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Viral Agents in Diarrhea

INTRODUCTION

Until the early 1970s, little evidence existed to strongly implicate viruses as important causes of human diarrhea. Earlier, there had been an occasional statistical association of enterovirus and adenovirus recovery from infants with diarrhea when compared to recovery rates of well children. In a small number of sporadic cases of illness, these viruses were shown to cause diarrhea, in view of virus isolation and documentation of a specific antibody rise during convalescence. Echovirus occasionally produced laboratory infection in adults[1,2] and epidemic diarrhea in newborn nurseries.[3] Adenoviruses were felt to play a role in wintertime diarrheal illness, where they were isolated from children with diarrhea and respiratory symptoms. Diarrhea was a known complication of measles and chicken pox in malnourished children,[4,5] and inflammatory changes in the intestinal mucosa of such children were reported.[6,7] Acute diarrhea associated with childhood exanthems is not a common problem in the United States, probably reflecting the importance of malnutrition in the evolution of intestinal complications.

Reimann *et al.*[8] demonstrated that a diarrheal illness could be transmitted to volunteers who ingested bacteria-free filtrates of stool suspensions and throat washings from individuals with illness, yet the filtrates contained none of the well-characterized viral agents known at that time. Gordon *et al.*[9] infected persons with the "Marcy agent" after passage through a series of volunteers. Homologous immunity was documented among those recovering from symptoms, yet Marcy-agent-immune volunteers developed illness when exposed to stool filtrates obtained in a sepa-

H. L. DuPont et al., *Infections of the Gastrointestinal Tract*
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rate outbreak,[10] suggesting that more than one virus was responsible for epidemic gastroenteritis.

In 1971 and 1972, a series of collaborative volunteer experiments were initiated by the National Institutes of Health and the University of Maryland which eventually established without doubt that viruses were important causes of gastroenteritis. The stool material employed was obtained from an adult patient with diarrhea acquired during secondary spread of illness at an elementary school in Norwalk, Ohio. The Norwalk agent was shown to produce illness in a high percentage of serologically unselected prison volunteers.[11] Homologous immunity was demonstrated when Norwalk material was again fed to volunteers following recovery from experimentally induced Norwalk disease. Kapikian *et al.*[12] were able to directly visualize the Norwalk agent by immune electron microscopy (IEM) and indicated that it was a 27-nm particle that morphologically resembled a parvovirus.

The most recent major new development in the area of viral etiology of acute diarrhea was presented by Bishop and her associates in 1973[13] when viral particles were identified in the duodenal mucosa of six of nine infants and children with diarrhea acquired in Australia. Since then, these viral agents (now termed "rotaviruses") have been shown to be the most commonly identified diarrheal agents with worldwide distribution. Table 7.1 lists the viral agents that have been associated with gastroenteritis, along with a brief statement of their importance and means of detection. Since the Norwalk-like agents and rotaviruses represent the only known important viral agents in diarrhea, they will be the focal points of this chapter.

NORWALK-LIKE AGENTS

The Norwalk agent was the first well-characterized enteric viral particle to be associated with diarrhea. A rectal swab specimen obtained during an outbreak of diarrhea in an elementary school in Norwalk, Ohio, produced illness in volunteers before and after serial passage through both human intestinal tract and fetal intestinal organ cultures.[11] A spectrum of clinical illness was produced, ranging from protracted vomiting and low-grade fever without diarrhea to mild watery diarrhea without vomiting or detectable fever (see Figure 7.1). Leukocytosis (with leukocyte counts as high as 18,400/mm^3) was noted in a few volunteers. The viral particles could be visualized by IEM when stool filtrates were incubated with serum obtained during convalescence from induced Norwalk infection.[12] The Norwalk agent was found by IEM to be a 27-nm particle, and both experimentally and naturally infected individuals were shown to develop serologic evidence of infection. Volunteers who recovered from

TABLE 7.1
Viral Agents in Diarrheal Disease of Humans

Viral agent	Role in human illness	Methods of detection
Norwalk-like agents Norwalk Montgomery County Hawaii "W" Ditchling Cockle	Cause of epidemic diarrhea among adults and children	Immune electron microscopy, radioimmunoassay
Rotavirus	Major cause of pediatric diarrhea	Electron microscopy, serology
Coronavirus	Unknown	Electron microscopy, organ culture
Enterovirus	Rare cause of diarrhea	Immune electron microscopy, tissue culture
Astrovirus	Unknown	Electron microscopy
Adenovirus	Rare cause of diarrhea	Electron microscopy, tissue culture
Minirotavirus	Unknown	Electron microscopy
Calicivirus	Unknown	Electron microscopy

