472,534,541,667,680,684,687, 715). As noted earlier, recent studies demonstrated that the gene encoding the outer capsid protein VP4 of the asymptomatic neonatal rotavirus strains is highly conserved and differs from the corresponding protein of symptomatic strains (188,194,237). Further study is required to determine if the highly conserved VP4 of asymptomatic neonatal rotaviruses plays an important role in the ability of these viruses to persist over a long period of time in newborn nurseries without causing significant disease. Rotavirus infection is also often subclinical in adults (365). However, it should be noted that rotavirus gastroenteritis has been documented in older children and adults, who may be important in the transmission of rotavirus infection to young children (and vice versa) (256,271,552,730).

Clearly, both males and females are susceptible to rotavirus gastroenteritis. The attack rate for males was slightly higher than for females in Washington, D.C. and in Toronto (69,452).

Malnutrition is thought to play an important role in increasing the severity of clinical manifestations in humans during rotavirus infection (79,415). This phenomenon has been demonstrated in an experimental mouse model (539). It has also been suggested that repeated diarrheal infections may be a precipitating factor in the development of malnutrition by damaging intestinal mucosa so that absorptive cells are compromised over an extended period (415,418). In addition, in rural Bangladesh, increasing distance from a treatment center correlated with more severe diarrheal dehydration on presentation (529).

Molecular Epidemiology

Initial investigations of the molecular epidemiology of human rotaviruses utilized gel electrophoresis of segmented genomic dsRNAs for strain identification (electropherotype) and comparison. For example, analysis of rotaviruses derived from 116 children hospitalized with gastroenteritis in Melbourne, Australia between 1973 and 1979 identified 17 different electropherotypes (542). Distribution of electropherotypes was not random during the 7-year study period but showed, instead, a sequential pattern of appearance and prevalence. Only a limited number of electropherotypes were detected at any one time. Characteristically, a single electropherotype was predominant, often accompanied by less common types. In contrast, only two closely related electropherotypes could be identified among rotaviruses from 72 newborn babies studied in seven different hospitals from 1975 to 1979. It was remarkable that these two electropherotypes had similar RNA patterns and that neither one was ever identified in viruses from children hospitalized for acute gastroenteritis. One of these electropherotypes was detected in nurseries in five different hospitals and persisted in one nursery for 4 years. This adds to the mystery of neonatal rotavirus infection because the year-round occurrence of rotavirus infection in the nurseries by viruses of a single distinctive electropherotype differed from the seasonal pattern involving multiple electropherotypes in the general population.

Similarly, in France, an epidemiologic survey over a 16-month period in a maternity unit revealed that all 184 rotavirus fecal samples obtained from 3- and 6-day-old newborns exhibited the same electropherotype. This pattern was in sharp contrast to the variable electropherotypes found in postneonatal infections (212). It should be noted that in a similar study in Venezuela, 62 (57%) of 108 children shed rotavirus within the first few days of life; furthermore, a similar gel migration pattern was observed for each of the 52 samples examined. Six of the 62 children had diarrhea, but only one required oral rehydration therapy (515).

In further studies in numerous countries, various patterns of electrophoretic diversity were observed in pediatric patients with diarrhea. However, numerous electropherotypes were detected in practically every location (5,17,87,98,102,117,165,198,218,231,374,484, 549,619,622,639,642,644,646). In addition to providing evidence for genetic diversity of human rotaviruses as well as heterogeneity of circulating rotaviruses, analysis of electropherotypes has also provided a method for tracing the spread of rotavirus through a population group. However, electropherotype cannot be used to predict serotype (19,29,218).

RNA–RNA hybridization studies employing labeled ssRNA viral transcripts as probes for genomic RNA identified two major families of human rotaviruses (193,196). The 11 genes of most human rotaviruses exhibit significant homology to the corresponding genes of either Wa (serotype 1) virus or DS-1 (serotype 2) virus. On several occasions, human rotaviruses were identified that had a mixed genotype, with some genes being DS-1-like while others were Wa-like. This was interpreted as evidence for gene reassortment during coinfection *in vivo*. Hybridization analysis also indicated that the corresponding genes of most animal viruses and human viruses lacked significant homology, providing evidence that human rotaviruses do not infect animals under natural conditions (or vice versa).

IMMUNITY

The mechanisms responsible for immunity to human rotavirus infections and illness are poorly understood. Nonetheless, animal studies have been particularly instructive in elucidating the relative importance of systemic and local immunity (27,70,76,300,389,395,493, 611,739). It was observed that newborn calves frequently developed rotavirus diarrhea despite a moderately high level of circulating rotavirus antibodies derived from colostrum (739). This observation was confirmed experimentally when five calves were fed colostrum containing rotavirus antibodies within 24 hr of birth and then challenged 7 days later with calf rotavirus. These calves developed a diarrheal illness despite the presence of a high level of serum rotavirus antibodies at the time of challenge. In contrast, two calves that were fed colostrum containing rotavirus antibodies 4 hr before challenge, and 4 and 24 hr after challenge with calf rotavirus failed to develop diarrhea within the normal incubation period. One of these calves developed a mild diarrheal illness that did not start until the 6th day after inoculation (76,739).

The relative role of local and systemic rotavirus antibodies was also examined in newborn lambs (611). A group of 1-day-old lambs was fed colostrum that contained rotavirus antibodies, and two other groups were fed either (a) the same preparation of colostrum on days 1 through 4 or (b) serum containing rotavirus antibodies on days 2 through 4. Following challenge with lamb rotavirus at 2 days of age, the first group of lambs developed diarrhea despite the presence of serum antibodies to rotavirus (acquired from the colostrum), whereas the other two groups of lambs did not. These observations suggest that antibody in the lumen of the small intestine is a major determinant of resistance to rotavirus illness. In addition, passively acquired gastrointestinal rotavirus antibodies, but not circulating antibodies, have been shown to protect newborn mice against experimental rotavirus disease (492).

In subsequent studies of immunity in experimental

animals, additional evidence for a role of rotavirus antibodies was obtained, but it also appears that other immune mechanisms may play an important role. Although serotype-specific immunity has been demonstrated convincingly in several experimental animal models, protection could not be explained on this basis in other studies (50,72,73,91,152,403,442,471,493,567, 609,675,703,740,741). For example, two avirulent bovine strains were evaluated in gnotobiotic calves for their ability to protect against disease following challenge with a virulent strain (72). Protection was conferred by prior infection with either avirulent virus even though one of them was not related significantly to the virulent challenge virus in cross-neutralization tests. Thus, mechanisms other than neutralizing antibodies, such as cytotoxic T lymphocytes, may also play a role in immunity to disease (72,73,496,540). It is also of interest that circulating rotavirus antibodies can appear in the gastrointestinal tract of neonatal calves if the level of circulating antibodies is sufficiently high; these mucosal antibodies can provide protection against experimentally induced infection and diarrhea (36).

Preliminary information concerning correlates of rotavirus immunity was obtained during a volunteer study in which human rotavirus serotype 1 ("D" strain) was administered to 18 individuals by the oral route (356,357). Five of the 18 volunteers shed rotavirus, and four of the five developed diarrheal illness. A preexisting high titer of serum neutralizing antibodies to the homotypic virus or to heterotypic serotype 2 human rotavirus correlated with resistance to diarrheal illness. The relationship of neutralizing activity in intestinal fluid to resistance was less clear. Volunteers with a high level ($\geq$1:100) of neutralizing activity in intestinal fluid to the homotypic virus tended to develop fewer diarrheal illnesses than those with lower levels ($<$1:100), but the difference was not statistically significant. The five volunteers who shed rotavirus developed a significant increase in intestinal-fluid neutralizing activity for the homotypic virus. Each of the ill volunteers also developed a serum neutralizing antibody response to the challenge strain and also to heterotypic human rotavirus DS 1 (serotype 2) as well as to bovine rotavirus NCDV (serotype 6). These cross-reactive responses may have implications for vaccine development. In another adult volunteer study, the level of homotypic serum antibodies did not correlate with protection (35,725).

The presence of serum antibody to rotavirus as measured by ELISA is not associated with resistance to infection or illness (55,110,262). Perhaps the failure of serum ELISA antibody to correlate with resistance should not be surprising because ELISA measures antibodies that are mostly directed at the rotavirus group antigen. However, rotavirus serum IgA levels were correlated with a protective effect on the severity of rotavirus illness (282). Recently, a correlation between serum homotypic neutralizing antibodies and resistance to disease was observed during a study of 1- to 24-month-old infants and young children residing in an orphanage (103). A serum neutralizing antibody titer of 1:128 or greater to human serotype 3 was associated with resistance to gastroenteritis due to that serotype, whereas serum antibody levels of 1:64 or less failed to correlate with significant homotypic protection. Serum antibodies may only correlate with, and hence reflect, local intestinal antibodies that are produced locally and/or enter the lumen of the intestinal tract by transudation (36,131,282,315,538,618,692,728,760).

Rotavirus reinfection has been documented frequently in adult contacts of children with rotavirus illness. Many of these reinfections are subclinical. Sequential rotavirus infections were described in a cohort of 45 infants and young children studied intensively during the first 3 years of life in Santa Maria Cauque, Guatemala: One individual experienced only one rotavirus infection, while 15 individuals had two rotavirus infections, 12 had three infections, 12 had four infections, four had five infections, and one had seven rotavirus infections (417). The distribution of virus serotypes and severity of illness associated with initial infection or subsequent reinfections were not described. In an earlier study, sequential rotavirus illnesses were observed in infants and young children, but these illnesses were associated with strains belonging to different subgroups (759).

During a prospective study of children in Winnipeg, 10 of 82 rotavirus infections represented reinfection (262). Seven of the 10 reinfections were associated with gastrointestinal symptoms, a rate similar to the overall rate of gastrointestinal symptoms observed for rotavirus infections in this study. Each of the seven children who had gastrointestinal symptoms during the second infection had also experienced gastrointestinal symptoms during their first infection. The three children who underwent silent reinfection also failed to develop gastrointestinal symptoms during their first rotavirus infection. The interpretation and significance of these observations awaits serotype analysis of the rotaviruses responsible for initial infection and reinfection. Only then will it be possible to define the extent and duration of homotypic or heterotypic immunity. Answers to some of these questions are emerging from studies such as those performed in the orphanage cited above, as well as from vaccine field trials to be described later.

As noted earlier, there is a relative sparing of neonates from rotavirus illness despite a high incidence and persistence of infection in certain newborn nurseries. Sparing cannot be ascribed to breast-feeding because both breast-fed and bottle-fed infants develop

subclinical infection (105,261). Possibly, a very high level of circulating rotavirus antibody acquired transplacentally is responsible for relative resistance to disease during neonatal infection, but this remains to be determined. It has also been suggested that qualitative or quantitative differences in intestinal enzymes required for activation of rotavirus infectivity may be responsible for this sparing effect (290). Finally, it should be noted that rotaviruses that persist in newborn nurseries without causing significant disease bear a highly conserved VP4 which is rarely encountered on rotavirus strains that cause diarrheal disease in older infants in the community (194,235). It has been suggested that viruses able to persist in newborn nurseries without causing significant disease are naturally attenuated by virtue of their VP4.

The important question of whether or not the immune response induced by asymptomatic or mild neonatal infection protects against rotavirus infection and illness later in childhood was addressed in an Australian study (46). Eighty-one babies, 44 of whom were rotavirus-positive, and 37 of whom were rotavirus-negative during the first 14 days of life, were kept under clinical and serologic surveillance for 3 years. The frequency of subsequent postnatal rotavirus infection was almost the same for both groups (54–55%). However, when postnatal rotavirus infection occurred, the children in the neonatal rotavirus-positive group developed significantly less illness, or if they did become ill their symptoms were less severe than those of their cohorts who had not been infected neonatally. Thus, neonatal rotavirus infection did not protect against reinfection, but it did confer partial resistance to disease during reinfection. The rotavirus strains responsible for neonatal infection in this study were of particular interest because their RNAs were indistinguishable by gel electrophoresis. One of the 20 neonatal strains was identified as serotype 3. This unusual electropherotype was not identified among the strains that induced illness in the study group during the postneonatal period. Thus, infection at birth with a rather uniform rotavirus conferred partial protection against disease during reinfection with other rotavirus strains that most likely included a variety of serotypes. This finding may be of importance to the development of an effective strategy for immunoprophylaxis of rotavirus disease.

Although there is a paucity of information from human studies, it appears that mainly homotypic immunity is induced following the first rotavirus infection; this is mediated, in large part, by VP7 and VP4 antibodies (82,297,402,494,497). Although the neutralizing antibody response to primary rotavirus infection in piglets is primarily homotypic, a lower-level heterotypic response commonly occurs involving serotypes 1, 3, and 4 (300). Subsequently, in both infants and experimental animals the predominantly serotype-specific response is expanded to include other serotypes following subsequent reinfection with the same or another serotype ("original antigenic sin") (80–82, 526,609). It should be noted that one or more neutralization epitopes on both VP4 and VP7 are shared among viruses of serotypes 1, 3, and 4 (405,406,650–652,656). Thus, it is not surprising that reinfection with a virus of the same serotype amplifies the cross-reactive neutralizing antibody response.

Serotype-specific immunity has been demonstrated under experimental conditions in lambs and rabbits (115a,609). Furthermore, immunization of cows with a single rotavirus serotype induced neutralizing antibodies to that strain and to heterotypic strains against which these animals had preexisting neutralizing antibodies but not to strains the animals had not been infected with previously (609). In other studies, immunization of cows with a single rotavirus serotype induced a marked antibody response to numerous serotypes (81).

As noted earlier, the VP4 and VP7 proteins independently induce neutralizing antibodies, and each outer capsid protein plays a role in resistance to disease (297,494,727). In a recent study employing an epitope-blocking assay, it was observed that a majority of infants and young children fed the live rhesus rotavirus candidate vaccine developed antibody to the major serotype-specific neutralization epitope on VP7 of RRV, and almost half of the vaccinees also responded to a major neutralization epitope on VP4 (588). This indicates that infection with an attenuated vaccine strain which is restricted in its capacity to replicate in the intestine can induce antibodies to neutralization epitopes on these protective antigens.

Observations made during two prospective studies suggest that breast-feeding confers partial resistance to rotavirus infection during infancy. Seven hundred fifty-one neonates who were breast-fed experienced fewer rotavirus infections (22%) than did 305 neonates in the same nursery who were bottle-fed (58%) (105). Furthermore, neonates who were breast-fed shed less virus when infected than did bottle-fed neonates. The effect of breast-feeding on rotavirus illness could not be determined because only 8% of infected neonates developed diarrhea or vomited. None of these infants was ill enough to require treatment. In another prospective study, rotavirus infection was documented in (a) 55% of 29 infants who were not breast-fed and (b) 39% of 75 infants who were breast-fed (262). This difference was not statistically significant. Serotype-specific antibodies have been demonstrated in breast milk (34). The effect of breast-feeding on the occurrence of infantile gastroenteritis needs additional study, since the available data do not permit a definite conclusion (125, 126, 200, 227, 228, 281, 358, 501, 503, 530, 660, 669,

679,681,682,731). In any event, if breast-feeding does prove to transfer immunity to newborns and young infants, its protective effect will probably be modest.

CLINICAL FEATURES

Rotavirus infection produces a spectrum of responses that vary from subclinical infection, to mild diarrhea, to a severe and occasionally fatal dehydrating illness. In a study at Children's Hospital National Medical Center (Washington, D.C.), a comparison was made between (a) the clinical manifestations of 78 patients hospitalized with rotavirus diarrhea and (b) 72 patients hospitalized with a diarrhea illness that could not be associated with rotavirus (Table 5) (547). The majority of both rotavirus and non-rotavirus patients had a temperature of 37.9°C or greater. However, the rotavirus group vomited and became dehydrated significantly more often than the rotavirus-negative group. The mean duration of vomiting as determined from history and hospital records was longer in the rotavirus group than in the rotavirus-negative group (2.6 days versus 0.9 days). Rotavirus diarrhea started later than vomiting but lasted longer (mean duration 5 days versus 2.6 days). After infants and children were hospitalized, diarrhea continued for a mean of 2.6 days (range: 1–9 days) in the rotavirus group and 3.8 days (range: 1–16 days) in the rotavirus-negative group. The duration of hospitalization ranged from 2 to 14 days, with a mean of 4 days for the rotavirus group. It is of interest that in Japan, rotavirus infection has been associated in some instances with severe diarrhea characterized by a milky white stool (hakuri) (478). Laboratory findings in the Children's Hospital National Medical Center study reflected the high frequency of vomiting and dehydration associated with rotaviral illness (547). A BUN value of greater than 18 ml per dl was observed in 58% of the rotavirus-positive group, and a urine specific gravity of 1.025 or greater was noted in 71%. These rates were significantly higher than in the nonrotavirus group. The frequency of acidosis and electrolyte imbalance did not differ in the two groups.

Over a 5-year period in a Toronto hospital, rotavirus gastroenteritis was responsible for the death of 21 infants and young children between 4 and 30 months of age (89). Ten of these children were dead on arrival at the hospital, and 10 were moribund and could not be resuscitated. One of the children was already hospitalized when he acquired the disease; this patient had been hospitalized with congestive cardiomyopathy which also contributed to his death. With the exception of this patient and one other child, each of the children who died had been healthy previously. Death occurred within 1–3 days of onset of symptoms. The major factor causing death was believed to be (a) dehydration and electrolyte imbalance in 16 and (b) aspiration of vomitus in 3. In the remaining 2, seizures were a contributing factor. Each of the 16 patients tested had a sodium level (serum or vitreous humor) in excess of 150 mEq/liter; 11 of the 16 had a sodium level in excess of 160 mEq/liter. The rapidly fatal course of untreated severe rotavirus gastroenteritis was underscored by the fact that the parents of 16 of the 20 children who were brought to the hospital had some contact with a physician during the course of the illness. Language difficulties, combined with the rapid progression of fluid depletion, contributed to fatal outcome.