Norwalk illness were immune to a second challenge.[14,15] Either acid (to a pH 2.7) or heat treatment (60° for 30 min) failed to inactivate the virus, as determined by infectivity in volunteers.[15]

Bacteria-free stool filtrates obtained from family outbreaks in Honolulu, Hawaii, and Montgomery County, Maryland, reproduced an illness similar to Norwalk disease in volunteers.[14] Cross-challenge studies with the three agents to look for induced immunity indicated that the Norwalk and Hawaii agents were antigenically dissimilar; disease produced by either agent failed to induce immunity to subsequent illness by the other. The Norwalk and Montgomery County agents appeared to be antigenically similar, in view of each agent's cross-resistance to the other following induced disease by either. Virus-like particles 26–27 nm in diameter were seen in stool in IEM after treatment with Genetron and concentration.[16] The particles of the Hawaii and Montgomery County agents were similar in size and buoyant density to the virus-like particles associated with Norwalk illness. As with Norwalk illness, antibody development was detected by IEM in outbreaks due to the Hawaii and Montgomery County agents.[17] These IEM studies supported the earlier volunteer challenge experiments indicating that the Hawaii agent did not share surface

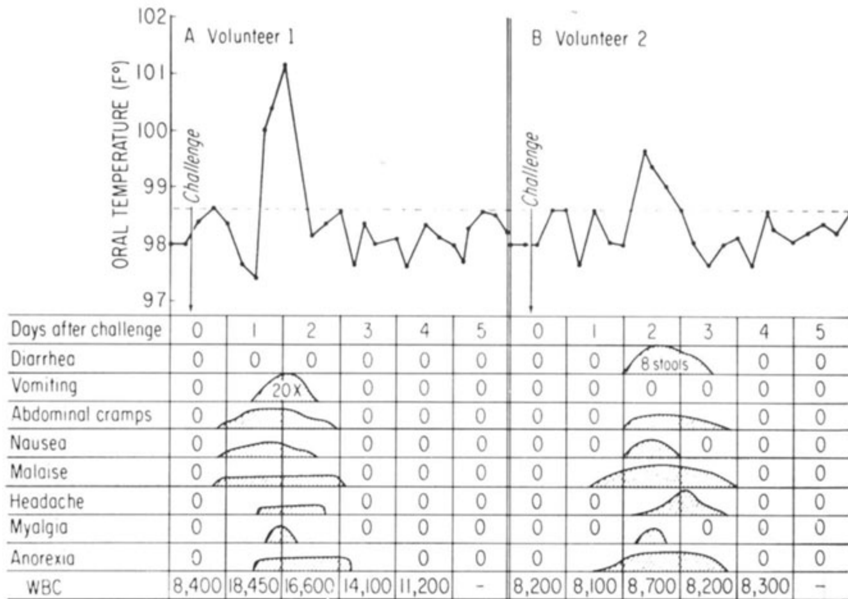

Figure 7.1. The response of two volunteers experimentally infected with the Norwalk agent. From Blacklow *et al.*[137]

antigens with the Norwalk particle, while the Montgomery County agent did share antigens with the Norwalk agent.[17] Workers in Great Britain offered evidence that a Norwalk-like agent that was distinct antigenically from the Norwalk and Hawaii agents was responsible for an outbreak of winter vomiting disease in a primary school.[17] Six Norwalk-like agents have now been associated with gastroenteritis[15–19]: Norwalk, Hawaii, Montgomery County, "W," Ditchling, and cockle. Each of these agents shows similar ultrastructural and morphologic characteristics: 25-27 nm in diameter, nonenveloped with an apparent cubic symmetry, and a buoyant density in cesium chloride of 1.38-1.40 g/ml. While cross-challenge studies in volunteers and serologic evaluations employing electron microscopy have indicated that the Norwalk agent is antigenically related to the Montgomery County agent and is distinct from the Hawaii particle, the W and Ditchling agents appear to be antigenically related to one another and distinct from the Hawaii or Norwalk agents.[17] The newly described cockle agent appears to be distinct from the other Norwalk-like agents.[19] The relative importance of these infectious particles in causing human illness is unknown. Norwalk infection has been shown to be common in all age groups with worldwide distribution.[20]

Recovery and, therefore, identification of the virus in these cases of gastroenteritis have been difficult. These viral agents have not grown in tissue culture, and humans and chimpanzees appear to be the only naturally susceptible hosts.[21] The virus is present in stools of infected persons in low titer.[22] While IEM is a sensitive test for detecting these viral agents, it is an expensive, tedious procedure requiring a skilled reader and is not adaptable to routine laboratory use. Two other serologic procedures for detection of the Norwalk agent and its antibody have been developed: immune adherence hemagglutination assay (IAHA) and microtiter solid-phase radioimmunoassay (RIA).[20] RIA was found to be more sensitive than IEM and far easier to perform, while IAHA was less sensitive than the other two.