TABLE 5. *Clinical characteristics of 150 children hospitalized with acute gastroenteritis*[a]

Clinical finding	Percent having each clinical finding: Rotavirus infection detected (72 patients)	Rotavirus infection not detected (78 patients)
Vomiting	96[b]	58[b]
Fever		
37.9–39°C	46	29
>39°C	31	33
Total	77	61
Dehydration	83[c]	40[c]
Hypertonic	5	16
Isotonic	95	77
Hypotonic	0	6
Irritability	47	40
Lethargy	36	27
Pharyngeal erythema	49	32
Tonsillar exudate	3	3
Rhinitis	26	22
Red tympanic membrane with loss of landmarks	19	9
Rhonchi or wheezing	8	8
Palpable cervical lymph nodes	18	9

[a] From ref. 547.
[b] $p < 0.01$ (two children not included).
[c] $p < 0.01$.

Rotaviruses can produce a chronic symptomatic infection in immunodeficient children. For example, chronic diarrhea associated with prolonged shedding of rotavirus has been described in children with primary immunodeficiency or T-cell immunodeficiency (581,735). A most unusual aspect of illness in the former group was the occurrence of rotavirus antigenemia.

Rotaviruses pose a special threat to individuals who are immunosuppressed for bone marrow transplantation. In one study of gastroenteritis in a bone marrow transplant unit, eight of 78 patients (average age, 20–21 yr) shed rotavirus as the sole pathogen, and five of these individuals died (763). The serious consequences

of various viral infections (including rotaviruses) were described in another study of 12 children with severe combined immunodeficiency (318). During chronic infection in immunocompromised children, rotavirus has been observed to undergo marked changes in its genome as indicated by abnormal electrophoretic migration patterns of rotavirus RNAs (10,153,309,434,512).

A temporal association of rotavirus infection with a variety of other conditions has been presented in descriptions of isolated illnesses or single outbreaks (86, 132,133,141,175,205,233,258,262,292,318,319,377,378, 392,429,432,468,485,498,547,556,557,568,570,577,581, 592,625,626,643,659,700,702,734,743,768,776). Because the occurrence of these conditions is extremely rare in comparison to the incidence of rotavirus infection, it appears that with the exception of (a) severe disease manifestations in immunocompromised patients and (b) necrotizing enterocolitis and hemorrhagic gastroenteritis in neonates, the association of most of the reported complications is temporal and not etiologic.

DIAGNOSIS

The clinical manifestations of rotavirus illnesses are not sufficiently distinctive to permit diagnosis on this basis alone. Therefore, diagnosis requires detection of virus or viral antigen and/or demonstration of a serologic response. The epidemiologic pattern may suggest the diagnosis, but laboratory confirmation is required.

Many assays have been developed for the detection of rotaviruses in stools. Specimens from the first to fourth day of illness are ideal for virus detection; however, shedding may continue for ~9–21 days, depending on the duration of symptoms (317). Viral shedding usually coincides with the duration of diarrhea, and diarrhea can continue for 2–3 days after the cessation of viral shedding (538). Initially, direct visualization of stool material by electron microscopy was employed for rotavirus detection (49,60,340,360). It had the advantage of high specificity because rotaviruses have a distinctive morphologic appearance. The electron microscope continues to be a mainstay in the diagnosis of rotaviral diseases and is frequently used as the final arbiter when discrepancies occur with other techniques. When only a few specimens are to be examined for rotavirus, electron microscopy is the most rapid diagnostic method because fecal specimens can be stained with phosphotungstic acid (PTA) and examined directly within a few minutes of collection. Direct electron-microscopic examination of stools permits detection of rotavirus in about 90% of the virus-positive specimens (68). If the specimen is centrifuged and the pellet is examined after PTA staining, electron microscopy is as sensitive as any other method for rotavirus detection (267,427). It has the added advantage of being able to detect the non-group A rotaviruses that do not share the common group A antigen (665). Immune electron microscopy is not necessary for the detection of rotaviruses because the particle has such a distinct morphologic appearance that it can be readily identified without the use of an immune serum.

The negative-staining procedure employed appears to be critical for the detection and characterization of rotavirus particles by electron microscopy. Three strains of predominantly double-shelled strains belonging to groups A, B, or C were degraded to single-shelled particles when stained with 2% PTA above pH 5.0 for 10 sec or more, whereas 1% uranyl acetate staining (pH 4.3) consistently yielded undegraded particles (481). In addition, staining of group B rotavirus with neutral PTA for more than 10 sec resulted in the disappearance of nearly all particles. It was recommended, therefore, that 1% uranyl acetate (pH 4.3) or 2% PTA at (pH 4.5) be used for negative staining of rotaviruses. For IEM studies, PTA (pH 4.5) was superior to uranyl acetate in that it allowed clearer visualization of antibody and virus. Degradation of group B rotavirus particles with PTA staining was prevented by fixation of the specimen with 0.1% glutaraldehyde (632).

Numerous methods are available for the detection of rotaviruses in stool specimens (336,359). The method of choice in many laboratories is the confirmatory ELISA, since it is highly sensitive, does not require specialized equipment, and has a "built-in" control for nonspecific reactions (41,68,348,360,761,765, 771). A preimmunization or postimmunization goat serum is employed as the solid-phase pre-coat; the preimmunization serum acts as the control because a specimen must react to a significantly greater extent with the well-coated post-immunization goat serum in order to be considered positive. Some laboratories routinely employ other methods for virus detection, such as counter-immunoelectro-osmophoresis (CIEOP), electrophoresis of rotavirus RNA in gel, reverse passive hemagglutination assay (RPHA), or latex agglutination (140, 225, 242, 244, 268, 276, 488, 502, 525, 527, 572,573,665,666,701). Commercial kits are available for the ELISA, latex agglutination, RPHA, and RNA electrophoresis assays. Assays without confirmatory reagents may yield false-positive results (106,532,683). Recently, a self-contained enzymic membrane immunoassay (SCEMIA) was developed for detection of rotavirus from clinical samples (764). It is rapid, sensitive, specific, and inexpensive. An enzyme immunoassay has also been developed for detection of group B rotavirus and for measurement of antibodies directed against this virus (479,480,528,631,720,769).

Two recently described methods for rotavirus detection deserve special attention. With newly devel-

oped procedures, it is now possible to grow human rotaviruses directly in cell cultures (43,273,475,579, 580,695,696,726,747,749). It appears that rotavirus can be grown from at least 75% of stool specimens known to contain the virus by other test procedures. The efficiency of recovery of rotaviruses from rectal swabs using cell culture is considerably less. Growth of rotavirus in tissue culture makes possible the determination of virus serotype by neutralization assay; however, serotype can also be determined by (a) ELISA or (b) solid-phase IEM using absorbed antisera or serotype-specific (VP7) monoclonal antibodies for each of the six human rotavirus serotypes (30,39,118,120, 121,220,275,457,590,616,652,655,668,688,699). When serotype-specific monoclonal antibody is employed, it is used in a sandwich procedure with one or more polyclonal antisera. Rotaviruses may also be "serotyped" by nucleotide sequence analysis of the gene encoding VP7, since there is a high degree of conservation of sequence among rotaviruses belonging to the same serotype (245–247). Serotyping has also been accomplished by hybridization in which single gene substitution (VP7) reassortants are used as probes (185,453). Subgroup analysis is also helpful in the characterization of rotavirus isolates; almost all human subgroup I viruses are also serotype 2, whereas viruses belonging to the other three epidemiologically important serotypes bear the subgroup II antigen. Although postinfection sera from gnotobiotic calves administered a subgroup 1 or 2 rotavirus were employed initially in subgrouping assays, these sera have been replaced by subgroup-specific monoclonal antibodies (251, 601,658).

Recently, a dot hybridization assay for detection of rotaviruses was developed which is based on the *in situ* hybridization of labeled rotavirus ssRNA transcripts to heat-denatured rotavirus RNA immobilized on nitrocellulose membranes (183). The method is highly specific, exhibiting excellent concordance with other tests such as electron microscopy, RNA analysis, and ELISA (154,183). In a comparative study of ELISA and dot hybridization for the detection of rotavirus in various dilutions of fecal specimens, the dot hybridization method was 10- to 100-fold more sensitive than the confirmatory ELISA (183). The limit of detection for purified RNA by the dot hybridization procedure was 8 pg in a homologous reaction. Rotaviruses were detected with high frequency in stool specimens that were dotted in Venezuela and mailed to the United States, where hybridization was performed 1 month later. The dot hybridization technique also was efficient for detection of rotaviruses from rectal swab specimens. A variety of other techniques have also been described for the detection of rotavirus (336,359).

There are many techniques for measuring serologic response to rotavirus infection, such as IEM, CF, immunofluorescence, IAHA, ELISA, neutralization, HI, and inhibition of reverse passive hemagglutination (26, 125, 223, 257, 263, 314, 338, 352, 369, 412, 423, 424, 436, 464, 495, 499, 574, 578, 599, 738, 745, 761, 762, 770, 772, 778). The CF method is about as efficient as the other methods for detecting an antibody response in patients between 6 months and 24 months of age, but it is not as efficient in adults or in infants below 6 months of age (263,770). In these age groups, immunofluorescence, ELISA IgG, ELISA IgM, and ELISA IgA are more efficient (135,402). An ELISA IgA has been particularly effective in demonstrating serologic responses in young infants who possess passively acquired maternal IgG antibodies, since IgA does not cross the placenta (135,402). This assay may also be used for examining stools for coproantibodies and saliva for local salivary antibodies (319). With this technique, it was shown that fecal IgA antibody levels correlate well with duodenal IgA antibody levels (257). Based on observations from a clinical study in infants and children, it has been suggested that rotavirus IgA in serum reflects the immunological status of the intestine with respect to this virus (131,280,282). Early diagnosis can also be made by ELISA, which can detect a specific IgM response as early as 5 or 6 days after onset of illness.

It is now possible to measure neutralizing antibodies by plaque reduction or by inhibition of cytopathic effect in roller tube culture (164,253,273,579,580,695, 696,749). Neutralization assays yield the most meaningful information with regard to the identity of the infecting rotavirus, and they also permit a determination of serotype-specific antibody response (135). The plaque reduction neutralization assay is more sensitive than tube neutralization for detection of antibody, although the latter is slightly more efficient for detecting a seroresponse (unpublished studies). A recently developed competition–solid-phase immunoassay that measures epitope-specific immune responses to individual rotavirus serotypes has proven to be especially useful in evaluating immune responses to individual epitopes. This technique employs the test sera as the blocking reagent and uses individual monoclonal antibodies as the detecting reagent (588).

Detection of rotavirus and/or the demonstration of a serologic response to rotavirus in an individual patient does not necessarily establish an etiologic association of rotavirus with that patient's illness (308,334). This is especially so in newborns and adults who commonly undergo inapparent infection.

TREATMENT

The primary aim of treatment of rotaviral gastroenteritis is the replacement of fluids and electrolytes lost

by vomiting and diarrhea. Intravenous fluid administration has been used successfully for many years in treating dehydration from diarrhea. Because facilities for parenteral administration of fluids and electrolytes are not readily available in many parts of the world, intensive efforts have been made to evaluate the efficacy of oral fluid replacement therapy (151,564,575). In a double-blind study, oral rehydration therapy for rotavirus gastroenteritis was successful using electrolyte solutions containing either glucose or sucrose (565). In another study of oral rehydration of infantile diarrhea of mixed etiology, all of the patients fed glucose electrolyte solution and 92% of those fed sucrose electrolyte solution were treated successfully (482). The sucrose group experienced a slower correction of electrolyte abnormalities, and a larger proportion of patients required more than 24 hr of therapy. It was concluded that sucrose could be employed as a substitute for glucose but that glucose-electrolyte solutions were preferable. The standard WHO oral glucose-electrolyte formula is made by adding the following to 1 liter of water: sodium chloride (3.5 g), trisodium citrate, dihydrate (2.9 g); potassium chloride (1.5 g); and glucose anhydrous (20 g). Sodium bicarbonate (2.5 g) may be substituted for the trisodium citrate, dihydrate (2.9 g). The efficacy of oral glucose-electrolyte solutions containing either 90 mmol of sodium per liter (WHO formula as above) or 50 mmol of sodium per liter, plus additional electrolytes, has been evaluated in well-nourished children who were hospitalized with acute diarrhea of various causes, including rotavirus but excluding cholera (576). Both sodium concentrations were found to be safe and effective. After correction of the initial calculated fluid loss by oral rehydration solution, either water or fluids without added electrolytes (such as breast milk or some other form of low-solute feeding) should be administered orally in addition to the oral rehydration solution; when this regimen is used, both continued diarrheal fluid and electrolyte losses will be replaced and normal daily fluid requirements will be maintained (575, 576). Of course, if the oral rehydration regimen is not able to correct the fluid and electrolyte loss or if the patient is severely dehydrated or in shock, intravenous fluids must be given.

Human milk containing rotavirus antibodies has been used successfully for treatment of immunodeficient children with chronic rotavirus infection and illness (400,581). In contrast, colostrum or milk concentrate from cows immunized with human rotavirus was not effective for the treatment of children with acute gastroenteritis, although a decrease in the duration of virus shedding was observed (145,279). Daily oral administration of rotavirus-antibody-containing colostrum, prophylactically, appeared to exert a protective effect when an orphanage outbreak of rotavirus diarrhea occurred (145).

The efficacy of several broad-spectrum antiviral agents have been examined as inhibitors of rotavirus replication *in vitro*. In this survey, various adenosine analogues were found to have antirotavirus activity, and it was suggested that this activity resulted from inhibition of *S*-adenosylhomocysteine hydrolase, an enzyme involved in regulating methylation required for maturation of viral mRNA (370).

PREVENTION AND CONTROL

Observations made during epidemiologic and hospital-based studies throughout the world clearly indicate the need for prevention of rotavirus disease (137,316,317,342,355). The aim of a rotavirus vaccine should be to prevent severe rotavirus gastroenteritis during the first 2 years, the period when rotavirus disease is most serious (95–97,342,343,355,752). Considerable evidence from studies in animals indicates that local intestinal immunity plays the most important role in resistance to rotavirus disease. These observations suggest that the effectiveness of a rotavirus vaccine will depend, in large part, upon its ability to stimulate intestinal IgA antibodies and other forms of local immunity. The most effective means of stimulating local immunity is thought to be infection involving the local site. For this reason, most current efforts in experimental rotavirus immunoprophylaxis are directed toward the development of live attenuated virus vaccines. Although progress has been made during the past few years in developing various live virus vaccine candidates, a number of important issues have been raised concerning the most effective strategy to be pursued.

First, how effective is homotypic immunity in preventing reinfection and disease? Reinfection within the first few years of life appears to be a common event in certain settings, but it has been observed that an infant who has a severe bout of rotavirus diarrhea will most often have a mild illness during subsequent rotavirus infection (204,417,759). However, it is not known whether the reinfecting virus usually or always represents a different serotype. For this reason, widespread use of newly developed methods for (a) identifying the serotype of strains recovered from infants and children under longitudinal surveillance and (b) determining epitope-specific antibody responses, coupled with a detailed clinical description of each illness associated with rotavirus infection, are required in order to define the effectiveness and duration of homotypic immunity.

Recently, new information with regard to this ques-

tion has emerged from two longitudinal studies of naturally occurring rotavirus infections. Homotypic immunity against both infection and illness caused by a serotype 3 virus was observed in residents of an orphanage who had a pre-outbreak serum homotypic neutralizing antibody titer of ≥1:128 (103). In another study, neonates who were infected in a newborn nursery during the first 14 days of life with what appeared to be a single strain of rotavirus experienced almost 50% fewer rotavirus diarrheal episodes during the next 3 years than did a cohort of infants who were not infected with rotavirus while residents of the nursery (38% versus 85%) (46). In addition, none of the 24 children infected neonatally developed severe diarrheal illness on reinfection, whereas eight of the 20 who had not been infected neonatally developed severe diarrheal illness associated with rotavirus infection. During the 3-year surveillance period, participants in the study were infected with rotavirus strains that exhibited at least two different electropherotypes, neither of which corresponded to that of the strain responsible for neonatal infection. These strains were not identified as to serotype; however, based on observations made in other studies, it is likely that at least two serotypes were involved, since both "short" and "long" RNA patterns were observed. Possibly, the 50% protective effect against any diarrheal illness observed in the longitudinal study reflected homotypic immunity induced by infection in the nursery, whereas the absolute protective effect against severe diarrheal illness reflected both homotypic and heterotypic immunity.

If, as suspected, homotypic immunity proves to be relatively effective against diarrheal illness, the next question that must be answered involves the protective efficacy of attenuated rotaviruses. Rotavirus infection is superficial and primarily involves the epithelium of the small intestine. Experience with mutants of other viruses that cause a mucosal infection, such as influenza A virus and RS virus, indicates that attenuation is usually a correlate of decreased viral replication in the target organ, which is also the site at which infection is initiated (97). Experience with attenuated mutants of mucosal viruses also indicates that decreased replication is associated with a diminished immune response. This means that a delicate balance must be achieved between acceptable attenuation and satisfactory immunogenicity. If the protective efficacy of wild-type virus infection is marginal, it may be difficult, if not impossible, to achieve this desired balance with a mutant that grows significantly less well in the intestines.

Again, observations made during the longitudinal surveillance of children infected during the neonatal period may be instructive (29). The neonatal infections that occurred in this study appeared to be attenuated, since they were primarily asymptomatic or associated, at most, with mild diarrhea. Nonetheless, neonatal infection induced immunity to rotavirus diarrhea for at least 3 years. Thus, there is a suggestion that a naturally occurring attenuated infection can protect.

The third question that must be answered involves heterotypic immunity. How many serotypes must be included in a rotavirus vaccine in order to provide maximum protection? There are at least four epidemiologically important human rotaviruses. This means that effective coverage against disease may require a quadrivalent vaccine (serotypes 1, 2, 3, and 4). However, there is a possibility that sufficient heterotypic immunity to protect against severe rotavirus disease could be induced by one or two serotypes. Epidemiologic studies have not been helpful in settling this issue.

In gnotobiotic calves and piglets, heterotypic immunity has been demonstrated between an animal rotavirus (bovine, serotype 6) and a human rotavirus (serotype 1) (755,757,780). Calves infected *in utero* with bovine rotavirus resisted challenge with human rotavirus at birth. However, in other studies in piglets and calves, only homotypic immunity was demonstrated, although in certain instances the severity of diarrhea was reduced following heterotypic challenge (60,215,471,736,740). Whether effective heterotypic immunity to each of the four major serotypes of human rotavirus can be induced in humans by infection with an animal rotavirus remains to be determined. Generally, heterotypic immunity to viruses is more transient than homotypic immunity, and this could limit the usefulness of a monotypic "Jennerian" approach to rotavirus immunoprophylaxis (342).

Current strategies for development of a rotavirus vaccine range from cell culture cultivation of strains obtained from humans or animals to the application of recently developed molecular biologic techniques. The most extensively evaluated approach is based upon the method first used by Edward Jenner in 1798 for vaccination of humans against smallpox (386). Jenner used a live related animal virus, cowpox, to immunize humans against the virus of smallpox, variola virus. The use of this strategy for human rotavirus vaccination was considered when early studies demonstrated that human and animal rotaviruses shared a common antigen (338,339,738). Thus, infants and young children who developed illness following rotavirus infection developed a serologic response by complement fixation not only to the infecting human strain but also to various animal strains (338). The feasibility of this approach was shown during animal studies in which calves administered a bovine rotavirus (serotype 6) *in utero* were protected from illness following challenge at birth with a human rotavirus of serotype 1 (757).

A high tissue culture passage, cold-adapted, serotype 6 bovine rotavirus NCDV strain vaccine (designated RIT 4237) was developed by Smith Kline RIT and was studied extensively for reactogenicity, antigenicity, dose–response, and protective efficacy (134–136,224,269,385,586,638,705,706,708–710,712–714,717). This nonreactogenic, orally administered vaccine virus induced over 80% resistance to clinically significant diarrhea in infants and young children 8–11 and 6–12 months of age in two separate efficacy trials in Finland (705,708). In addition, vaccination of neonates appeared to modify the severity of rotavirus diarrhea during the 16-month follow-up period (714). However, it failed to induce protection against rotavirus diarrhea in trials in certain developing countries; as a result, it was withdrawn from further clinical evaluation (136,269).

A related serotype 6 bovine rotavirus strain designated WC3, developed by the Wistar Institute, was evaluated during a clinical trial in Philadelphia in 3- to 12-month-old infants (109,111). Its protective efficacy during an outbreak period when serotype 1 strains were predominant was shown to be 76% against any rotavirus diarrhea and 100% against moderate to severe rotavirus diarrhea. Currently, more extensive studies are underway with this and related vaccines (113).

Another animal rotavirus strain, rhesus rotavirus (RRV) strain MMU 18006, which is antigenically similar to human rotavirus serotype 3 by neutralization, is also under intensive study (12,107,108,184,190,192,341–346,349,353,401,402,516,535,536,638,709,711,722,744,753,754). The RRV vaccine has been evaluated in both developed and developing countries and appears to be a promising candidate. Although in some settings at the 10^4 PFU dose it induces a mild, transient, febrile response in about one-third of infant vaccinees, it appears to be more antigenic than the other attenuated strains as determined by the frequency of serum antibody response following vaccination (190,709). However, the RRV vaccine has proven to be nonreactogenic in neonates (184). This may be of some importance because vaccination soon after birth may be necessary in developing countries, where immunization is needed most, because 10–40% of neonates do not have contact with a health care provider beyond the newborn period (265).

In a field trial in 1- to 10-month-old infants in Venezuela, the vaccine's overall efficacy rate was 64% against any rotavirus diarrhea (192). Moreover, in the 1- to 4-month age group, vaccine efficacy was 82% against any rotavirus diarrhea, whereas its efficacy was 90% against the more severe illnesses encountered in the entire study group. Results of efficacy trails were also encouraging in Maryland, Sweden, and Finland, but the vaccine failed to protect 2- to 4-month-old infants in Rochester or 2- to 5-month-old infants in Arizona (107,240,341,345,346,576a,711). Variability of protective efficacy was difficult to understand until the strains collected during various trials were serotyped. The predominant strains responsible for disease in the Venezuelan study were serotype 3, the same serotype as the vaccine strain, whereas in the Rochester study almost all the strains recovered from ill infants were serotype 1; moreover, in the Arizona study almost all typed strains belonged to serotypes other than serotype 3. These observations suggest that serotype-specific immunity is required for protection against rotavirus diarrhea in young infants not primed by previous rotavirus infection (402). Protection against heterotypic strains in Finland and Sweden was observed in older infants and young children who may have been primed by previous rotavirus infection (240,711). Nonetheless, the effectiveness of a rotavirus vaccine will probably be judged by its ability to induce, in very young infants, protective antibodies to all of the epidemiologically important rotaviruses.

Failure of the RRV vaccine to induce protection in young infants against heterotypic rotavirus strains suggested that the strategy for development of an RRV vaccine required modification. As a consequence, the Jennerian approach to rotavirus immunoprophylaxis was modified to include the development of a quadrivalent vaccine that incorporated the VP7 specificity of each of the four epidemiologically important serotypes coupled to the attenuation phenotype of rhesus rotavirus (341,345,346,454,455,514). This approach took advantage of (a) the segmented genome of rotaviruses and (b) the ability of these viruses to undergo gene reassortment with high efficiency during coinfection (230,250,255,420). Virus reassortants were isolated from tissue cultures coinfected with a cultivatable, wild-type animal virus (rhesus rotavirus or bovine UK rotavirus) and a "noncultivatable" human rotavirus (i.e., a human rotavirus that had not been adapted to cell culture, except for serotype 4) (454,455). Monospecific antiserum directed against the animal rotavirus parent or a set of monoclonal antibodies directed against the VP7 of the animal rotavirus parent was used to select for virus reassortants with human rotavirus VP7 neutralization specificity. Using this selective pressure, it was possible to isolate reassortants that derived only the gene segment coding for VP7 from the human rotavirus parent, whereas the remaining 10 genes were derived from the animal rotavirus parent. In this manner, single human rotavirus gene substitution reassortants were isolated which possessed the human rotavirus gene for VP7 of serotype 1, 2, 3, or 4 on a background of 10 animal rotavirus genes. As expected, these reassortants exhibited the neutralization specificity of the human rotavirus parent (454,455). Since RRV belongs to serotype 3, it was not

necessary to prepare a RRV–human rotavirus serotype 3 reassortant. Phase 1 studies of the individual RRV–human rotavirus reassortants have been completed (190,264,341,342). Each of the reassortants was observed to be similar to its RRV parent with respect to attenuation and immunogenicity. Currently under evaluation is a quadrivalent rotavirus vaccine formulation containing single VP7 gene substitution human–RRV reassortants with serotype 1, 2, or 4 specificity and also containing RRV itself as the serotype 3 representative (514). The quadrivalent vaccine is as safe and as attenuated as its RRV parent. Various dosage schedules are now being evaluated for antigenicity for each serotype.

Another approach to rotavirus immunoprophylaxis involves the use of a neonatal human rotavirus strain that appears to be naturally attenuated. The impetus for evaluating this strain comes from the observation that neonates who have experienced a subclinical rotavirus infection in a nursery during the first 14 days of life were protected against clinically significant rotavirus diarrhea for up to 3 years. The neonatal rotavirus strain appeared to be satisfactorily attenuated and immunogenic during a phase 1 trial in infants (K. Midthun et al., *unpublished observations*). A cold-adapted strain of a human serotype 1 virus is also under consideration as a vaccine candidate (426).

Other approaches to immunization are also possible. Among these is the use of (a) synthetic viral proteins or (b) viral proteins expressed by cloned rotavirus cDNA (13–15,163,195,203,391,433,562,595,627). As noted earlier, the genes encoding the VP7 and VP4 of a number of rotavirus strains have been cloned, and full-length cDNA is available for expression of the outer capsid protein VP7 or VP4 in prokaryotic or eukaryotic cell systems. For example, 45% of VP4 (amino acids 42–387) has been expressed as part of a chimeric protein in *Escherichia coli*. However, the level of neutralizing activity induced by this protein was only one-twentieth of the activity stimulated by the virus (14,15). Thus, it is unlikely that proteins produced in this manner will stimulate an effective primary local intestinal immune response, especially if the antigen is administered by a parenteral route. However, parenteral inoculation of such a protein might prove useful in priming an immune response if administered before a live attenuated virus vaccine, or it might serve to boost an immune response if given after a live virus vaccine (159,640,641).

Finally, passive immunization has been shown to be effective in preventing rotavirus illness in animals (27,76,445,492,494,594,611,612,739). Similar studies have now been carried out in infants and young children with promising results (23,145). Various sources of passively administered antibodies (such as eggs or raw or pasteurized milk) have been suggested (766,767). However, except for special circumstances as in immunocompromised individuals, passive immunization is not practical for protection against rotavirus illness, since it requires repeated administration of antibody.

It is apparent that major advances have been made in the quest for a rotavirus vaccine. Available vaccines have yielded encouraging signals that rotavirus disease can be prevented or modified. If such a vaccine is developed, several major issues remain to be addressed, such as the optimal dosage and route of administration, the optimal age for administration, the effect of lactation and acid pH of stomach acid on vaccine "takes" in an infant, the need for methods to enhance local immune response, the feasibility of immunization of pregnant women to enhance the level of rotavirus antibodies in breast milk, the possible emergence of new serotypes in nature, the effect of a vaccine on malnutrition and on mortality in developing countries, and, ultimately, the effect of a rotavirus vaccine on dual rotaviral bacterial or rotaviral infections, as well as the delivery of vaccine into areas of the world where it is most needed.

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CHAPTER 24

Norwalk Group of Viruses

Albert Z. Kapikian and Robert M. Chanock

The 27-nm Norwalk virus is a major cause of epidemic viral gastroenteritis, a disease that occurs in family or community-wide outbreaks and predominantly affects school-aged children, adults, and family contacts (13,18,43,47,101,106,107,210,215). Gastroenteritis is an important public health problem in both developed and developing countries. Infectious gastroenteritis was the second most common disease recorded in the Cleveland Family Study among some 25,000 illnesses over a 10-year period, accounting for 16% of the illnesses studied (46). Undoubtedly, the Norwalk virus or a related virus was responsible for a substantial portion of these illnesses. However, the Norwalk virus is not associated with severe viral diarrheal illnesses of infants and young children (13,22,43,79,124,192); these have been linked predominantly with the 70-nm rotaviruses described in Chapter 49. Thus, these two distinct groups of agents (namely, Norwalk and related viruses and the rotaviruses) are of major importance as etiologic agents of infectious gastroenteritis, and each has its own unique epidemiological characteristics.

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HISTORY

Bacteriologic and parasitologic discoveries made during the past century resulted in elucidation of the etiology of a small portion of diarrheal episodes, leaving a major segment that could not be associated with any etiologic agent. During the 1940s and 1950s, it was assumed by exclusion that viruses were responsible for the major portion of these illnesses. Because viruses responsible for acute enteric disease could not be propagated *in vitro*, volunteer studies were initiated in order to determine whether bacteria-free stool filtrates derived from outbreaks of gastroenteritis would induce

illness; if they did, intensive efforts could be initiated to isolate the agent from the infectious fecal filtrate.

In 1945, Reiman et al. (177) reported that enteric illness could be induced in volunteers by aerosolization of bacteria-free throat washings or fecal suspensions derived from patients with gastroenteritis. Illness was not produced by oral administration of throat washings or fecal suspensions. Two years later, Gordon et al. (69) described the induction of an afebrile diarrheal illness in volunteers who were fed bacteria-free fecal filtrates or throat washings from patients with gastroenteritis. This infectious inoculum was derived from pooled diarrheal stools obtained from two individuals who became ill during an outbreak of gastroenteritis at Marcy State Hospital near Utica, New York. This agent, designated the *Marcy strain*, was successfully passaged serially seven additional times in volunteers, indicating that it had multiplied *in vivo* and was not merely a filterable toxin (71). Both short-term (several weeks) and longer-term (9–15 months) immunity to this agent were demonstrated by homologous rechallenge (69,70,72).

During this same period, Japanese investigators also attempted to determine the etiology of nonbacterial gastroenteritis. In 1948, Kojima et al. (122) induced gastroenteritis in volunteers by administering bacteria-free fecal filtrates derived from cases of diarrhea in the Niigata Prefecture and other districts. Serial passage was successful, and short-term immunity was demonstrated following rechallenge. In the same year, Yamamoto et al. (217) described the induction of diarrheal illness in volunteers (and cats as well) who were fed bacteria-free fecal filtrates derived from a gastroenteritis epidemic in the Gumma Prefecture. In 1957, Fukumi et al. (64) established a relationship between the Niigata Prefecture strain and the Marcy strain by cross-challenge studies in volunteers. Initially, intraduodenal administration of the Marcy strain induced diarrheal illness. Two months later, challenge with the Niigata strains by the same route did not induce illness, suggesting that the two strains were antigenically related.

In 1953, Jordan et al. (98) induced a febrile gastroenteritis in volunteers who were fed a bacteria-free fecal filtrate derived from a participant in the Cleveland Family Study who developed gastroenteritis. The agent was designated the *FS strain* and was passaged serially in volunteers. Cross-challenge studies with the Marcy and FS strains revealed that they were distinct. Also, the incubation period of these two agents, as well as their clinical presentation, were different.

During the course of the aforementioned studies, fecal specimens were identified that contained filterable agents capable of inducing acute gastroenteritis. In many instances, these specimens were studied intensively in the laboratory in an attempt to detect an etiologic agent, presumably a virus. Despite extensive use of the tissue culture techniques developed during the 1950s, a specific etiologic agent was not identified.

This historical description of viral gastroenteritis has focused on that form of the disease which characteristically occurs in outbreaks in communities, schools, or families and affects school-aged children and adults. Concurrent studies of the etiology of nonbacterial infantile diarrhea also failed to reveal an etiologic agent as described in Chapter 49.

By the beginning of the 1970s, the etiology of most community outbreaks of gastroenteritis still could not be established (218). This was particularly frustrating to virologists because it was assumed that the nonbacterial gastroenteritides were most likely caused by viruses. Also, during the preceding 20 years, which represented the beginning of the tissue culture era, more than a hundred new cultivatable viruses were discovered; many of these grew to high titer in the enteric tract, but not one of the newly recognized "enteric viruses" could be implicated as an important etiologic agent of infectious gastroenteritis (35,37, 99,158,215,218).

During the late 1960s, new techniques were developed for detection of fastidious viruses. These techniques included the use of organ culture, which preserved cells in their normal state of differentiation and architecture. The first human coronavirus was recovered in human embryonic tracheal or nasal organ cultures (206). This success in growing the fastidious coronaviruses stimulated renewed efforts to cultivate the heretofore elusive etiologic agents of "viral" gastroenteritis. These attempts, which employed the newer methods of organ culture as well as standard tissue culture techniques, also failed to yield an etiologic agent. Although the specimens tested in these studies came from patients with nonbacterial gastroenteritis, the infectivity of these fecal samples had not been demonstrated by transmission of disease to volunteers. Thus, failure to test proper specimens might have been responsible for failure to detect viruses responsible for gastroenteritis. Fecal suspensions of known infectivity containing the Niigata, Marcy, or FS agents could not be used for this purpose because these virus-containing materials had been exhausted or were not available at that time. For this reason, volunteer studies were initiated to identify fecal suspensions that contained infectious agents capable of inducing acute gastroenteritis and that could be used in an intensive effort to identify viral enteric pathogens (21).

Specimens collected during a gastroenteritis outbreak in Norwalk, Ohio were of particular interest. This outbreak, which was investigated by the Centers for Disease Control (CDC), began in October 1968 (1). During a 2-day period, acute gastrointestinal illness developed in 50% of 232 students and teachers of an

elementary school, and a secondary attack rate of 32% was observed among family contacts of primary cases. The disease lasted about 24 hr and had an incubation period of approximately 48 hr and was designated *epidemic vomiting disease* because it resembled this syndrome first described by Zahorsky in 1929 (219). Although some of the patients had diarrhea, the predominant clinical manifestations were vomiting and nausea (1). An intensive laboratory study of clinical specimens from the Norwalk outbreak failed to reveal an etiologic agent. A bacteria-free filtrate was prepared from a rectal swab specimen obtained from a secondary case of gastroenteritis, and subsequently this filtrate was administered orally to three volunteers (50). Two of these individuals developed gastroenteritis. The agent was serially passaged to other volunteers and induced a gastroenteric illness in approximately 50% (49). Although stool filtrates containing an infectious agent were identified, all attempts to cultivate the agent in cell or organ culture or to transmit it to various experimental animals were unsuccessful (21,49,50).

The "W" agent, derived from an outbreak of illness characterized by vomiting, fever, and mild diarrhea which occurred in a boys' boarding school in the United Kingdom, was identified by transmission in volunteers shortly after the Norwalk agent was transmitted to volunteers in the United States (34). Attempts to propagate the "W" agent in cell or organ cultures were also unsuccessful.

Subsequently, in 1972, Kapikian et al. (108) discovered virus-like particles in fecal material derived from the Norwalk outbreak. These particles were 27 nm in their shortest diameter and 32 nm in their longest diameter (referred to as 27-nm particles) (Fig. 1). This was achieved by application of the technique of immune electron microscopy (IEM), which involves the direct observation of antigen–antibody interaction by electron microscopy (3,4,11,103). IEM was described for the first time in 1941 but did not achieve its fullest potential for many years (4,11). The IEM technique has been particularly useful in visualizing viruses that are present in low concentration in feces, because antibodies aggregate virus particles and thus act to concentrate such viruses. Antibodies also aid in identification of virus particles by producing a characteristic antibody coating of particles (103).

Spherical particles, 27 nm in diameter, were visualized in a fecal filtrate with known infectivity which was derived from a volunteer who developed gastroenteritis following oral administration of bacteria-free fecal material obtained from one of the two volunteers who had developed illness in the first Norwalk challenge study (108). This was achieved by incubating the fecal filtrate, which had induced gastroenteritis in 6 of 10 volunteers following oral administration, with convalescent phase serum from one of these six volunteers. Following centrifugation of the mixture, the sediment was examined by electron microscopy (EM), and 27-nm particles coated with antibody were observed. The IEM technique was then adapted to the detection and measurement of antibodies that reacted with the 27-nm particle. A serological response was observed when paired sera from four of the ill volunteers were tested by IEM. Certain individuals who developed illness during the original Norwalk outbreak also developed a seroresponse to the 27-nm particle by IEM. In each of these instances, convalescent-phase serum from certain naturally or experimentally infected individuals contained significantly more antibody detectable by IEM than did acute-phase or preinfection serum. These observations and the temporal pattern of virus shedding suggested that the 27-nm particle was the etiologic agent of the Norwalk outbreak (108,202).