Clinical Aspects

The incubation period of Norwalk illness as induced in volunteers is 18–48 hr, and symptoms persist from one to three days. Viral particles are found in stool specimens during the illness, with the greatest concentration at the onset of illness, and are absent, or present in low titers, just before illness and 60 hr after onset of disease.[22] Intestinal biopsy of volunteers with Norwalk disease[23,24] and illness induced by the Hawaii agent[25] demonstrated histologic changes of the small bowel during infection. These changes included blunting of villi, shortening of microvilli, dilatation of endoplasmic reticulum, increase in the number of intracellular multivesiculate bodies, crypt hypertrophy, increase in the number of epithelial cell mitoses, and mucosal inflammation. Abnormal mucosal findings developed a few hours before clinical illness and persisted for at least four days[24] but less than two weeks.[23] At the time of illness, the brush-border enzymes alkaline phosphatase, sucrase, and trehalase were decreased.[23] Although vomiting is a prominent symptom of illness, a gastric histopathologic lesion is not normally present.[26]

Figure 7.1 shows the clinical response of two volunteers with illness induced by oral inoculation of the Norwalk agent.[11] Note that the first volunteer experienced vomiting and low-grade fever without diarrhea, at which time there was a leukocytosis, while in the second person, watery diarrhea without discernible fever or vomiting characterized the illness. These two cases define the extremes of clinical illness. Vomiting is the most common finding, followed by diarrhea and low-grade fever. A variable degree of abdominal cramps, malaise, anorexia, headache, and myalgias also were reported. In addition to occasional leukocytosis, transient lymphopenia may occur.[27]

Immunity

Experimental infection with the Norwalk agent was shown to result in resistance to reinfection with the same agent for at least 14 weeks.[15] In a separate series of rechallenge studies, certain volunteers were shown to maintain long-term immunity to the Norwalk agent, in view of a lack of symptoms after an initial challenge as well as after a subsequent oral challenge inoculum administered 34 months later, while others who had earlier developed induced clinical illness were immune to clinical illness 27–42 months after recovery from the primary infection.[28] The Norwalk and Hawaii agents are antigenically distinct and do not afford cross-protection, as determined in volunteer studies.[14] Circulating antibody as determined by IEM does not appear to play a major protective role, in view of the lack of a relationship between the level of antibody and susceptibility to infection.[12,28] Studies designed to characterize the local intestinal immune response to infection need to be carried out. Jejunal IgA synthesis occurs during both symptomatic and asymptomatic infection[29]; however, specificity of this intestinal antibody has not been determined. Undoubtedly, delayed hypersensitivity will play a role in susceptibility and immunity.

ROTAVIRUSES (RVs)

The 70-nm particle generally known as rotavirus (RV) because of its morphologic appearance—resembling a wheel with radiating spokes[30]—was first associated with diarrhea in children by Bishop *et al.*[13] It originally was considered to be an orbivirus on morphologic grounds and has been variably called reovirus-like agent, orbivirus, duovirus, rotavirus, and infantile gastroenteritis virus. The virus resembles a reovirus and was shown not to cross-react serologically with a number of orbiviruses or human reoviruses types 1, 2, and 3.[31] Similar RVs have been described as causative agents of diarrhea in the newborn of various animal species,[32] including mice,[33] calves,[34] lambs,[35] monkeys,[36] pigs,[37] and horses.[38]

In the initial report from Melbourne, six of nine infants and children with diarrhea had viral particles detected in duodenal mucosa, while a separate report by the same group published the following year offered evidence that the viral particles could readily be seen by direct examination of stool by electron microscopy.[39] Since these observations, most of the data derived concerning the importance of RV in the etiology of diarrhea and other studies designed to determine the epidemiology of RV

gastroenteritis have relied upon the electron microscope for detection of virus.

The organism belongs to the family Reoviridae on the basis of morphologic, biologic, and biochemical properties as well as the presence of double-stranded RNA. RVs are visualized by electron microscopy as particles 60 nm (without an outer shell) and 75 nm (with a double-shelled capsid) in diameter.[40] The virus contains a hexagonal inner core which is approximately 37–38 nm in diameter. The surface in cesium chloride is 1.29–1.30 g/ml for empty shells and 1.36–1.37 g/ml for complete particles.[41–43] The calf RV is stable, remaining viable and infectious at room temperature for seven months.[44] Morphologically intact human RV (HRV) particles were detected when stool samples were stored at −20°C for up to nine years.[45] In 1975, the Reoviridae working team was established under the sponsorship of the World Health Organization/Food and Agriculture Organization Comparative Virology Program. The generic name "rotavirus" was adopted for the reovirus-like agents associated with diarrhea in humans and animals, and the Nebraska calf diarrhea virus strain (NCDV) was selected as the reference strain.[46] Figure 7.2 shows RV particles as visualized by electron microscopy.