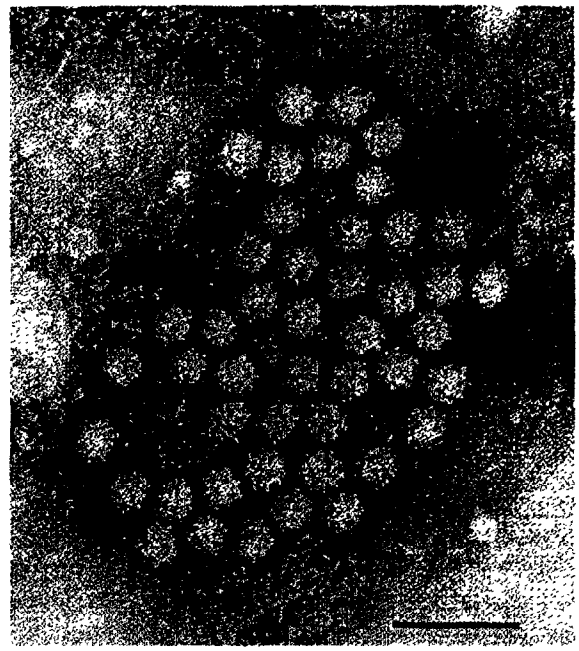


FIG. 1. An aggregate observed after incubation of 0.8 ml of Norwalk (8FIIa) stool filtrate with 0.2 ml of a 1:5 dilution of prechallenge serum of a volunteer and further preparation for electron microscopy. The quantity of antibody on these particles was rated as 1+. Bar = 100 μm. (From ref. 108 with permission.)

The IEM method used for the discovery of the fastidious noncultivatable 27-nm Norwalk particle (and later the 27-nm hepatitis A virus as well) bypasses (by necessity) tissue culture and experimental animals for virus detection (61,102,103,109). Instead, specimens are examined directly by EM, and distinctive virus particles that react with antibodies in convalescent-phase serum are sought. Moreover, this method extends beyond virus detection because it can be used for further

characterization of the particle by serological, epidemiological, and biophysical techniques.

INFECTIOUS AGENTS

Classification

The Norwalk virus is the prototype strain of a group of fastidious 23- to 34-nm nonenveloped viruses associated with outbreaks of gastroenteritis. These agents share certain characteristics: (a) They were detected in feces of gastroenteritis patients; (b) they cannot be cultivated in cell cultures by standard techniques; (c) their nucleic acid content is not known; (d) they have a buoyant density of 1.36–1.41 g/cm^3 in CsCl; and (e) they lack a distinctive morphologic appearance by EM (7,10,13,28,29,43,53,79,95,104,108, 109,160,203). Table 1 lists various members or possible members of this group along with their properties. The possible members have similar properties, except their density differs or has not been reported.

The antigenic relationships among these viruses have been evaluated primarily by IEM, employing sera from infected individuals and antigens present in fecal suspensions. There appear to be at least four distinct serotypes: the Norwalk, Hawaii, Ditchling (or W) and Snow Mountain viruses (Table 1) (7,53,103,108,146, 212). The cockle agent is distinct from Norwalk virus, but its relationship to the other serotypes is not known (10). The Taunton agent has not been tested serologically against any of the established serotypes (28,29), and the Parramatta agent has only been shown to be distinct from Norwalk virus (33).

The Norwalk group of viruses has not yet been classified into any family of viruses because essential information such as their nucleic acid content is lacking. Initially, it was suggested that the Norwalk virus was parvovirus-like on the basis of its size, morphology, acid, ether, heat stability, and buoyant density (49,104,108). However, studies of the polypeptides of Norwalk virus indicate that it is not parvovirus-like. Rather, it appears to be "calicivirus-like" because it

TABLE 1. *Characteristics of Norwalk virus, Norwalk-like viruses, and other possibly related agents identified in specimens from patients with acute epidemic nonbacterial gastroenteritis*[a]

Virus [initial source and ref.]	Size (nm)	Density in CsCl (g/cm^3)	Growth in cell culture	Administration[b] of virus induces illness in		Particle detected by	Serologic responses detected by	Antigenic relationships
				Humans	Animals			
Norwalk [elementary school GE outbreak in USA (49, 50,81,93,94,104,105,108, 212)]	27 × 32[c]	1.38–1.41	No	Yes	No	IEM, RIA, IAHA, ELISA	IEM, RIA, IAHA, ELISA	Distinct
Hawaii [family GE outbreak in USA (51,108,203,212)]	26 × 29[c]	1.37–1.39	No	Yes	No	IEM, ELISA	IEM, ELISA	Distinct
Montgomery County (MC) [family GE outbreak in USA (108,203,212)]	27 × 32[c]	1.37–1.41	No	Yes	No	IEM	IEM	Related to Norwalk virus by IEM and cross-challenge studies
Ditchling [primary school GE outbreak in England (7)]	25–26	1.38–1.40	No	NT	No	EM	IEM	Ditchling and "W" agents related to each other but appear to be distinct from Norwalk and Hawaii viruses by IEM
"W" (Wollan) [boys' boarding school GE outbreak in England (7,34,103)]	25–26	1.38–1.40	No	Yes	NT	EM	IEM	
Cockle [GE outbreak after ingestion of cockles in England (10)]	25–26	1.40	No	NT	NT	EM	IEM	Appears distinct from Norwalk virus by IEM
Taunton (UK) [hospital GE outbreak in England (28,29,137)]	32–34	1.36–1.41	No	NT	NT	EM	IEM	Distinct from a human astrovirus by IEM
Parramatta [primary school GE outbreak in Australia (33)]	23–26	NT	No	NT	NT	EM	IEM	Distinct from Norwalk agent by IEM
Snow Mountain (Colorado) [waterborne GE outbreak in resort camp in USA (25,53,54,146,160,168)]	27–32	1.33–1.34	No	Yes	NT	IEM, RIA, ELISA	IEM, RIA, ELISA	Distinct from Norwalk, Hawaii, and Marin County agents by IEM or RIA

[a] ELISA, enzyme-linked immunosorbent assay; GE, gastroenteritis; IEM, immune electron microscopy; IAHA, immune adherence hemagglutination; NT, not tested; RIA, radioimmunoassay.
[b] Alimentary route.
[c] Shortest × longest diameter.

has a single primary virion-associated protein with an estimated molecular weight of 59,000 daltons (76,79). In addition, a single soluble protein of 30,000-dalton molecular weight is present in stools of infected individuals (76,79). It cannot be considered a parvovirus because the latter have three structural proteins with molecular weights ranging between 60,000 and 85,000 daltons (183). In addition, it is not a picornavirus because these viruses have four structural polypeptides with molecular weights ranging from 8,000 to 35,000 (36). The caliciviruses, however, have a single structural protein with a molecular weight of about 65,000 daltons (185). Some caliciviruses also have a small virion-associated protein of 15,000-daltons molecular weight and a nonstructural virus-associated protein with a molecular weight of 29,000 daltons (185). It should be noted that the Norwalk virus bears some resemblance morphologically to the caliciviruses and has a similar buoyant density in CsCl as described below. Further evidence that the Norwalk virus may indeed be a calicivirus is provided by studies of paired sera from individuals with serologic evidence of infection with a human calicivirus: 12 of 20 patients with gastroenteritis associated with human calicivirus UK4, as well as two of eight with gastroenteritis associated with human calicivirus UK2, developed a seroresponse by radioimmunoassay (RIA) to Norwalk virus (39). A relationship could not be shown by IEM, which probably measures serotype-specific antigens, whereas the RIA likely recognizes a group antigen. Therefore, definitive classification of the Norwalk group of agents is not possible at this time.

Comparison Between the Norwalk Group of Viruses and Other Small, Round Particles Detected in Feces of Patients with Gastroenteritis

The Norwalk virus is a nonenveloped circular particle that averages 27 × 32 nm in its shortest and longest diameters (Fig. 1) (108). A definite substructure is not readily apparent, although there is a suggestion of small indentations on its surface (79). These indentations are not as pronounced as the cup-like depressions of conventional caliciviruses (39,185). Because in most instances antibodies are required for the concentration and visualization of Norwalk virus, it is impossible that certain features of virus surface substructure are obscured by antibody. In addition, the Norwalk virus characteristically has a somewhat indistinct rough outer edge; whether this is a result of the attachment of antibody or is a feature of its surface structure is not certain.

An interim scheme of classification of small, round, fecal viruses has been proposed based primarily on their morphological appearance (28). The viruses, which were all examined in the same laboratory under similar conditions, were divided into two main groups: featureless and structured. The former included viruses that had a smooth outer edge without a defined surface structure. The latter included those with a defined surface structure and/or a ragged edge. The featureless strains included cocksackie B5 (a picornavirus), hepatitis A (a picornavirus), mink enteritis (a parvovirus), and the gastrointestinal viruses "W" (Wollan), Ditchling, cockle, and Parramatta, which were considered to be possible parvoviruses. The structured particles included the Norwalk, Hawaii, Taunton, astro-, calici-, and Harlow viruses. The Norwalk, Hawaii, and Taunton viruses comprised a subgroup designated *small, round, structured viruses*; these were readily distinguishable from the astroviruses (which exhibit a five- or six-pointed surface star that is stained in its center) and the calciviruses (which have a six-pointed surface star with a hollow at the center, along with surface cup-like depressions) (28,140). It is of interest that the Harlow virus, which did not have the typical astrovirus surface structure, was subsequently identified as an astrovirus by IEM and by examination of infected cell cultures by the immunofluorescence (IF) technique (7,8,28).

Table 2 summarizes the major differences between the Norwalk virus (and its related viruses) and other small, round, virus-like particles that have been detected in stools of infants and young children with acute gastroenteritis. Astroviruses, which have a distinctive appearance by EM, have characteristically been detected in stools of infants and children with mild gastroenteritis (12,55,118,130,142,144,145). Astroviruses have a positive-strand RNA genome (127). The electrophoretic mobility pattern of their polypeptides suggests that they belong to the picornavirus family, since preliminary evidence indicates that they possess four polypeptides with molecular weights ranging from 26,500 to 52,000 (127,130). The buoyant density of human astroviruses has been reported variably at 1.33–1.34, 1.35–1.37, or 1.38–1.42 g/cm^3 in CsCl (123,127,130,139). Currently, five distinct serotypes, each of which can be grown in cell culture, are recognized by IF and immunosorbent electron microscopy with serotype 1 strains predominant (92,127–130, 133,135). The Marin County virus, derived from an outbreak of gastroenteritis in a convalescent home for the elderly and initially considered to be a Norwalk-like virus, is now classified as *astrovirus type 5* (91,92,157,168). Astroviruses are only rarely associated with disease serious enough to require hospitalization (22,56,124). Astroviruses have been associated with outbreaks of mild gastroenteritis in a newborn nursery, a kindergarten, and a pediatric ward (123,132). In the the latter two instances, adult contacts were also affected. In addition, astroviruses

TABLE 2. *Comparison of Norwalk group of viruses with other small, round particles detected in feces from patients with acute gastroenteritis*[a]

Virus or virus-like particle	Size (nm)	Morphology by EM (negative stain)	Genome nucleic acid	Viral protein(s)	Density in CsCl (g/cm³)	Growth in cell culture	Evidence of infection of humans by seroresponse (type) or transmission (T)	Implicated as important cause of severe infantile gastroenteritis	Implicated as important cause of epidemic gastroenteritis of children and adults
Norwalk virus[b] (43,104,106,108, 109,212)	27	Spherical, edge not sharply defined; suggestion of surface indentations	Not known	Single structural polypeptide (58–62 kd)	1.33–1.41	No	Yes (EM, RIA, ELISA, IAHA, T)	No	Yes
Astroviruses (90,123,127, 128,130,131, 133,134, 139–142)	28–30	Five- or six-pointed surface star without a central hollow (10% of particles); triangular surface hollows; smooth circular edge	RNA	Four polypeptides (26.5–52 kd)	1.33–1.34, or 1.35–137, or 1.38–1.42	Yes	Yes (IEM, IF, T)	No	No
Calicivirus (31,32,140,143, 163,196)	30–40	Six-pointed surface star with central hollow; circular oval surface hollows; scalloped feathery outer edge	RNA	Single structural polypeptide (62 kd)	1.37–1.40	Yes	Yes (IEM, RIA)	No	No
Minireovirus (156,193)	30	Spherical; some particles have double capsid; others appear as rim-like structures with external projection 2–3 nm long	Not known	Not known	Not known	No	No	No	No
Otofuke agent (and antigenically related Sapporo agent; a morphologically similar virus, Osaka agent, has also been detected (166,199)	33–40	Spherical with surface projections; 20 round or rod-shaped capsomers seen by rotational enhancement	Not known	Not known	Not known	No	Yes (IEM)	No	No
SRVs (other than Norwalk group) (6, 16,26,27,60,62,121, 156,171–174,216)	20–30	Round particles with smooth edge; surface structure not distinctive	Not known	Not known	Not known	No	No	No	No

[a] EM, electron microscopy; IEM, immune electron microscopy; IF, immunofluorescence; RIA, radioimmunoassay; ELISA, enzyme linked immunosorbent assay; SRVs, small, round viruses.
[b] See Table 1 for other members or possible members of Norwalk group.

have been associated with acute gastroenteritis in the elderly in a nursing home and on a geriatric ward (42). Antibody prevalence studies indicate that >70% of children acquire astrovirus serum antibody by 5 years of age (128). Astrovirus infection was induced in 13 of 16 adult volunteers, but only one person developed a diarrheal illness (131). Astroviruses have also been associated with (a) diarrhea in lambs, dogs, cats, and turkeys and (b) hepatitis in ducks (73,96,130,149,150, 152,190,191,208). The lamb astrovirus is not related antigenically to human or calf astrovirus (190,191). The calf astrovirus does not induce illness in gnotobiotic calves, whereas the lamb astrovirus induces diarrhea in gnotobiotic lambs (74,191,211). The lamb astrovirus contains single-strand RNA and has two major capsid polypeptides (90).

Caliciviruses are icosahedral viruses with a positive-strand RNA genome which have a single structural polypeptide of 62-kd molecular weight and possess a distinctive appearance when viewed by EM (140,185). At least four distinct strains of human calicivirus have been identified (39). In addition, a one-way serologic relationship with the Norwalk virus has been observed by RIA but not by IEM (39). Particles with classic calicivirus morphology have been associated with pediatric gastroenteritis (31,38,41,63,143,153,154,156, 193,196), but these agents are only rarely associated with severe infantile gastroenteritis requiring hospitalization. However, they were detected in the small bowel of a 22-month-old child who died of acute gastroenteritis (63). They have also been detected in outbreaks of gastroenteritis in (a) a nursery housing infants and very young children, (b) children in a primary school, and (c) children attending day-care centers (31,41,153,154). Serologic evidence of infection was demonstrated by IEM. Calicivirus-like particles have also been associated with outbreaks of gastroenteritis in homes for the elderly (38,42,75). During a survey of 647 pediatric patients hospitalized with gastroenteritis in Japan, calicivirus-like particles were visualized in the stools of 1.2% of individuals tested (196). Antibody prevalence studies demonstrate a rapid acquisition of

serum antibody to a calicivirus-like agent by 5 years of age (40,162,165,182). A bovine calicivirus-like particle (Newbury agent) that induced diarrhea, villous atrophy in the small intestine, and decreased D-xylose absorption in gnotobiotic calves has also been described (24,211). Calicivirus-like particles have also been detected in piglets (181). A RIA for a human calicivirus has been developed (163); serum antibody measured by this assay was found to correlate with resistance to illness in infants and young children (164).

In Japan, the 34- to 48-nm Otofuke agent, which has a density of 1.35–1.37 g/cm^3 in CsCl, was associated with an outbreak of mild gastroenteritis in a work training center for mentally deficient adolescents and adults (199). As with the calicivirus-like agents, serologic evidence of infection was detected by IEM. Virus-like particles (Sapporo agent) 33–39 nm in size were associated with an outbreak of gastroenteritis in infants and young children in an orphanage (120). The Sapporo agent, which has a density of 1.37–1.40 g/cm^3 in CsCl, was shown by IEM to be antigenically related to the Otofuke agent but distinct from Norwalk, Hawaii, and W agents as well as a human enteric calicivirus-like particle (120). The 35- to 40-nm Osaka agent, which has been associated in Japan with sporadic infantile gastroenteritis and with outbreaks of gastroenteritis in a nursery and in primary schools, resembles the Otofuke agent by EM (166).

The 30- to 32-nm "minireoviruses" have been detected in pediatric patients with nosocomial gastroenteritis as well as gastroenteritis requiring hospitalization (156,178,193). "Small, round viruses" (SRVs) 20–30 nm in diameter have also been detected in stools of some pediatric patients with gastroenteritis, but their significance is not clear because definite evidence of infection was not obtained (5,6,16,26,27,156,167,171–174,178,188,216). One SRV associated with gastroenteritis in infants and young children was shown by IEM to be antigenically related to the Sapporo agent (60,121). A recent analysis of SRVs observed over a 4-year period indicated that most were misclassified, since many could be categorized into known groups such as parvovirus-like and astrovirus (167).

Although astroviruses, calicivirus-like particles, Otofuke agent, minireoviruses, and SRVs (other than Norwalk and related agents) have been detected in stools of patients with acute gastroenteritis, none of these viruses or virus-like particles appears to be an important cause of either sporadic pediatric gastroenteritis or epidemic gastroenteritis that affects both children and adults. Further studies are needed to place these agents in proper perspective as etiologic agents of acute gastroenteritis (13,16,43,100,106,170).

The roles of rotaviruses and enteric adenoviruses as etiologic agents of viral diarrhea are described in Chapters 49 and 61.

Physicochemical Properties of Norwalk Viruses and Related Viruses

With the exception of the less dense Snow Mountain virus, the buoyant density of the Norwalk group of viruses ranges from 1.36 to 1.41 g/cm^3 as determined by the distribution of particles in a $CsCl_2$ gradient (Table 1) (7,10,28,34,104,146,147,203). The Norwalk virus retained infectivity for volunteers following exposure to pH 2.7 for 3 hr at room temperature, and both the Norwalk and "W" agents were stable following treatment with 20% ether at 4°C for 18 or 24 hr (34,49). In addition, the Norwalk virus retained infectivity for volunteers after it had been incubated at 60°C for 30 min (49). Norwalk virus is resistant to inactivation following treatment with 3.75–6.25 mg of chlorine per liter (free residual chlorine of 0.5–1.0 mg/liter), a chlorine concentration consistent with that which might be present in a drinking water distribution system (117). However, Norwalk virus is inactivated following treatment with 10 mg of chlorine per liter, a concentration that is used to treat a water supply system after contamination has been detected. It should be noted that Norwalk virus was more resistant to inactivation by chlorine than was poliovirus type 1, human rotavirus (Wa), simian rotavirus (SA11), or f2 bacteriophage (117).

Norwalk virus and Norwalk soluble antigen have been purified from fecal specimens obtained from infected volunteers (76,79). When these purified materials are immunoprecipitated by convalescent-phase serum antibodies and are then dissociated and examined by polyacrylamide gel electrophoresis, a single virion-associated protein and a single soluble protein with estimated molecular weights of 59,000 and 30,000 daltons, respectively, are resolved. In addition, one major structural protein with an estimated molecular weight of 62,000 has been identified in the virion of the Snow Mountain agent (147). The single virion-associated protein of the Norwalk and Snow Mountain viruses suggests that these agents are related to the caliciviruses (201).

Propagation and Assay in Cell Culture

Despite intensive efforts, none of the Norwalk group of viruses has been propagated in cell culture or in human embryonic intestinal organ culture (7,10,21,33,34,49,109).

Infection of Experimental Animals and Host Range

Numerous attempts have been made to induce illness in experimental animals. The Norwalk virus failed to induce illness following introduction into the alimentary tract of mice, guinea pigs, rabbits, kittens, calves, chimpanzees, baboons, rhesus monkeys, marmosets, owl monkeys, patas monkeys, or cebus monkeys (21,49,50,81,213,214; R.G. Wyatt et al., *unpublished studies*). However, chimpanzees inoculated with Norwalk virus developed a seroresponse as detected by IEM and RIA (81,213). In addition, inoculated chimpanzees shed soluble Norwalk antigen, which was detectable by RIA (81).

PATHOGENESIS AND PATHOLOGY

Biopsies of the jejunum of volunteers who developed gastrointestinal illness following challenge with the Norwalk or Hawaii agent exhibited histopathological lesions (2,51,186,187). When viewed by light microscopy, there was broadening and blunting of the villi of the proximal small intestine, although the mucosa itself was histologically intact (Fig. 2). Infiltration with mononuclear cells and cytoplasmic vacuolization were also observed. When viewed by transmission EM, the epithelial cells were intact but there was shortening of the microvilli. Biopsies obtained during the convalescent phase of illness were normal. Neither virus was detected by EM in epithelial cells of the mucosa. It is of interest that the characteristic jejunal lesion has also been observed in volunteers who were fed Norwalk or Hawaii virus but who did not become ill (155,186,187). Histologic lesions were not observed in the gastric fundus, antrum, or rectal mucosa of volunteers with Norwalk virus-induced illness (207).

A transient malabsorption of fat, D-xylose, and lactose was observed during experimentally induced Norwalk virus illness (21,186). Levels of small intestinal brush-border enzymes (trehelase and alkaline phosphatase) were significantly decreased when compared to baseline and convalescent-phase values, whereas adenylate cyclase activity in the jejunum was not elevated following Norwalk or Hawaii virus-induced illness (2,136). In addition, interferon was not detected in serum, jejunal aspirates, and jejunal biopsies of Norwalk or Hawaii virus-infected volunteers (48). Gastric secretion of hydrochloric acid, pepsin, and intrinsic factor did not appear to be altered during Norwalk virus illness (155).

Marked delay in gastric emptying was observed in infected volunteers who became ill or who were asymptomatic but developed the typical jejunal mucosal lesion (155). It has been proposed that abnormal gastric motor function is responsible for the nausea and vomiting associated with these viral agents (155).

FIG. 2. A: Normal-appearing jejunal tissue from biopsy of a volunteer prior to challenge with Norwalk agent. H&E, X90. **B:** Broadened and flattened villi in jejunal biopsy tissue from same volunteer during illness with Norwalk-induced gastroenteritis. H&E, X90. (From ref. 2 with permission.)

EPIDEMIOLOGY

Prevalence and Incidence

Following the development of an immune adherence hemagglutination assay (IAHA) and a RIA for detection of antibodies to the Norwalk virus, it became practical to perform seroepidemiologic surveys for the prevalence of these antibodies (81,105). The prevalence of Norwalk virus antibodies measured by IAHA was compared with that of rotavirus antibodies in infants, children, and adults (Fig. 3) (105). The pattern of acquisition of antibodies in various groups in the United States differed for these two viruses. Norwalk

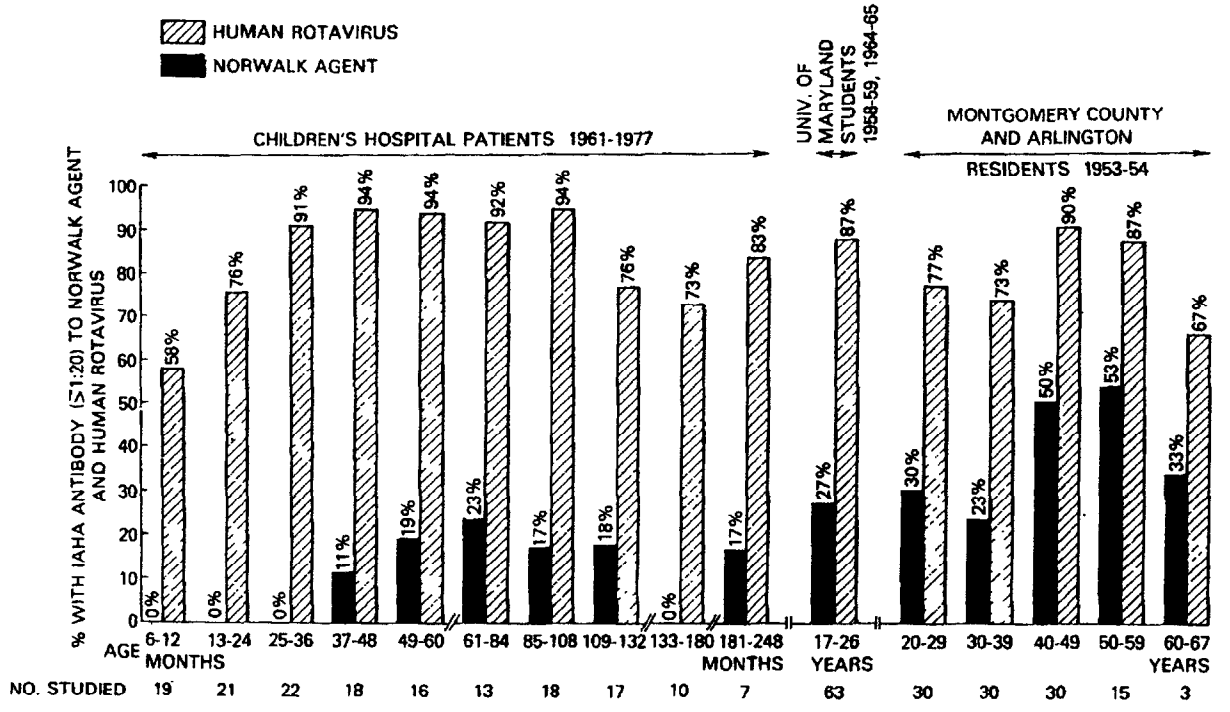


FIG. 3. Prevalence of antibody to Norwalk agent and rotavirus by IAHA in three groups. (From ref. 105 with permission.)

antibodies were acquired gradually in childhood, and acquisition accelerated during the adult years so that 50% of individuals had such antibodies by the fifth decade. In contrast, antibodies to rotavirus were acquired rapidly during infancy and early childhood so that >90% of individuals had such antibodies by the 36th month of age. This pattern of acquisition of Norwalk virus antibodies is similar to that described for hepatitis A and certain rhinovirus serotypes (89,197,198). The paucity of antibodies to the Norwalk virus in infants and young children in the United States suggested that this virus was not an important cause of infantile diarrhea. Later studies confirmed this observation (22,105,124,175,195).

The prevalence of Norwalk virus antibodies in various groups of adults from different parts of the world (including the United States, Ecuador, Belgium, Switzerland, Yugoslavia, Bangladesh, and Nepal) was studied by the RIA blocking (RIA-BL) technique (77). The widespread distribution of Norwalk virus in both developed and developing countries was apparent from these studies, because a majority of adults in each country possessed these antibodies. It was of interest that none of the adults sampled in a highly isolated Ecuadorian Indian tribe in Gabaro possessed serum antibodies to Norwalk virus. This was in sharp contrast to three other, less isolated Ecuadorian villages in which 90% of adults had Norwalk virus antibodies. Male and female homosexuals in the United States had a prevalence of Norwalk antibody comparable to other adults.

The prevalence of antibodies to the Norwalk virus in pediatric populations from various parts of the world was also studied by an RIA-BL assay (17,44,58,77, 180). In this age group, antibodies to Norwalk virus were acquired more rapidly in developing countries such as Ecuador, Bangladesh, Thailand, the Phillipines, and Panama than in the United States, Taiwan, or Yugoslavia. For example, in two Matlab villages in Bangladesh, 43% of infants and children 2–49 months of age possessed Norwalk virus serum antibodies. The prevalence of antibodies was only 7% in the 2- to 7-month-old age group but increased to 100% in 4-year-old children (17).

Specific incidence data for illness associated with the Norwalk virus or related agents are not available in the United States. However, the importance of the Norwalk virus group is suggested from data on the incidence of enteric illnesses in family studies, which indicate that, on the average, each family member experiences more than one such illness per year (46,159). Because the Norwalk virus and related agents are a major cause of epidemic gastroenteritis, it is likely that a significant proportion of such illnesses is associated with these viruses.

In a longitudinal study of infectious diseases and nu-

trition in rural Bangladesh, diarrhea was the major cause of morbidity among children less than 5 years of age (17). The incidence was over five episodes per child per year. In this population, the annual incidence of a significant increase in antibodies to Norwalk virus (measured by RIA) was 29 per 100 children, with a peak incidence in the December to March cool, dry period. Thirty (24%) of 127 children experienced a single seroresponse, whereas three had two seroresponses during the year. Children who lacked detectable Norwalk virus antibodies in their serum at the start of the study developed a seroresponse significantly more often than those who had preexisting antibodies. The age-specific incidence of serologic responses was low in infants and then rose in the 14- to 19-month age group and remained high through the third year of life.

This longitudinal study also provided the first evidence that Norwalk virus infection may cause diarrhea in infants and young children. A comparison of the incidence of diarrhea not associated with other pathogens during periods when a significant seroresponse to Norwalk virus did or did not occur revealed that children who had a seroresponse experienced an excess incidence of 0.3 diarrheal episodes per 100 days. Thus, 1–2% of the diarrheal episodes in these children (who averaged 5.6 episodes per year) were estimated to be due to Norwalk virus. However, it should be noted that Norwalk virus infection is not usually associated with dehydrating diarrheal illness in Bangladesh. Only one of 31 children under 10 years of age who were treated for dehydrating diarrhea in a hospital and who did not have rotavirus, enterotoxigenic *Escherichia coli, Salmonella, Shigella,* or *Vibrio cholerae* in their stools had a seroresponse to Norwalk virus (17). This is consistent with observations made at the Children's Hospital National Medical Center, Washington, D.C.; none of 51 rotavirus-negative patients admitted to the hospital with diarrhea developed a seroresponse to Norwalk virus (105). Over the 8 years of this study, particles that were Norwalk virus-like in morphology were detected in stools of only 1.6% of 1,537 patients hospitalized with gastroenteritis (22). In a 6½-year cross-sectional study of pediatric patients hospitalized with gastroenteritis in Japan, caliciviruses or SRVs were detected by EM in only 0.9% of 1,910 patients (124). Also, in a prospective family study in Texas, none of 28 infants and young children studied from birth to 2 years of age developed serologic (RIA-BL) evidence of Norwalk virus infection (176).

A seroepidemiologic study of children under 5 years of age from two Panamanian San Blas Islands indicated that the prevalence of Norwalk virus antibodies increased rapidly with age, and the frequency of a seroresponse was 35% over a 9-month interval (180,189). Infection was detected most often during the second and third years of life. In addition, children who had a seroresponse to Norwalk virus experienced a significantly greater number of diarrheal episodes than those who failed to develop such a response. This suggested that the Norwalk virus was associated with mild gastroenteritis in this pediatric group, which had an incidence of diarrheal disease of 32 per 100 children per year.

In a longitudinal study of infants and children in three northern North American communities, the incidence of Norwalk infection was highest among the neonates in the only community that had a relatively unsafe water supply (0.15 infections per child per year) (88). There was a suggestion that some of these infections were associated with an episode of gastroenteritis.

Epidemic Pattern of Infection

The Norwalk group of viruses are major causes of outbreaks of nonbacterial gastroenteritis outbreaks that occur in communities, schools, institutions, camps, and families and that primarily affect adults and school-age children. As noted previously, members and probable members of this group were detected initially in specimens derived from gastroenteritis outbreaks in schools (Norwalk, Ditchling, W, and Parramatta), families [Hawaii and Montgomery County (MC)], a resort camp (Snow Mountain), a hospital (Taunton), and dining places (cockle). Each of these outbreaks occurred in the fall or spring season. However, more recent studies of other Norwalk virus outbreaks in the United States indicate a year-round occurrence (78,111).

The setting of the outbreaks in which the Norwalk virus and related agents were detected provides information bearing upon the natural history of these agents in the community. These viruses have been a prominent cause of acute nonbacterial gastroenteritis. For example, the Norwalk virus was derived from a specimen from a secondary case from an outbreak of gastroenteritis in an elementary school in Norwalk, Ohio which was studied extensively by the CDC (1). On October 30 and 31, 1968, acute gastrointestinal illness developed in 50% of 232 students and teachers in an elementary school. The majority of illnesses occurred between noon on October 30 and noon on October 31. Symptoms lasted 12–24 hr in the majority of cases and seldom longer than 24 hr. The secondary attack rate among family contacts of primary cases was 32%; these occurred mostly on November 1–3, with an average incubation period of 48 hr. The W agent was derived from a specimen from an outbreak of gastroenteritis in a boys' boarding school in England which affected approximately one-fourth of the students in

residence between March 14 and March 22, 1963 (34). During the outbreak, only one teacher and one member of the kitchen staff were affected. The attack rate was higher in the younger boys than in the older boys. The Ditchling agent, which is antigenically related to the W agent, was associated with an outbreak of gastroenteritis in a primary school in Ditchling, England (7). Twenty-four percent of 138 individuals were affected between October 3 and October 7, 1975. The Parramatta agent was detected in specimens from a primary school outbreak in Sydney, Australia in which 54% of 381 children and nine of 18 teachers were affected over a 1-month period, July 18 to August 18, 1977 (33).

Infection can also be introduced into the family by an adult, as illustrated by the Hawaii and MC viruses. These viruses were derived from specimens collected during family outbreaks of gastroenteritis (203). The Hawaii family outbreak occurred over a 4-day period and involved first the mother, then a 2½-year-old child, then the father, and finally a student who lived with the family. The MC family outbreak occurred over a 7-day period, initially affecting the father, then a 4-year-old child, then the mother, and finally a 5-year-old child.

Large-scale outbreaks of infection by viruses of the Norwalk group have also been associated with contamination of shellfish. For example, the cockle agent was detected in specimens from two outbreaks of gastroenteritis in England, which affected groups of individuals 24–30 hr after consumption of cockles between December 21, 1977 and January 10, 1978 (10). Also, a large gastroenteritis outbreak, which involved more than 2,000 persons who ate raw oysters during the winter months of June and July 1978 in Australia, was associated with Norwalk virus by demonstration of serologic response (83,84). In other instances, contamination of bakery products or salad has been implicated in large-scale common-source outbreaks of Norwalk virus gastroenteritis (126,138).

Other forms of common-source contamination also play a role in outbreaks of disease. The Snow Mountain agent was derived from a gastroenteritis outbreak that affected 55% of 760 persons at a resort camp in Colorado during December 1976 (160). A contaminated water supply was suspected as the source of the outbreak. The secondary attack rate in household contacts of ill individuals was 11% of 772. This virus was also implicated as the cause of several additional outbreaks of gastroenteritis (25,85). Common-source outbreaks of Norwalk virus disease have also been associated with contamination of the water supply of cruise ships (87).

Finally, outbreaks can occur in a hospital. For example, the Taunton virus was detected in specimens from an outbreak of gastroenteritis in patients and staff of a hospital in England (28,29). The agent was detected in the feces of nine of 19 (47%) affected patients.

The Importance of Norwalk Virus in Outbreaks of Epidemic Gastroenteritis

The development of an RIA-BL test for detection of Norwalk antibodies made it possible to define the role of this virus in outbreaks of nonbacterial gastroenteritis (14,15,30,68,78,81,82,86,87,111,112,114,115,125, 135,138,161,200,209). Until the development of this assay, the importance of the Norwalk virus could not be evaluated fully because the only method available for detection of Norwalk antibodies was IEM, a specialized, time–consuming technique that requires relatively large amounts of antigen and thus could not be used for large-scale epidemiological surveys despite its sensitivity and specificity (108).