Antigenic Relationship of RVs

As stated earlier, RVs have been identified in a wide range of mammalian species. These RVs are not antigenically related to other known members of Reoviridae. They do, however, share a common antigen, as demonstrated by complement fixation, immunofluorescence, immunodiffusion, and IEM.[47] RVs of various species are distinguishable on the basis of virus neutralization and variation in RNA segments and structural proteins.[48,49]

RVs have a distinctive outer capsid with a more sharply defined edge than that of reoviruses or orbiviruses.[48] As determined by gel electrophoresis, RVs have an 11-segment genome, in contrast to the 10-segment genome of the known reoviruses and obviruses.[41,50–55] Gel electrophoresis of viral RNA can be used to distinguish members within the RV group.[52,53,55] HRV and bovine RV vary by as many as three to five RNA
genome of the known reoviruses and obviruses.[41,50–55] Gel electropho-
lamide gels.[52,53] Ovine RV differs in one segment from porcine RV,[51] while porcine and bovine RNA segments may be similar.[54] Strains of RV isolated from the same animal species also may show RNA segment variation. The molecular weights of RNA segments of an RV isolated

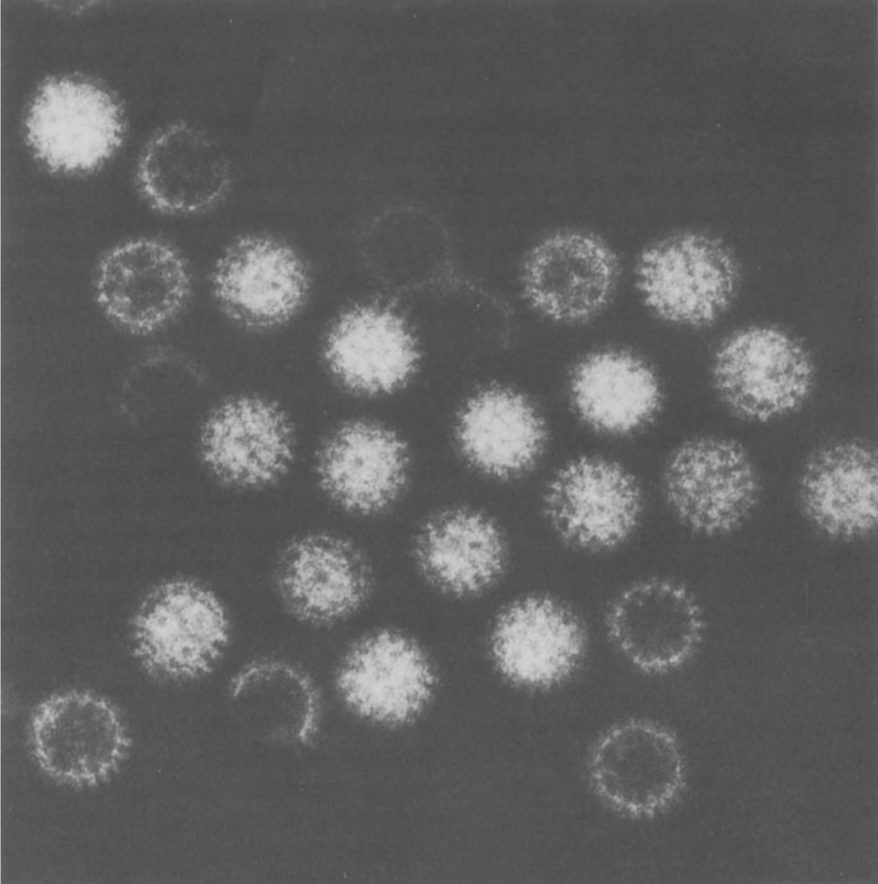

Figure 7.2. Electron micrograph of virus particles with partially removed capsids in a diarrheal stool specimen (phosphotungstic acid ×238,000). Prepared by J. J. Vollet, III, Ph.D.

from a calf with illness differed from the cell-cultured-adapted NCDV,[55] and small differences in electrophoretic mobility of RNA segments have been noted with HRV strains.[53]