Twenty-four (34%) of 70 gastroenteritis outbreaks were associated serologically with Norwalk virus infection (78). Most of these outbreaks had been studied previously for pathogenic bacterial agents, and none were found. Characteristics of the outbreaks are shown in Table 3. It was surprising that such a high proportion of outbreaks could be associated with a single member of this group of agents. A review of 74 gastroenteritis outbreaks investigated by the CDC from 1976 to 1980, including most of the 70 studied above and a few more, confirmed the importance of this virus (111). In this survey, Norwalk virus was associated with 42% of the outbreaks studied. In addition, the Norwalk virus or a related virus was provisionally associated with another 23% of the outbreaks, while the remainder were considered definitely unrelated to Norwalk virus.

The relative importance of the Norwalk virus group in all outbreaks of acute gastroenteritis regardless of etiology was estimated by reviewing the records of 642 unselected outbreaks reported to the CDC from 1975 to 1981 (110). Sufficient data were available from 558 of the outbreaks to suggest their etiology based on clinical and epidemiologic characteristics. Fifty-four (9.7%) of the unselected outbreaks resembled outbreaks previously linked to Norwalk virus by laboratory study. These included 23% of 96 water-borne outbreaks, 4% of 430 food-borne outbreaks, six of nine nursing home outbreaks, three of five summer camp outbreaks, and five of 18 cruise ship outbreaks. Fourteen of the 54 outbreaks that met the clinical and epidemiological criteria for a Norwalk-like pattern were investigated serologically for evidence of Norwalk virus infection. Seven (50%) were associated serologically with Norwalk virus infection (i.e., at least 50% of the paired sera tested showed a fourfold or greater rise in serum antibody). In addition, in three other out-

TABLE 3. *Epidemiologic characteristics of 24 Norwalk virus-associated gastroenteritis outbreaks*[a]

Location or source of outbreak	Number of outbreaks	Month of occurrence	Age range of affected individuals
Recreational camps	4	March, June, August, November	Children (4–15 years) and adults
Cruise ships	4	January, April	Adults
Contaminated drinking or swimming water	4	May, July (×2), October	Children (>4 years) and young adults
Community or family	4	February, June, August, December	Children and adults
School (elementary or college)	3	May, October, November	Children and young adults
Nursing homes	2	November, April	Elderly adults
Shell food	1	June (Australia)	All ages
Other	2	August?	Adults

[a] From ref. 106 (modified from ref. 78). Clinical characteristics consisted of mild to moderate nausea, vomiting, or diarrhea, or a combination thereof.

breaks, at least one affected individual developed a significant antibody rise in response to the Norwalk virus antigen, suggesting a role in the outbreak for a Norwalk-like virus.

The role of Norwalk virus infection in outbreaks of diarrheal illness in families was evaluated during a 1-year prospective study of 28 families who were enrolled at the time of birth of an infant (176). Fourteen of the families experienced one outbreak of diarrheal disease which could not be associated with a bacterial enteropathogen. Two of these outbreaks were associated serologically with Norwalk virus infection. None of the 28 infants and young children in the study developed serologic evidence of Norwalk virus infection.

Traveler's Diarrhea

Norwalk virus does not appear to be an important cause of traveler's diarrhea. Its estimated role in four studies ranged from 0% to 15% (57,59,116,179).

Transmission

Studies in volunteers have demonstrated that the Norwalk, Hawaii, MC, W, and Snow Mountain viruses infect and produce disease when administered by the oral route (34,49,50,136,160,194,212). In one study, a $10^{-4.7}$ dilution (the highest dilution tested) of a stool containing Norwalk virus induced illness in volunteers (R. Dolin, *unpublished studies*). Transmission via the respiratory route appears unlikely for this group of agents, although it has been suggested as a possible route in a large gastroenteritis outbreak in a hospital (184). Nasopharyngeal washings from a volunteer with experimentally induced Norwalk gastroenteritis did not induce illness in three other volunteers (49). It is of interest that Norwalk virus was detected by IEM in vomitus obtained from infected volunteers (80).

The explosive nature of some Norwalk virus outbreaks in which a large number of persons become ill within 24–48 hr suggests that infection is often acquired from a common source. Indeed, this was suggested in the original Norwalk virus outbreak, but a common-source exposure could not be identified (1). Later, a review of 38 Norwalk virus-associated outbreaks suggested that a common source of infection was likely in 31 (82%) (111). The vehicle of transmission could be identified in 17 of the 31 outbreaks, including water in 13 instances and food in four others. Water-borne infection was attributed to a municipal water system (2), semipublic water supply (7), stored water on a cruise ship (2), and recreational swimming (2). The food-borne outbreaks were associated with the ingestion of oysters or salad. A large outbreak of gastroenteritis in Minnesota was traced to contamination of cake frosting by Norwalk virus (100). Primary person-to-person transmission occurred in seven of the 38 outbreaks (111). Sufficient data were available from 26 of the outbreaks to permit estimation of secondary attack rates (111). In 20 of 23 common-source outbreaks and in each of three person-to-person outbreaks, secondary transmission was observed, with attack rates ranging from 4% to 32%. In a large outbreak in which age data were available, the secondary attack rate was highest in children under 10 years of age. The median duration of the 38 outbreaks was 7 days (ranging from 1 day to 3 months). The number of individuals who became ill ranged from 2 to 2,000, with the attack rate being higher in common-source outbreaks (median 60%, range 23–93%) than in primary person-to-person outbreaks (median 39%, range 31–42%).

In the Colorado outbreak caused by the Snow Mountain agent, 61% of the 418 illnesses began on a single day (53,160). A water-borne agent was suggested as the etiologic agent, since the attack rate was correlated

with the amount of water or ice-containing beverages consumed. In addition, the water supply was inadequately chlorinated and was contaminated by a leaking septic tank.

Incubation Period

In volunteer studies with the Norwalk virus, the incubation period ranged from 10 to 51 hr, with a mean of 24 hr (21,49,50,194,212). Illness usually lasted less than 24–48 hr. The incubation period was also recorded in 22 naturally occurring outbreaks of Norwalk virus gastroenteritis (111). The mean (and median) incubation period was between 24 and 48 hr in 20 of the outbreaks, and the range was from 4 to 77 hr. During experimental infection of volunteers, virus shedding as detected by IEM coincided with the onset of illness and usually did not extend more than 72 hr after the first symptoms (202). The incubation period of experimentally induced Snow Mountain virus illness ranged from 19 to 41 hr, with a mean of 27 hr (53).

Geographic Distribution

The available information concerning antibody prevalence and laboratory-identified outbreaks suggests that the Norwalk virus has a world-wide distribution. Norwalk virus antibodies have been detected in the serum of residents of the United States, Ecuador, Belgium, Switzerland, Yugoslavia, Bangladesh, Nepal, Taiwan, Phillippines, Indonesia, Japan, Australia, Panama, and Thailand (17,44,58,77–79,81,83,105,113, 180). Only the very isolated Gabaro Indians in Ecuador lacked detectable antibodies (77,113). Outbreaks of Norwalk virus gastroenteritis have now been documented in 16 states in the United States (111).

Temporal Distribution

Outbreaks of Norwalk virus gastroenteritis occur throughout the year in the United States (78,111). Analysis of 34 Norwalk outbreaks revealed that 11 (32%) occurred in the spring (March, April, May), 10 (29%) in the summer (June, July, August), 7 (21%) in the fall (September, October, November), and 6 (18%) in the winter (December, January, February) (111). This distribution was surprising because prior to this analysis, it was thought that Norwalk virus outbreaks occurred predominantly in the cooler months of the year. It is of interest that during a 1-year prospective study in Bangladesh, seroresponse to Norwalk virus was most frequent in the cool, dry periods of the year (17).

IMMUNITY

Immunity to Norwalk virus is poorly understood and does not resemble the pattern of most other viruses. Adults consistently demonstrate a high degree of susceptibility to both naturally occurring and experimentally induced Norwalk illness. In some outbreaks, more than 80% of adults became ill (111). In addition, approximately 50% of unselected adult volunteers consistently develop illness following challenge with Norwalk virus (21,49,194,212). This level of susceptibility cannot be attributed to lack of previous exposure to the agent.

Because serum or intestinal secretory neutralizing antibodies cannot be measured in tissue culture, most of our information on immunity comes from volunteer studies. These studies have established that there are two forms of resistance to Norwalk virus: one is short-term and the other is long-term (49,169,212). Short-term immunity, which follows the traditional pattern, is serotype-specific; thus, volunteers who become ill following Norwalk virus challenge are usually resistant to rechallenge with this agent 6–14 weeks later. However, challenge of such volunteers with the heterotypic Hawaii virus induces illness. Similarly, volunteers who recently became ill following infection with Hawaii virus are susceptible to challenge with the Norwalk virus.

Long-term immunity, however, deviates from the traditional pattern. Twelve volunteers who were challenged with the Norwalk virus on two occasions, 27–42 months apart, exhibited one of two different patterns to sequential challenge (169). Six volunteers developed gastrointestinal illness following the initial challenge, and they developed it again following rechallenge 27–42 months later. In contrast, six other individuals failed to become ill following the initial challenge and were also resistant after rechallenge 31–34 months later. Serological studies in which prechallenge serum antibodies to Norwalk virus were measured by IEM and RIA failed to provide an explanation for the difference in susceptibility (45,79,169). Paradoxically, volunteers who did not become ill had little, if any, antibody to Norwalk virus measurable by IEM in either prechallenge serum specimen (169). Also, they failed to develop a significant seroresponse following each challenge. However, volunteers who became ill following each challenge developed a seroresponse after each challenge.

Additional evidence for nonimmunological factors in resistance was obtained when prechallenge serum and local jejunal antibody levels were determined in 23 additional volunteers who were challenged with Norwalk virus (79). The geometric mean Norwalk antibody titer (by RIA-BL) in serum or jejunal fluid did not correlate with resistance to illness. However, paradoxically, in-

dividuals who developed illness had a mean prechallenge jejunal fluid antibody titer which was significantly higher than that of volunteers who did not become ill. They also tended to have serum antibody titer which was higher than that of volunteers who did not develop illness.

Several explanations for these observations have been proposed (18,19,43,169). One explanation proposes a genetically determined variation in virus receptors in the intestinal tract, which may influence susceptibility (14,19,43,169). Clustering of susceptibility to Norwalk virus illness in families exposed to contaminated water suggests that genetic susceptibility operates under natural as well as experimental conditions (125). Another explanation proposes that several infections may be necessary to induce the eventual immunity, antibody response, or even the susceptibility that is exhibited by ~50% of adults (18,19,43). This hypothesis is consistent with the natural history of Norwalk virus illness in the United States because neither infection nor illness is common in infancy and early childhood (22,105). In addition, antibody is acquired gradually with increasing age (105). However, this explanation is not consistent with antibody acquisition patterns in a developing country such as Bangladesh or Panama (17,180). In addition, in contrast to the data from the volunteer study, in these developing countries the presence of serum antibodies to the Norwalk virus correlates with resistance to Norwalk virus infection (17,180).

CLINICAL FEATURES

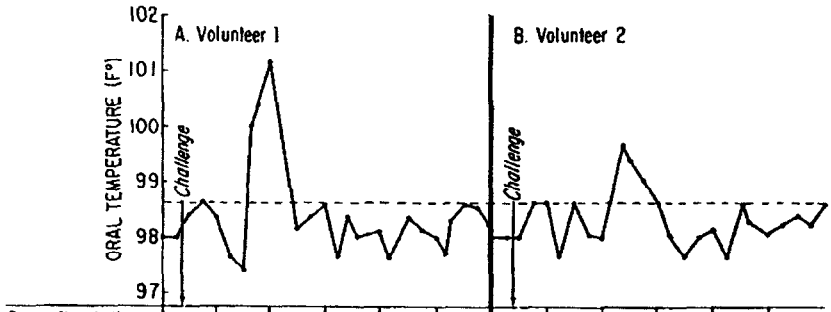
Clinical manifestations observed in 38 outbreaks associated with Norwalk virus included the following (expressed as the median percentage of patients): nausea (79%), vomiting (69%), diarrhea (66%), abdominal cramps (30%), headache (22%), fever [subjective] (37%), chills (32%), myalgias (26%), and sore throat (18%) (111). Bloody stools were not reported. Of interest, vomiting occurred more frequently than diarrhea in children, whereas in adults the reverse was observed. The duration of illness in 28 outbreaks ranged from 2 hr to several days, with a mean or median of between 12 and 60 hr in 26 of the 28 outbreaks. In six outbreaks, illness lasted more than 3 days in up to 15% of the affected individuals. The attack rates did not differ significantly with age or sex in six outbreaks where this was studied (111). Usually, naturally occurring Norwalk illnesses are mild. However, a few exceptions have been noted: Three middle-aged persons were hospitalized for severe dehydration in two outbreaks; two elderly debilitated patients died on the third and seventh days after onset of gastroenteritis (diffuse atherosclerosis was considered the cause of death); and three patients in a nursing home outbreak required intravenous fluids but were not hospitalized (111).

The spectrum of clinical manifestations observed in two volunteers who developed illness following Norwalk virus challenge is shown in Fig. 4 (50). Although both individuals received the same inoculum, one volunteer developed an illness characterized by vomiting without diarrhea, whereas the other had diarrhea without vomiting. Clinical manifestations observed in 31 experimentally infected volunteers who became ill included the following: fever >99.4°F (45%), diarrhea (81%), vomiting (65%), abdominal discomfort (68%), anorexia (90%), headache (81%), and myalgias (58%) (212). The illnesses were characteristically mild and usually lasted 24–48 hr; however, one volunteer was given parenteral fluid because he vomited 20 times within a 24-hr period. In another study with Norwalk virus in which 34 volunteers became ill, each volunteer had nausea, 91% vomited, and 56% developed diarrhea (194). Blood, mucus, and leukocytes are not characteristically present in stools (21,47). Fourteen of 16 volunteers who developed illness following Norwalk or Hawaii virus infection developed transient lymphopenia which affected T-, B-, and null-cell lymphocyte subpopulations (52). This was attributed to a redistribution of circulating lymphocytes to the site of viral infection in the small intestine. The lymphocytes remaining in the circulation responded normally or exhibited an exaggerated response to mitogenic stimuli.

Illnesses induced by the Hawaii, MC, W, and Snowmountain agents in volunteers cannot be distinguished clinically from those caused by the Norwalk virus (34,53,212). Subclinical infections with Norwalk virus have been documented under experimental and natural conditions (111,194). For example, in one study, eight of 25 (32%) volunteers challenged with Norwalk virus underwent a silent infection (194). It should be noted that the characteristic jejunal lesion may develop during asymptomatic infection with Norwalk or Hawaii virus (155,186,187).

DIAGNOSIS

Differentiation of illness caused by Norwalk virus from that caused by other viruses of this group cannot be made on clinical grounds. However, a provisional diagnosis of infection by a member of this group can be made in an outbreak situation if the the following criteria are met: (a) bacterial or parasitic pathogens are not detected; (b) vomiting occurs in more than 50% of the cases; (c) the mean or median duration of illness ranges from 12 to 60 hr; and (d) there is an incubation



Days after challenge	0	1	2	3	4	5	0	1	2	3	4	5
Diarrhea	0	0	0	0	0	0	0	0	8 stools		0	0
Vomiting	0	20+		0	0	0	0	0	0	0	0	0
Abdominal cramps	0			0	0	0	0	0			0	0
Nausea	0			0	0	0	0	0		0	0	0
Malaise	0			0	0	0	0				0	0
Headache	0			0	0	0	0	0			0	0
Myalgia	0			0	0	0	0	0		0	0	0
Anorexia	0				0	0	0				0	0
WBC	8,400	18,450	16,600	14,100	11,200	–	8,200	8,100	8,700	8,200	8,300	–

FIG. 4. Response of two volunteers to oral administration of stool filtrate derived from volunteer who received original Norwalk rectal swab specimen. The height of the curve is directly proportional to the severity of the sign or symptom. Volunteer 1 had severe vomiting without diarrhea, whereas volunteer 2 had diarrhea without vomiting, although both received the same inoculum. (From ref. 50 with permission.)

period of 24–48 hr (87). The illnesses of 81–100% of affected individuals in 38 Norwalk virus outbreaks met these four criteria (110).

A specific diagnosis of infection with any of the agents still remains essentially a research project because none of the viruses can be grown in tissue culture nor can they induce illness in a laboratory animal. Although EM was used exclusively for the diagnosis of infection with the Norwalk virus group in early studies, RIAs or enzyme immunoassays are now being used in most investigations for detection of infection with the Norwalk, Snow Mountain, and Hawaii viruses (25,81,105,148,205).

EM

None of the Norwalk group of viruses has a distinctive enough morphologic appearance to enable identification by EM (9,23,67,102,109,178). Direct examination of stool material without concentration is of limited value in screening for these 27-nm particles because they are usually present in low concentration. Furthermore, the significance of particles that are visualized directly cannot be established without other tests because stools contain many small, round objects whose size is similar to that of viruses of this group. For this reason, putative Norwalk virus and related agents must be identified by IEM. In the case of astroviruses and caliciviruses, particles may be present in sufficient concentration to permit detection by direct EM. The characteristic morphology of these particles makes possible their identification by direct EM (140).

IEM

IEM remains a mainstay for the detection and identification of 27-nm gastroenteritis agents in stools (102,103,108,109,178). A stool suspension that fails to yield a recognizable viral agent (e.g., rotavirus) by direct EM should be examined further by IEM. The patient's stool suspension is incubated for 1 hr with his or her convalescent serum (1:5) or with immune human serum globulin (1:5) if convalescent serum is not available. The mixture is then centrifuged at 35,000 *g* for 90 min, after which the pellet is resuspended and negatively stained with phosphotungstic acid (PTA). Antibody directed against a particle in the stool can be visualized readily on the surface as a "fuzzy" coating. In addition, under certain conditions, the antibody may cause aggregation, which further facilitates recognition. However, the detection of convalescent-phase serum antibodies on the particle does not necessarily constitute serologic evidence of recent infection. Therefore, it must be determined whether the patient developed a serologic response to the particle by repeating the above procedures, under code, with paired acute and convalescent sera. An example of a seroresponse to the Norwalk virus is shown in Fig. 5. In this illustration, it is clear that there is more antibody on the particles incubated with convalescent serum (Fig. 5B and C) than on those incubated with the preillness serum (Fig. 5A). This patient was considered to have developed a seroresponse to the Norwalk virus. A 1+ difference in antibody on a 1+ to 4+ rating scale is considered a significant rise in antibody (108).

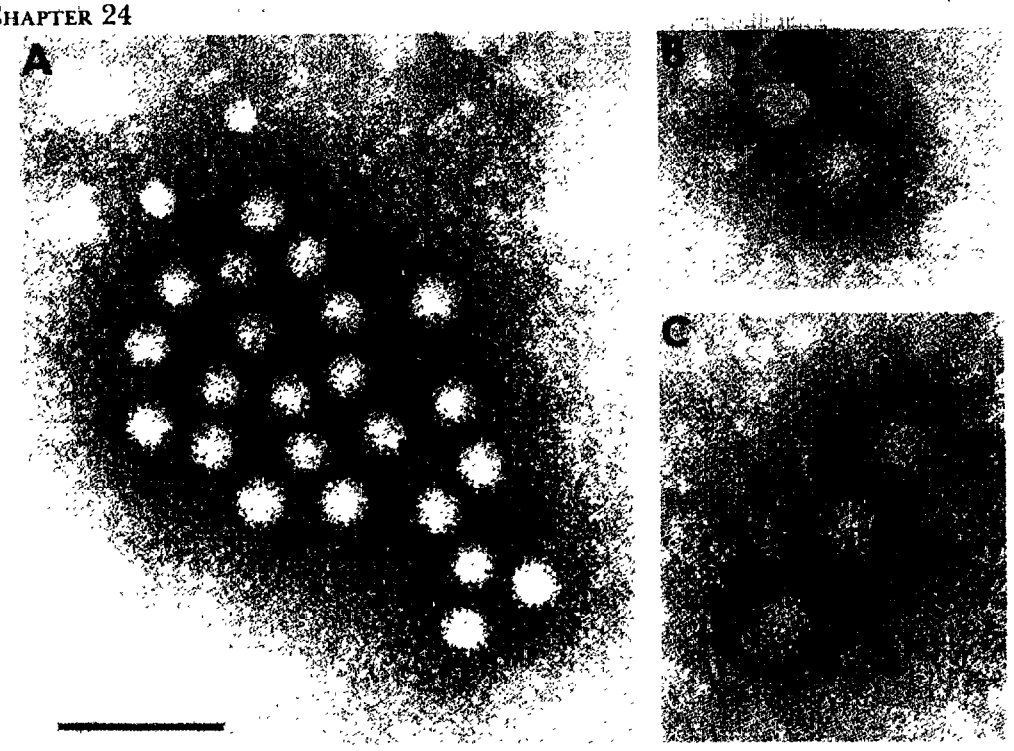


FIG. 5. A: An aggregate observed after incubation of 0.8 ml of Norwalk (8FIIa) stool filtrate with 0.2 ml of a 1:5 dilution of a volunteer's prechallenge serum and further preparation for electron microscopy. This volunteer developed gastroenteritis following challenge with a second-passage Norwalk filtrate which had been heated for 30 min at 60°C (49). The quantity of antibody on the particles in this aggregate was rated 1–2–2+, and this prechallenge serum was given an overall rating of 1–2+. **B:** A single particle and **C:** three single particles observed after incubating 0.8 ml of the Norwalk (8FIIa) stool filtrate with 0.2 ml of a 1:5 dilution of the volunteer's postchallenge convalescent serum and further preparation for EM. These particles are very heavily coated with antibody. The quantity of antibody on these particles was rated 4+, and the serum was also given an overall rating of 4+. The difference in the quantity of antibody coating the particles with the prechallenge and postchallenge sera of this volunteer is clearly evident. Bar = 100 nm and applies to **A, B,** and **C.** (From ref. 103 with permission.)

The time of stool collection is critical to success in virus detection. For example, Norwalk virus was detected in the stools of 11 of 23 volunteers who developed illness following challenge (202). Virus was detected in 26 of 54 specimens collected during the first 72 hr after onset of illness and in two of 11 collected after this interval. In addition, 27-nm particles were detected in only five of 24 outbreaks associated serologically with Norwalk virus (111). Furthermore, only 36 of 106 samples from these five outbreaks were Norwalk-virus-positive by IEM. It should be noted that the detection of a virus-like particle in stool of patients with gastroenteritis, along with the demonstration of an antibody response to the particle, does not establish an etiologic association; however, this is a necessary first step to be followed by appropriate epidemiological studies (97,100).

Norwalk virus particles can be identified if the appropriate paired reference sera are available. The IEM technique may also be employed to detect seroresponses in paired sera to a specific antigen as noted above. The reader is referred to reviews of the IEM techniques (102,103,109,119). Variations on the IEM technique may facilitate visualization of particles such as reacting the virus–antibody complex on a solid phase (immunosorbent EM) or using electron-dense markers such as colloidal gold (119,137).

RIA and ELISA

RIA, which detects both particulate and soluble Norwalk antigens, is more efficient and sensitive than IEM for detection of Norwalk antigen (20,79,81). However, even with this technique, Norwalk antigen was detected in the stools of only 12 of 20 volunteers who developed illness following challenge with Norwalk virus (194). As shown in Fig. 6, the RIA is based on the differential binding of Norwalk virus antigen (present in feces) to microtiter wells coated with convalescent-phase or preinfection serum (20,79,81).

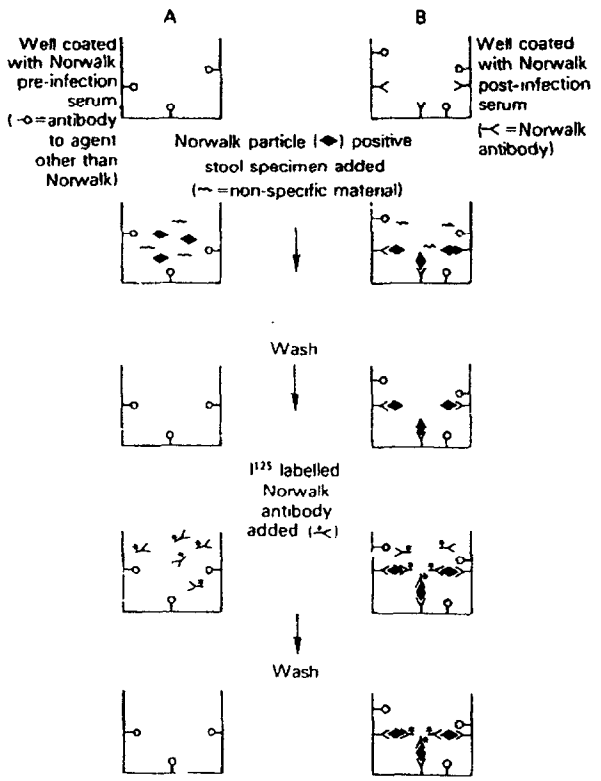


FIG. 6. Radioimmunoassay for Norwalk antigen detection. If counts per minute of B/A was ≥ 2, then specimen was considered positive for Norwalk agent. (From ref. 109 with permission.)

An ELISA which is based on the same general principles as the RIA but which does not employ radioactively labeled reagents has recently been developed for detection of the Norwalk virus (65,66,93,94,148). It is also more efficient and sensitive than IEM for detection of Norwalk antigen but is not as efficient as serologic methods for detection of infection. An RIA and an ELISA have also been developed for detection of the Snow Mountain agent, and an ELISA has been developed for detection of the Hawaii agent (148,205).

The development of an RIA-BL test (and, more recently, an ELISA-BL test) for Norwalk virus antibody has made it possible to study a considerable number of gastroenteritis outbreaks (20,66,79,81,94). This method is based on the ability of a test serum to block the binding of the ^{125}I-labeled immunoglobulin G (IgG) fraction of a Norwalk volunteer's convalescent-phase serum to Norwalk antigen which is attached to the precoat in the solid phase (Fig. 7) (81,109). The RIA-BL, ELISA-BL, and IEM techniques are comparable in efficiency for detection of a Norwalk virus seroresponse (20,66,79,81,94). These serologic assays detect more Norwalk virus infections than do methods that rely on identification of virus particles or antigen in stool. The RIA and ELISA are more practical than IEM because they are less time-consuming, require less antigen and antibody, and can be carried out routinely. It should be noted that RIA-BL and ELISA-BL tests have been developed for the Snow Mountain agent and that an ELISA-BL test has been developed for the Hawaii agent (55,205). In addition, a monoclonal antibody specific for the Snow Mountain agent was recently described (204). This antibody should prove useful in serodiagnosis and virus purification.

IAHA

IAHA can also be used for detection of Norwalk virus and its antibodies, but this technique is not efficient for detection of virus or antigen in stool (105). Both IEM and RIA were slightly more efficient than IAHA for detecting a serologic response. This test has not been used routinely for Norwalk virus serologic studies because it requires a higher concentration of purified antigen than does RIA.

TREATMENT

As noted earlier, the Norwalk group of viruses characteristically induce a mild, self-limited gastroenteritis that normally resolves without complications (21,47,49,50,194,212). Oral fluid and electrolyte replacement therapy with isotonic fluids is usually sufficient to replace fluid loss (47). However, parenteral administration of fluids may be necessary if severe vomiting or diarrhea occur. As noted earlier, hospitalization for severe dehydration, although rare, can occur with Norwalk virus gastroenteritis. In addition, deaths from Norwalk virus gastroenteritis in debilitated elderly patients have been documented; however, these fatalities were considered to be due, in large part, to other causes (111).

Oral administration of bismuth subsalicylate after onset of symptoms significantly reduced the severity and duration of abdominal cramps during experimentally induced Norwalk virus illness (194). In addition, the median duration of gastrointestinal symptoms was reduced from 20 to 14 hr. The number, weight, and water content of stools, as well as the extent of virus excretion, were not significantly affected by treatment.

CONTROL AND PREVENTION

Specific methods are not available for the prevention or control of Norwalk virus infection or illness. Because this agent is highly infectious, effective handwashing and disposal or disinfection of contaminated material may decrease transmission within a family or

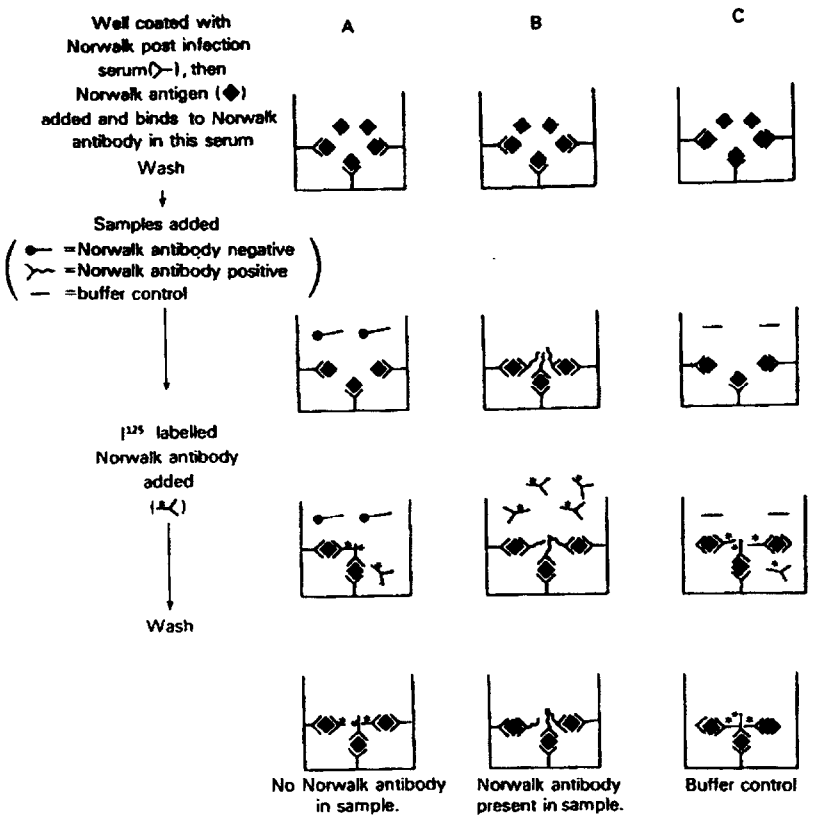


FIG. 7. Blocking radioimmunoassay for Norwalk antibody measurement. If counts per minute of A/C was > 0.5, then A is considered negative for Norwalk antibody. If counts per minute of B/C was ≤ 0.5, then B is considered positive for Norwalk antibody. (From ref. 109 with permission.)

institution. Special care must also be given to the hygienic processing of food in view of the frequent occurrence of food-borne outbreaks of Norwalk virus disease. Measures that increase the purity of drinking water or swimming pool water should also decrease the frequency of Norwalk virus outbreaks.

It is premature to formulate a strategy for immunization against Norwalk virus and its related viruses. First, the basis for long-term immunity is not understood. Second, these viruses have not been successfully grown in cell culture. However, if a safe and effective vaccine could be produced, it would undoubtedly reduce the incidence of epidemic viral gastroenteritis. In addition, it might reduce the number of episodes of gastroenteritis in children who live in developing countries. Although Norwalk gastroenteritis tends to be a mild illness, a reduction in diarrheal episodes may be important in the debilitated, malnourished infant because it has been suggested that repeated diarrheal episodes may be a precipitating factor in the development of malnutrition through damage to the intestinal mucosa (151).

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REVIEW

Epidemiology of Norwalk Gastroenteritis and the Role of Norwalk Virus in Outbreaks of Acute Nonbacterial Gastroenteritis

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Outbreaks of Norwalk gastroenteritis, which may involve persons of all ages, occur during all seasons and in various locations. Waterborne, foodborne, and person-to-person modes of transmission have been described, and secondary person-to-person transmission is common. Outbreaks generally end in about 1 week; longer outbreaks occur only when new groups of susceptible persons are introduced, usually in the setting of a persistent common source of infection. The illness is generally mild and characterized by nausea, vomiting, diarrhea, and abdominal cramps. Vomiting is the predominant symptom among children, whereas diarrhea is commoner among adults. Forty-two percent of 74 outbreaks of acute nonbacterial gastroenteritis investigated by the Centers for Disease Control from 1976 to 1980 were attributed to the Norwalk virus. The rest resembled Norwalk outbreaks clinically and epidemiologically and were probably caused by 27-nm viral agents similar to the Norwalk virus.

SIGNIFICANT ADVANCES have occurred over the past decade in our understanding of the role of viruses in outbreaks of acute nonbacterial gastroenteritis. Although earlier studies had implicated an infectious agent as the cause of nonbacterial gastroenteritis (1-3), no virus was definitively associated with this condition until 1972. Using immune electron microscopy, Kapikian and coworkers (4) observed increased aggregation of virus-like particles with convalescent serum, when compared with acute serum, in the stools of volunteers who developed gastroenteritis after oral challenge with a stool filtrate from an outbreak in Norwalk, Ohio, in 1968 (4). This association between particles and gastrointestinal illness was followed by the application of immune electron microscopy to serologic testing in other outbreaks. In 1978 the development of a radioimmunoassay blocking technique for Norwalk antibody provided a test that was both sensitive and specific for Norwalk infection (5). Since that time, much has been learned about the role of Norwalk virus in sporadic illness and in outbreaks of acute nonbacterial gastroenteritis (6-18). It has also become apparent that viral particles structurally similar to, but antigenically distinct from, the Norwalk virus are capable of causing outbreaks of gastroenterities (19-21). In this review, we describe the Norwalk outbreaks that were reported through 1980 and assess the role of Norwalk virus in outbreaks of acute nonbacterial gastroenteritis that have been investigated by the Centers for Disease Control (CDC) since 1976.

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Methods

We reviewed the records of outbreaks of gastroenteritis that had been investigated for a viral cause of illness by the CDC from 1976 through 1980. All outbreaks of acute nonbacterial gastroenteritis (acute onset of vomiting or diarrhea with stool cultures negative for bacterial pathogens) in which serologic tests for Norwalk virus were done on acute and convalescent serum specimens were included. (Serologic testing of most of these outbreaks was done at the National Institute of Allergy and Infectious Diseases, National Institutes of Health.) For each outbreak the following data were recorded: the date, location, and duration of the outbreak, the number of persons who became ill, the attack rate (including age-specific and sex-specific information when available), the prevalence of symptoms among cases, the duration of illness, and reports of severe illnesses requiring hospitalization. An outbreak was attributed to a common source of infection if a vehicle of transmission was incriminated by epidemiologic analysis or if the peak onset of illness occurred during the first 2 days of the outbreak. Primary person-to-person transmission was presumed when no vehicle of transmission could be found and when the peak onset of illness occurred after the second day of the outbreak; this was shown in some outbreaks by geographic clustering of cases. Secondary person-to-person transmission was evidenced in both types of outbreaks by the finding of illness in family members or roommates not exposed to the primary location of the outbreak.

The incubation period was determined by measuring either the interval between exposure to a common source and onset of illness or the intervals between onsets of illness in primary and secondary cases. The results of serologic testing for antibody to the Norwalk agent by radioimmunoassay and results of stool testing for viral particles by immune electron microscopy or radioimmunoassay were recorded.