Sera from infants and children who had recovered from illness contained immunofluorescent RV antibodies which failed to neutralize the calf virus.[30] Antisera to porcine RV did not neutralize bovine RV.[32,56] A lack of cross-neutralization also was noted between calf and lamb RV,[57] between human and simian RV,[58] and between human, porcine, and murine RV.[59]

RV Detection

Studies designed to determine the importance of RV in human illness have been limited because of an inability to efficiently grow the virus in cell culture systems. Identification of viral particles in stool specimens can be enhanced by incubation with serum containing antibody to the virus or by IEM, which encourages aggregation of the virions.[60] Due to the size of the RV (65–75 nm) and the high density of particles in diarrheal stools of children, direct visualization is possible without employing IEM (Figure 7.2). In adults, whose stool may contain a lower concentration of virus or by IEM, which encourages aggregation of the virions.[60] Due to been developed for detecting RV in stool specimens, including complement fixation,[62] counterimmunoelectroosmophoresis (CIEOP),[62–65] free viral immunofluorescence (IF) utilizing light microscopy,[66] a cell culture technique combined with IF,[67] solid-phase RIA,[68,69] a fluorescent virus precipitin test (FVPT),[70] ELISA,[71,72] and an enzyme-linked immunofluorescent assay (ELFA).[73] The procedures adaptable for efficient processing of large numbers of samples have been CIEOP, IF, FVPT, RIA, and ELISA. CIEOP may[65,74] or may not[62,64] be as sensitive as electron microscopy, while RIA appears to retain the sensitivity of electron microscopy.[69] The most important development to date in the area of RV detection has been ELISA. Here, alkaline phosphatase or other enzymes (not a radioisotope) is linked to anti-RV antibody. Antisera prepared against HRV or against the homologous virus probably should be used to assure optimal sensitivity.[75] Antibody is bound to the solid phase, a test solution containing antigen is added, and a second enzyme-labeled antibody is added. A substrate is then added, and enzyme activity is related to antigen concentration. A color change occurs which can be quantitated spectrophotometrically or read visually. ELISA is as sensitive as RIA and electron microscopy and as efficient as RIA.[72] ELISA can be used to differentiate RVs of the various animal species by employing a blocking technique[71] and to distinguish between the two major HRV types.[76] The procedure requires simple equipment and is adaptable to field conditions. Commercial colorimetric readers designed for ELISA plates are currently available. ELFA may prove to be more sensitive than the comparable ELISA due to sensitivity of detecting the fluorescent end product in a fluorocolorimeter.[73]

Pathogenesis of HRV Diarrhea

Although HRV was first identified in the duodenal mucosa of children with diarrhea,[13] much of the information on disease pathogenesis

has been derived from animal studies in which similar intestinal changes following infection with the animal-adapted RV resulted.[77,78] Colostrum-deprived and gnotobiotic calves infected with NCDV (a calf RV) developed alterations of the upper small bowel during early infection: Villous epithelium was comprised of low columnar to cuboidal cells, denuded tips of many villi were visible, and there was an increase in the number of reticulum-like cells in the lamina propria. Transmission electron microscopy of villous epithelium revealed that a large number of viral particles were present in the cytoplasm and that epithelial cells contained a large quantity of viral antigen. In infants with infection, an indirect fluorescent antibody technique was used to localize RV in duodenal biopsy.[79] The virus was present only in the cytoplasm of villous epithelial cells and not in the lamina propria. The distribution of virus in epithelial cells was patchy, and virus rarely was identified in mucosal biopsies obtained more than four days after the onset of illness. Immunofluorescence testing revealed viral antigen in duodenal and upper jejunal epithelial cells but not in stomach, large bowel, or mesenteric lymph nodes.[80] Virus replication appeared to be confined to columnar epithelium of duodenum and upper jejunum.

Gnotobiotic calves have been infected successfully with HRV.[81] The sequence of events in small-intestinal infection included invasion of the absorptive villous epithelial cells, replacement of the tall columnar villous epithelial cells with cuboidal and squamous cells, shortening of villi, enlargement of reticular cells, and lymphocytic infiltration of the villous lamina propria.

It has been suggested that lactase in the brush border of intestinal epithelial cells may act as receptor site and uncoating enzyme for HRV.[82] This postulate was forwarded because of the ability of purified β-galactosidase to remove the outer capsid layer of virus *in vitro,* possibly explaining the tendency for HRV to infect only gut epithelial cells. However, lactase-deficient persons remain highly susceptible to HRV infection, casting doubt on this theory.[83]

Tallet *et al.*[84] found high fecal output with sodium and chloride loss during early illness, yet adenyl cyclase and cAMP levels were not increased. Decreased levels of mucosal disaccharidases were documented, as well as reduced Na^+, K^+, and ATPase activities reflected in sugar malabsorption and defective sodium absorption. A failure of glucose-stimulated sodium absorption was shown. Similar enzymatic and absorptive defects were noted in a piglet model used to study transmissible gastroenteritis virus.[85] In a study of 57 male infants and children in Bangladesh with RV diarrhea, 28 were given oral therapy with a sucrose electrolyte solution and 29 were given a glucose electrolyte preparation. A

control group of 44 additional children received only intravenous hydration.[86] These groups did not differ clinically in the rate of rehydration or the rate of stooling, despite a documented defect of carbohydrate absorption.

Propagation of HRV

HRV has not grown efficiently in cell culture in studies carried out through 1979. A limited number of strains of NCDV have been propagated in cell culture, as well as SA11 (simian RV) and the "O" agent (which is found in sheep and cattle). Murine RV does not grow well in cell culture but has been grown in mouse embryonic intestinal organ cultures.[87] HRV was cultivated *in vitro* in human fetal intestinal organ cultures employing immunofluorescent staining techniques to detect viral antigens[88,89] and has been successfully passed in cultures of bovine,[90] human embryonic kidney,[91] and African green monkey (AGMK)[92] cell lines. Wyatt *et al.*[92] have reported in 1980 the efficient growth of HRV type 2 in primay AGMK cells. This finding should be of profound importance to our being able to characterize the agent further.

HRV was fed to germ-free and conventional piglets,[93] resulting in diarrhea in some of the animals. The virus was found in stool specimens and mucosal epithelial cells of the small intestine. Cross-infection between some of the conventional piglets was documented. HRV was transmitted successfully to gnotobiotic piglets after three serial passages.[94] Excretion of virus in stool was documented, and serum antibody development occurred. Colostrum-deprived one-day-old rhesus monkeys have been infected experimentally with HRV, while eight-day-old and juvenile monkeys were shown to resist clinical infection.[95]

Clinical, Epidemiologic, and Microbiologic Aspects of HRV Infection

The incubation period of HRV infection ranges from 48 hr to four days.[80,96,97] The most common symptoms are profuse watery diarrhea accompanied by low-grade fever and vomiting. A high frequency of vomiting (up to 80%) is the most characteristic clinical finding of HRV infection. Clinical illness generally lasts five to eight days but may rarely last as long as a month.[44] As with infection by Norwalk-like agents, transient disaccharidase deficiency and carbohydrate malabsorption may follow HRV infection.[13] While RV characteristically produces a transient diar-

rhea, it may cause a severely dehydrating illness necessitating hospitalization. Fatalities secondary to HRV infection have been documented.[80,96] HRV infection shows primarily a wintertime distribution in temperate climates, as determined by studies in Australia,[96] England,[68] Japan,[97,99] Canada,[80] and Washington, D.C.,[100] whereas there may be a less striking association with time of year in warmer climates.[101,102]

During the first several years of study, it was felt that HRV was a cause of diarrhea primarily among infants and children between the ages of six months and three years.[97,100] More recently, evidence has accumulated to suggest that persons of all ages are susceptible to infection. Although there is an increased resistance among infants less than six months of age,[98] newborns commonly become infected,[103,104] and parents of infected children often become infected secondarily or perhaps serve as primary sources of infection.[100,105,106] Infection by HRV among newborn infants is not usually associated with clinical symptoms and is more common in bottle-fed children than in those who are breast-fed.[107] Diarrhea among adults from the United States who travel to Mexico may occasionally be secondary to HRV infection,[61] and epidemics of RV diarrhea in adults have been described.[108] Undoubtedly, one reason that earlier studies failed to indicate the frequency of infection among adults relates to the insensitivity of the study techniques. Viral replication probably is limited in adults, and electron microscopic examination of stool may not document the low levels of viral excretion.

While most studies have shown that HRV is the most commonly discovered agent associated with endemic diarrhea among infants and children, epidemics of HRV infection also have been well documented in open pediatric populations,[97] in confined hospital pediatric populations,[44,109] in newborn nurseries,[103,104] and in populations of school children.[110] During hospital outbreaks, adult hospital personnel may become infected.[104]