An outbreak of gastroenteritis was considered to be caused by the Norwalk virus if at least 50% of the serum pairs from cases had a fourfold or greater rise in Norwalk antibody titer between specimens from acute and convalescent phases. Twenty-eight outbreaks fulfilled this criterion. The geometric mean titers of acute and convalescent serum specimens from these outbreaks were 1:117 and 1:986, respectively. In 20 outbreaks, fewer than 50% of the serum pairs showed a fourfold rise in antibody titer to the Norwalk agent. Three of these 20 were included in the Norwalk category because the geometric mean convalescent titers (1:919, 1:1514, and 1:3200) strongly suggested infection with Norwalk virus. The remaining 17 were classified as "possibly" due to Norwalk virus. Twenty-six outbreaks were designated as not caused by Norwalk virus, because none of the serum pairs tested showed an antibody response. To characterize the experience to date with Norwalk outbreaks more comprehensively, seven additional outbreaks of Norwalk gastroenteritis confirmed through 1980 at the National Insti-

Table 1. Thirty-eight Outbreaks of Gastroenteritis Caused by the Norwalk Virus

Location (Reference)	Setting	Date	Positive Serum Pairs*	Total Serum Pairs from Cases	Geometric Mean Acute Titer	Geometric Mean Convalescent Titer
			n			
Florida (6)	College	November 1967	3	5	1:158	1:2512
Ohio (7)	Elementary school	October 1968	3	5‡	...	...
Maryland† (8)	Family	June 1971	1	1‡	...	...
Sea	Cruise ship	September 1972	6	6	1:631	1:6310
Japan†	Elementary school	February 1976	6	7	1:251	1:3162
North Carolina	College	November 1976	4	7	1:37	1:135
Japan†	Elementary school	December 1976	2	4	1:200	1:673
Sea (9)	Cruise ship	April 1977	7	8	1:219	1:2476
Ohio	Swimming pool	June 1977	4	7	1:200	1:800
Maryland†	Family	December 1977	1	1	1:200	1:3200
South Dakota	Nursing home	April 1978	4	7	1:978	1:4750
Pennsylvania (10)	Recreational area	May 1978	3	5	1:115	1:303
Washington (11)	Elementary school	May 1978	2	3	1:40	1:200
Pennsylvania (10)	Camp	June 1978	3	3	1:40	1:400
Australia† (12)	Community	June 1978	22	30	1:187	1:919
Sea	Cruise ship	January 1979	3	6	1:79	1:356
Sea	Cruise ship	March 1979	5	5	1:152	1:2111
New York	College	March 1979	1	2	1:100	1:400
Washington	Day camp	March 1979	5	5	1:33	1:919
Georgia	Elementary school	April 1979	10	11	1:47	1:1056
South Dakota	Restaurant	April 1979	5	10	1:66	1:230
Connecticut	Recreational area	July 1979	4	5	1:76	1:400
Michigan (13, 14)	Recreational area	July 1979	6	13	1:469	1:1514
Pennsylvania	Camp	July 1979	1	4	1:1345	1:3200
North Carolina	Camp	August 1979	13	14	1:58	1:882
New Jersey	Nursing home	October 1979	4	6	1:45	1:503
Florida	Restaurant	November 1979	1	1	1:200	1:3200
New Jersey (15)	Restaurant	December 1979	6	9	1:233	1:1270
New Jersey	Nursing home	December 1979	9	11	1:146	1:1600
Florida† (16)	Family	January 1980	5	5	1:528	1:3676
California†	Recreational area	January 1980	8	10	1:76	1:985
Minnesota	Elementary school	May 1980	11	14	1:78	1:346
South Dakota	Elementary school	May 1980	7	10	1:162	1:696
Arizona	College	May 1980	8	10	1:107	1:696
Pennsylvania	Restaurant	July 1980	3	5	1:400	1:1393
Georgia	Community	August 1980	12	19	1:40	1:346
New York	Camp	August 1980	1	5	1:606	1:919
Florida	Nursing home	October 1980	1	2	1:71	1:200

* Fourfold or greater rise in antibody titer (radioimmunoassay) to the Norwalk agent between specimens from the acute and convalescent phases of illness.
† Investigated through the National Institutes of Health.
‡ Serologic tests done by immune electron microscopy.

tutes of Health but not investigated by the CDC were included in the Norwalk category.

Results

ANALYSIS OF NORWALK OUTBREAKS

The 38 confirmed Norwalk outbreaks (including the seven not investigated by the CDC) are listed in Table 1. Ten of these outbreaks occurred in camps and recreational areas, seven in elementary schools, four on cruise ships, four in nursing homes, four in colleges or universities, four in restaurants, three in small families, and two in larger communities. Outbreaks occurred in all months of the year. Three outbreaks occurred in other countries, four on cruise ships at sea, and the rest occurred in the United States.

Evidence for a common source of infection was present in 31 of the 38 outbreaks. A vehicle of transmission was implicated in 17 of these—water in 13 outbreaks and food in four. The sources of the water included municipal water systems in two outbreaks, semipublic water supplies in seven, stored water on cruise ships in two, and recreational swimming in two outbreaks. Two foodborne outbreaks were associated with oysters, and two with salad. Primary person-to-person transmission was believed to have occurred in seven outbreaks. Secondary person-to-person transmission (attack rates 4% to 32%) was evidenced in 20 of the 23 common-source and in three of three person-to-person outbreaks for which sufficient information was available. The secondary attack rate was highest among children less than 10 years of age in the single outbreak in which such information was available (13).

The median duration of the outbreaks was 7 days (range, 1 day to 3 months). Of the 24 common-source outbreaks for which information was available, 12 lasted 5 to 9 days; the eight outbreaks of longer duration included seven in which successive weekly outbreaks occurred among newly introduced populations and one nationwide epidemic associated with eating raw oysters (12). The

Table 2. Prevalence of Symptoms in 38 Norwalk Outbreaks

Symptom	Outbreaks	Patients with Symptom*
	n	%
Nausea	30	79 (51-100)
Vomiting	34	69 (25-100)
Diarrhea	34	66 (21-100)
Abdominal cramps	30	71 (17-90)
Headache	22	50 (17-80)
Fever†	29	37 (13-71)
Chills	14	32 (5-74)
Myalgias	14	26 (11-73)
Sore throat	7	18 (7-32)

* Only outbreaks in which the percentages of ill persons with given symptoms were reported are included. Values are expressed as the median percent of patients with symptoms; the range of percentages are in parentheses.
† Reporting of fever was subjective in each outbreak.

our outbreaks of shorter duration included three in vhich ascertainment of late onset cases was incomplete.)f the five outbreaks initiated by person-to-person trans-ıission for which information was available, four lasted to 9 days.

The number of persons ill in the outbreaks ranged ·om 2 to 2000. The largest outbreaks occurred in com-ıunities, schools, recreational areas, and on cruise ships median, 348 cases; range, 19 to 2000 cases), and the nallest occurred among families and in nursing homes median, 19 cases; range, two to 43 cases). Common-ɔurce outbreaks involved more cases (median, 236 cas-s; range, 6 to 2000 cases) than those initiated by person-) on transmission (median, 38 cases; range, two to 5. ases). The attack rates were higher in common-ɔurce outbreaks (median, 60%; range, 23% to 93%) ıan in those attributed to primary person-to-person ansmission (median, 39%; range, 31% to 42%). At-ıck rates did not differ significantly with age or sex in ıe six outbreaks in which information was available. ·ospitalization of a total of three middle-aged persons ır severe dehydration was reported in two outbreaks. lso, in one nursing home outbreak, two elderly debili-ted patients died on the third and seventh days after ıset of acute gastroenteritis, respectively (both deaths ere attributed to diffuse atherosclerosis). In another ırsing home outbreak, three patients needed intrave-ɔus fluids but were not hospitalized.

The prevalence of symptoms among ill persons is shown in Table 2. Illness was generally characterized by nausea, vomiting, abdominal cramps, diarrhea, and headache. Fever was frequently reported but rarely documented. Myalgias, chills, and sore throat were less commonly observed. Bloody stools were never found. The age-specific prevalence of symptoms, reported in five outbreaks, indicated that vomiting occurs more frequently than diarrhea among children, but diarrhea is more frequent than vomiting among adults. This finding was also apparent when the outbreaks were analyzed by location. In six elementary school outbreaks, vomiting occurred in 75% (median) and diarrhea in 46% (median) of ill children. No data from the nursing home outbreaks were available, but in four outbreaks on cruise ships (affecting mostly adults), vomiting and diarrhea occurred in 51% and 85% of cases, respectively.

The duration of illness, recorded in 28 outbreaks, ranged from 2 hours to several days. The mean (or median) was between 24 and 48 hours in 19 outbreaks and between 12 and 60 hours in 26 of the 28 outbreaks. In six outbreaks, a small percent of persons (15% or less) were ill longer than 3 days.

The incubation period of illness was recorded in 22 outbreaks. The range was 4 to 77 hours, and the mean (or median) was between 24 and 48 hours in 20 of the 22 outbreaks.

Laboratory data on the outbreaks are shown in Table 1. Serum specimens from the acute phase were generally obtained in the first few days of illness, those from the convalescent phase 3 to 4 weeks later. The geometric mean titers of specimens from acute and convalescent phases (for all 38 outbreaks) were 1:140 and 1:924, respectively. In 15 outbreaks, serum pairs (specimens from acute and convalescent phases) from healthy persons (controls) were also submitted for testing. The geometric mean titers of specimens from the acute and convalescent phases were 1:158 and 1:248, respectively. In six of the outbreaks in which control serum specimens were submitted, 17 of 63 serum pairs showed a fourfold rise in antibody titer. Retrospectively, we realized that controls in these outbreaks had been chosen from the population at risk and, therefore, had been exposed to Norwalk infection.

Immune electron microscopy was done on stool samples from 24 outbreaks; 27-nm particles were seen in samples from only five. In two instances, these particles were aggregated by convalescent phase serum specimens

ıble 3. Duration of Illness, Symptoms, and Incubation Period in 81 Outbreaks of Acute Nonbacterial Gastroenteritis*†

utbreak ategory	Outbreaks	Outbreaks with				
		Duration of Illness from 12 to 60 Hours	Vomiting ≥ 50% of Cases	Diarrhea ≥ 50% of Cases	Headache ≥ 50% of Cases	Incubation Period from 24 to 48 Hours
	n	% (*n*)				
orwalk infection*	38	93(28)	89(27)	74(27)	50(18)	91(22)
ɔssibly Norwalk infection	17	92(12)	90(10)	70(10)	25(4)	80(5)
ot Norwalk infection	26	84(19)	50(18)	94(18)	38(13)	78(9)

* of these outbreaks were investigated solely by the National Institutes of Health; the rest were investigated through the Centers for Disease Control.
† t of outbreaks with the characteristic described: *n* = number of outbreaks in which information was available.

from patients, and in one, Norwalk antigen was detected in the stool by radioimmunoassay; in two instances, the small number of particles seen precluded adequate testing. In the five outbreaks in which particles were observed, they were found in only 36 of 106 samples.

ANALYSIS OF OUTBREAKS POSSIBLY CAUSED BY THE NORWALK VIRUS

The 17 outbreaks included in this category occurred in all seasons of the year. Eleven occurred in nursing homes, three in camps or recreational areas, two in elementary schools, and one in a college. Of 15 outbreaks in which information was available, six were associated with a common source of infection; one of these was waterborne. Nine outbreaks were attributed to primary person-to-person transmission; geographic clustering of cases was documented in two of these. Secondary transmission (attack rates, 33% to 40%) occurred in two of the three common-source outbreaks and in all six person-to-person outbreaks in which information was available. Outbreaks in the "possibly Norwalk" category were similar to those in the Norwalk category in duration of illness, prevalence of symptoms, and incubation period (Table 3).

The geometric mean titers of Norwalk antibody in acute and convalescent specimens from these outbreaks were 1:142 and 1:262, respectively. Virus-like particles were seen in stool specimens in two of the 11 outbreaks in which samples were submitted. However, these particles were so few that they could not be tested adequately to show a relation with the illnesses observed.

ANALYSIS OF NORWALK-NEGATIVE OUTBREAKS

The 26 outbreaks in this category also occurred in all seasons of the year. Five occurred in nursing homes, five in restaurants, five in residential communities, four on cruise ships, three in hospitals, two in camps or recreational areas, and two in colleges. Fourteen of 21 outbreaks in which information was available were related to a common source; seven were waterborne and three were foodborne (salads were implicated in all three instances). Seven outbreaks were attributed to primary person-to-person transmission; geographic clustering was found in two of these. Secondary transmission (attack rates 11% to 48%) occurred in all seven common-source outbreaks and in both person-to-person outbreaks for which information was available. Outbreaks not due to Norwalk virus were also similar to those in the Norwalk category in duration of illness, prevalence of symptoms, and incubation period (Table 3). The geometric mean titers of acute and convalescent specimens in these outbreaks were 1:164 and 1:179, respectively. Particles, 27 nm, were seen in stool specimens in two of 17 outbreaks in which samples were tested. In one of these outbreaks, a stool filtrate containing these particles produced illness in volunteers (22); in the other, the small number of particles precluded adequate testing.

A summary of the outbreaks of acute nonbacterial gastroenteritis is shown in Table 3. Thirty-one of the 74 outbreaks (42%) investigated by the CDC from 1976 to 1980 were caused by the Norwalk virus. Seventeen outbreaks (23%) showed evidence for possible involvement of the Norwalk agent, and 26 (35%) were not due to Norwalk virus.

Discussion

EPIDEMIOLOGY OF NORWALK GASTROENTERITIS

Our review indicates that outbreaks of Norwalk gastroenteritis have occurred in all months of the year and in various locations, including elementary schools and colleges, camps and recreational areas, nursing homes, restaurants, cruise ships, small families, and larger communities. In addition to primary person-to-person transmission, both water (drinking and swimming) and food have been clearly established as vehicles in the transmission of Norwalk infection. Secondary person-to-person transmission is a nearly universal feature of Norwalk outbreaks. It was found in 20 of 23 common-source outbreaks and in all three outbreaks initiated by primary person-to-person transmission in which information was available.

Norwalk infection produces a brief illness characterized primarily by nausea, vomiting, diarrhea, and abdominal cramps. Although these symptoms are seen in patients in all age groups, children are likely to present primarily with vomiting, whereas adults are more likely to present with diarrhea. The vomiting associated with Norwalk infection may be caused by a decrease in gastric motility, which has been shown in adult volunteers (23). The possibility that this abnormality may be more pronounced in children, therefore accounting for a higher prevalence of vomiting in this age group, has not been investigated.

The mildness of Norwalk illness is indicated by the fact that hospitalization of only three ill persons was recorded in the 38 outbreaks. However, two deaths were reported in patients in a nursing home, suggesting that Norwalk infection may occasionally hasten the death of an elderly debilitated person.

The characteristic duration (5 to 9 days) of the outbreaks in this review suggests that outbreaks of Norwalk gastroenteritis terminate naturally in about 1 week. In eight outbreaks (the nationwide outbreak associated with eating oysters and seven outbreaks in camps or cruise ships), continuing exposure to a common source was responsible for longer outbreaks or repeated waves of illness when new susceptible populations were introduced. Four of these outbreaks may have been interrupted by early recognition and correction of deficient water systems (10; Unpublished data), suggesting that interruption of these longer outbreaks is possible. However, there is no evidence that the course of a typical week-long epidemic has been altered by preventive measures.

ROLE OF NORWALK VIRUS IN OUTBREAKS OF ACUTE NONBACTERIAL GASTROENTERITIS

Our review of the experience at the CDC indicates that 42% of the outbreaks of acute nonbacterial gastroenteritis investigated from 1976 to 1980 were caused by the Norwalk virus. This prevalence is even higher than the 32% and 34% figures recorded previously (17, 18), and

it further suggests that only a few viral serotypes cause most outbreaks of acute nonbacterial gastroenteritis.

A comparison of the data for the outbreaks of Norlk, "possibly Norwalk," and non-Norwalk infection indicates that there are no epidemiologic or clinical criteria that will clearly separate these categories. Outbreaks in each category occurred in all seasons of the year and in similar locations. The duration of illness, prevalence of symptoms, and incubation period were similar in all groups (Table 3), and secondary person-to-person transmission was common among outbreaks in each of the categories.

The organisms that caused the outbreaks in the "possibly Norwalk" and non-Norwalk categories are unknown. As noted earlier, three outbreaks initially classified as "possibly Norwalk" were placed in the Norwalk category, because the magnitude of the convalescent antibody titers suggested that illness was caused by the Norwalk virus. Failure to show a rise in titer in most cases in these outbreaks was related to a delay in obtaining the acute blood specimens; in each of these outbreaks acute specimens were obtained 1 week or more after onset of symptoms, and the geometric mean titers of the acute specimens were also elevated (1:606, 1:469, and 1:1345, respectively). In the rest of the outbreaks in the "possibly Norwalk" category, however, the geometric mean titers of the convalescent specimens were not elevated. Some outbreaks in the "possibly Norwalk" category may have been caused by the Norwalk virus; perhaps additional sero-responses in these outbreaks would have been shown

l the serum specimens been titered at lower dilutions

n those commonly tested (14). Alternatively, because the epidemiologic and clinical characteristics of these outbreaks are similar to those of the Norwalk outbreaks (Table 3), these outbreaks may have been caused by 27-nm viral agents antigenically related to the Norwalk virus. Such serologic cross reactivity between antigenically related enteroviruses has been observed (24).

The epidemiologic and clinical characteristics of the non-Norwalk outbreaks similarly suggest that they were caused by agents resembling, but antigenically distinct from the Norwalk agent. Particles measuring 27 nm were seen in stool specimens from two of these outbreaks, and in one of these outbreaks oral administration of a stool filtrate produced illness in volunteers (22). Several 27-nm particles that are associated with gastroenteritis and are antigenically distinct from the Norwalk virus have been described (19-21).

The difficulty in identifying the organisms that caused the "possibly Norwalk" and the non-Norwalk outbreaks of acute nonbacterial gastroenteritis reflects the current deficiencies in our understanding of the 27-nm particle viruses. The Norwalk virus has not yet been completely characterized and classified. No 27-nm gastroenteritis agent has been successfully propagated in the laboratory; the antigenic material used in the Norwalk radioimmunoassay is derived from stool filtrates from volunteers and chimpanzees. Other recognized 27-nm particle

nts (Hawaii, W, Snow Mountain) have been successfully transmitted to volunteers (8, 22, 25), but serologic tests using the respective antigenic materials have not been developed and applied to serum specimens from outbreaks. Clearly, accurate information on the identity of these agents and their role in causing acute nonbacterial gastroenteritis will be welcome.

In the meantime, additional information must be acquired to define fully the epidemiology of gastroenteritis from 27-nm viruses. Infection appears to occur in all age groups, but the rates of symptomatic and asymptomatic infection and the risk factors involved in transmitting and acquiring infection have not been defined. Transmission is presumed to occur primarily by fecal-oral spread, but some evidence suggests that respiratory transmission occurs (26); this possibility needs clarification. There is currently no method for detecting the 27-nm particle agents in water; the concentration of the virus needed to produce human illness and its susceptibility to chlorination remain unknown. Finally, immunologic measurements of susceptibility to Norwalk infection remain unknown because neither humoral nor secretory antibody appears to correlate with immunity to infection (18, 27). Careful investigation of future outbreaks of acute nonbacterial gastroenteritis may help resolve these questions.

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