Originally, it was thought that a single HRV was responsible for the illness, in view of seroconversion to a common complement-fixing antigen among infected children from various parts of the world. The notion was further supported by the finding that older children and adults had serum antibody and reduced susceptibility. However, multiple outbreaks of HRV diarrhea were reported among populations under study.[110] In the latter report, serologic differences were reported among the children with infection due to HRV. Zissis and Lambert[111] subsequently described two HRV serotypes. The same group of investigators[112] described two sequential outbreaks of diarrhea in a confined population. During the first epidemic, children were infected with HRV type 1 and during the second outbreak, which occured one year later, with HRV type 2. A separate report documented two attacks of HRV gastroenteritis with an 11-month

separation in an infant.[113] HRV type 1 was identified during the first episode, when the infant was 12 weeks old, and seroconversion to HRV type 1 occurred; then, when the infant was 14 months old, a second illness was associated with intestinal excretion of HRV type 2. To determine the relative importance of the two known serotypes of HRV, an ELISA technique was employed to differentiate serotype-specific HRV antigen and antibody in pediatric diarrhea from various parts of the world.[76] HRV type 2 was implicated in 77% of 414 HRV isolations over a five-year period, while the remainder were due to type 1. Most children studied in the Washington, D.C., area had acquired antibody to both types of HRV by two years of age. Sequential illnesses usually indicated infection by the different serotypes where one agent did not produce immunity against the other. Recently, a third[114] and a fourth[115] HRV serotype have been identified.

Infected infants and children excrete the virus during the late incubation period and during the acute phase of the illness in densities of 10^7–10^{10} particles/g of stool. Virus excretion is greatest during the third and fourth days of illness and is rarely detectable after the eighth day,[96,97,116] although on occasion it may be found nearly a month after the onset of infection.[44] While HRV is occasionally identified along with other enteropathogens in stool of children,[100] there does not appear to be a relationship between HRV infection and infection by other agents in humans.[61,98,117,118]

Serology of HRV Infection

The most convincing evidence of the importance of the virus as a cause of illness in humans is the common development of humoral antibody during infection.[31,97,119,120] By complement-fixation testing, antibody has been detected as early as day 3 of illness, with peak antibody titers occurring during the second and third weeks of illness.[97] High titers of antibody are present 14 days after the onset of infection and persist without a fall for one to two years after infection.[120] The sequence of antibody acquisition may be similar to that seen with other viral agents,[121] with IgM antibody appearing rapidly and reaching maximum levels five to ten days after infection and IgG antibody becoming detectable later, reaching a peak two to three weeks after the onset of illness. The initial antibody in HRV infection, which develops two to three days after the onset of symptoms, is IgM,[79] while antibody resistant to 2-mercaptoethanol (probably IgG) is produced approximately ten days after the onset of disease.[97] Employing ELISA, it was verified that anti-HRV IgM antibody occurred early in the course of infection, while anti-HRV IgG

antibodies were documented later in convalescence.[122] These findings, plus the observation that anti-HRV IgG occurred in 95% of adults without anti-HRV IgM, suggest that anti-IgM in the absence of anti-IgG signifies a recent infection with HRV. It appears that HRV has at least two antigenically unrelated capsid layers. The inner capsid layer may be common to all RVs, while the outer capsid layer may be type- or species-specific.[30,32] Antigen from the HRV outer capsid more accurately measures serum antibody during infection as compared to whole HRV employed as antigen.[100,118,119,123]

The ubiquity of HRV agents is suggested by the finding of an age-related antibody among children from six to 18 months of age, indicating that infection by HRV is common early in life.[123] This kind of age-related serotype response is characteristic of other ubiquitous viral agents.[124] Surveys among populations in Melbourne,[120] Washington, D.C.,[119] and Toronto[64] have shown that most children have experienced HRV infection by age three. Levels of antibody do not rise with age beyond this point.

A complement-fixation test was developed employing HRV antigen from stool specimens of infected children.[31] Because of the difficulty in collecting large quantities of diarrheal stools containing HRV, and because many stools possess anticomplementary activity, other serologic procedures have been developed. Table 7.2 lists many of the published studies on RV serology, detailing the viral antigens used and the specific serologic procedures. Morphologically related animal RVs have been shown to be antigenically similar to HRV, and infected children often have an increase in antibody to a common complement-fixing antigen.[125] SA11 virus, recovered from a verret monkey, O (offal) agent, originally recovered from intestinal washings of sheep and cattle, NCDV, and EDIM (epizootic diarrhea of infant mouse) virus have been used as substitutes for HRV. O agent was found to be the most efficient substitute antigen for HRV.[125] NCDV has been employed by many laboratories because of its cross-antigenicity with HRV and its availability.[30,119,123,126] Although NCDV is not as efficient as HRV as an antigen for viral detection, when a serologic procedure employing NCDV is coupled with direct examination of stools by electron microscopy, the diagnosis is usually made with accuracy. In addition to complement fixation, specific serologic procedures employed include IEM, indirect IF, ELISA, and hemagglutination inhibition (see Table 7.2 for references). Advantages of the ELISA technique are that it is easily performed, does not require tissue culture techniques or subjective readings, and can be performed with a single dilution of serum, using a volume as small as 3 μl.[122] In the indirect IF described by Davidson *et al.*,[79] human duodenal mucosal absorptive cells from infants infected with HRV were used as the antigen. Other sources of antigen have been human fetal intestinal organ

TABLE 7.2
Serologic Procedures Employed to Measure Humoral Immune Response to HRV

	Serologic procedure					
Antigen employed	Complement fixation	IEM	Indirect IF	ELISA	Hemagglu-tination inhibition	References
HRV	×					31, 97, 100, 106, 109, 120, 123
HRV		×				31, 97
HRV			×			79, 88
HRV				×		122
NCDV	×					100, 119, 123
NCDV			×			100, 119, 123, 132
NCDV					×	126
EDIM	×					31, 125
"O" agent	×					125
SA11	×					125

culture infected with HRV[88] and infant rhesus monkey intestine infected with HRV.[95]

In documenting HRV infection, serologic evidence of infection should be sought along with identification of viral agents in stool specimens.[100,120] There exists an 86% concordance between detection of HRV in stools and serum complement-fixing antibody response.[100] In children older than six months, serology is slightly more reliable than electron microscopic visualization, since viral replication in the gastrointestinal tract decreases with age. In contrast, infants younger than six months may have a poor antibody response to infection.[120]

New techniques by which HR may be propagated to high titers in cell culture[92] hold the potential for development of a standardized serologic procedure with wide-scale application.

Immunity in HRV Infection

Infants from six months to two years of age are the most susceptible to HRV infection. The lower incidence of infection among infants less

than six months of age may relate to immunity acquired from the mother. The greatest resistance to infection appears to be among children above the age of two years and adults. Thus, it appears that natural immunization occurs, probably through repeated exposures to the virus or to an antigenically similar organism. The protective factor induced by infection has not been identified. It has been shown that there is no relationship between the level of serum antibody and susceptibility.[100,105] Immunity may be more related to resistance to clinical illness rather than resistance to infection, in view of the common finding of asymptomatic infection among adults and newborn infants.

Calves recovering from RV infection have been shown to be immune to reinoculation of virus from several days to four weeks after the initial infection.[77] The calf RV was attenuated by passing it approximately 140 times on bovine fetal kidney cells at 37°C and an additional 60 times on the same cell culture at 29–30°C. The strain was then employed as a vaccine. In a laboratory study it appeared to confer immunity to 6- to 7-hr-old calves, and in field studies carried out in 14 animal herds, it was shown further to protect calves when the animals were immunized within 24 hr of birth.[77,127] In view of the natural resistance of infants and because of possible limitations of active immunization,[128] passive immunization is being sought as a means of controlling infection. Two approaches have been taken in animals: immunization of the pregnant dam or artificial feeding of nonsuckling neonates with an antibody-containing preparation.[129] Antibody to RV is normally secreted in high concentrations in the colostrum of cattle and sheep during the first day postpartum but falls to negligible amounts three days later.[130,131] When high-titered (anti-RV antibody) colostrum was fed to calves, they were protected from infection by calf RV, as were lambs given sheep colostrum or human γ-globulin when exposed to lamb RV or HRV.[132,133] When fed daily to newborn animals, colostrum successfully prevented infection and clinical illness by RV. Use of antibody-containing colostrum may prove to be of value during the first few days to several weeks of life, when the newborn animals remain at high risk. Calves immunized *in utero* with NCDV were shown to resist challenge after birth by HRV type 2 or NCDV.[134] Similarity in the two viruses justifies further study of NCDV as a potential immunizing agent against human illness. Human colostrum and breast milk contain HRV-neutralizing antibody, and solid-phase RIA was employed to confirm that anti-HRV IgA antibody persisted in human milk for as long as nine months after delivery.[136] IgA antibody to HRV was found in colostral specimens from women living in Bangladesh, Guatemala, Costa Rica, and Washington, D.C.[76] Of the specimens, 88% had IgA antibody to HRV type 1 and 91% IgA antibody to HRV type 2. Infants who are breast-fed show a greater resistance to HRV infection

than those who are bottle-fed, and when infection occurs, it generally produces no symptoms.[107]

Further studies are now needed to document the relative immunity of humans to HRV infection, considering that there are at least two important serotypes. The next step will be to compare antigenic similarities between HRVs identified in distinctly differing regions of the world. Finally, that efficient propagation of HRV to high titers in cell culture systems appears feasible, it should be possible to evaluate the possibility of controlling infection through immunophylaxis. The ubiquity of the virus and the profound magnitude of its impact on diarrhea, malnutrition, and death indicate the importance of pursuing the development and testing of immunizing agents.

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