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Human Viral Gastroenteritis

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INTRODUCTION

Acute viral gastroenteritis is a very common illness which occurs in both epidemic and endemic forms. It affects all age groups worldwide and also includes some of the commonly encountered travelers diarrhea. This syndrome is recognized as being second in frequency only to the common cold among illnesses affecting U.S. families under epidemiological surveillance. The clinical presentation of the illness is variable, but in general it is selflimited, has an explosive onset, and is manifested by varying combinations of diarrhea, nausea, vomiting, low-grade fever, abdominal cramps, headache, anorexia, myalgia, and malaise. It is not only responsible for a great deal of misery and time lost from school and work, but can be severe, indeed fatal, in the infant, elderly, or debilitated patient. Due to associated malabsorption, viral gastroenteritis may trigger or enhance the morbidity associated with malnutrition in marginally nourished populations.

Identification and characterization of gastroenteritis viruses and a clear understanding of their epidemiology and immunology are necessary to be able to prevent their transmission and to develop appropriate vaccines. This has proven to be an especially formidable task since all currently recognized or suspected agents of human viral gastroenteritis share the property of being initially non-cultivatable by conventional in vitro techniques or in laboratory animal hosts. It has been long recognized that the commonly isolated cytopathic enteroviruses, including polio-, coxsackie-, and echoviruses, do not produce significant amounts of diarrheal illness in humans. The viral etiology of acute infectious nonbacterial gastroenteritis was only uncovered in the past 12 years by electron microscopic examination of human clinical specimens. Further progress in the study of some electron microscopically observed agents has been accomplished by the use of such techniques as immunoassays, human volunteer studies, and the persistent search for complete or partial viral replication by a variety of unusual culture techniques.

At this time, only two viruses, rotavirus and Norwalk virus, are recognized as medically important etiological agents of human gastroenteritis. Rotavirus has been serially propagated in culture. Its place in the viral classification scheme and many aspects of its antigenic nature, basic virology, epidemiology, and pathogenesis have been elucidated. Although our understanding of the immunology of rotaviral illness is incomplete, the need for disease prevention is recognized as being clear and urgent enough to warrant vaccine trials. Much information is available on the epidemiology and pathogenesis of Norwalk virus, but its characterization is likely to await its cultivation in vitro. Enteric or fastidious adenoviruses have recently emerged as strong candidates for an important etiological role in human diarrheal illness. These viruses have now been serially propagated and are classified as new serotypes of adenovirus. A host of other viruses or virus-like particles have been observed by electron microscopic examination of human diarrheal stool specimens. Establishment of their roles in human disease requires further research.

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A number of reviews have dealt with aspects of viral gastroenteritis (23, 24, 102, 106, 126, 142, 161, 283, 308, 323, 326). Our aim in this article is a comprehensive presentation of the topic which will serve to summarize and place into perspective many recent developments in this field, most of which were reported by September 1983.

ROTAVIRUS

Human rotavirus (HRV) was first detected in 1973 in Australia by thin-section electron microscopic examination of duodenal biopsies obtained from children with acute diarrhea (19) and was observed by electron microscopy soon afterwards in diarrheal stool specimens from various parts of the world. The virus is 70 nm in size, contains segmented double-stranded (ds) RNA as its genome, and has an inner and outer capsid but no envelope. It is classified as a separate genus of the Reoviridae family. The name of the virus is derived from the Latin word "rota," meaning wheel, which it resembles in appearance. Initially the virus was also called orbivirus, duovirus, reovirus-like agent, and infantile gastroenteritis virus. HRVs are morphologically similar to and share certain common antigens with animal rotaviruses. Special techniques are required for the cultivation of HRV, but many animal rotaviruses are readily propagated. A variety of rotaviruses have been isolated from mammalian and avian hosts in which they cause neonatal diarrhea. HRV is a major cause of infantile gastroenteritis.

Mapping of the Rotavirus Genome

An understanding of the molecular biology of rotaviruses has become important in the planning of strategies for disease prevention and control. This is because several serotypes of rotavirus are now recognized with a possible lack of cross-protection among serotypes, and there is also some indication that variant or recombinant viruses may occur in nature. We are currently in the process of understanding the role that each of the rotavirus genes plays in determining viral growth, infectivity, and induction of host immunity.

Rotaviruses possess a genome consisting of 11 segments of dsRNA. To devise gene assignment maps for rotaviruses, biochemical, gene cloning, genetic, and immunological approaches have been taken by groups of investigators working independently in several countries including the United States, Mexico, Australia, New Zealand, and England. In one biochemical approach, genomic dsRNA was isolated from purified virions and fractionated by gel electrophoresis. The fractionated RNA was heat denatured and translated in vitro, and the translation products were compared with polypeptides isolated from virions or infected cells (7, 80, 207, 276). In an alternative biochemical approach, viral RNA transcripts were synthesized in vitro by using the endogenous viral RNA polymerase. The transcripts were fractionated by gel electrophoresis, and the gene product of each transcript was identified by in vitro translation. The genomic RNA segment coding for each transcript was determined by RNA-RNA hybridization (108, 201). In other studies cloned DNA copies of dsRNA genomic segments were used to determine gene coding assignments and complete nucleotide sequences for several rotavirus genes (31, 32, 78, 147, 207). The genetic approach has taken advantage of the high frequency of reassortment which occurs during mixed infection of two rotaviral strains. Analysis of the phenotypic properties of the reassortants obtained and identification of parental origin of the viral genes have permitted the assignment of some properties to specific genes (107, 119, 150, 152). Monospecific antibodies have also been used to probe the functions of specific viral proteins (12, 121, 124).

The major findings with regard to rotavirus gene coding assignments and viral proteins are summarized in Table 1. VP7, the product of gene 9 of simian rotavirus, is an outer capsid glycoprotein which has been identified as the major rotavirus neutralization antigen. (Genomic segments 7, 8, and 9 are difficult to resolve by gel electrophoresis and the order of migration of these genes varies from strain to strain.) A lesser neutralization function is associated with another outer capsid protein, VP3, the product of gene 4. This protein, which is cleaved by trypsin, is responsible for restriction of growth in cell culture and protease-enhanced plaque formation and also serves as the viral hemagglutinin. VP6, the product of gene 6, is an inner capsid protein which has distinct epitopes for subgroup specificity and a common rotaviral antigen.

Extensive study of the genetics and function of the proteins of the taxonomically related reoviruses has yielded much information with regard to viral virulence and tissue tropism in mice (99, 138). It is hoped that similar studies with rotavirus will result in a better understanding of the pathogenesis and immunity of this pathogen.

Morphogenesis of Rotavirus

Rotaviruses are unusual among nonenveloped viruses in that they contain at least two glycoproteins, bud through the membranes of the endoplasmic reticulum, and pass through a temporary enveloped phase during their assembly process. The viral envelope is lost before release of the progeny virus by cell lysis.

Samples of rotavirus isolated from either feces or tissue culture fluid have been noted to contain a mixture of two different sized particles which can be separated by CsCl density gradient centrifugation. The class of larger particles bands at a density of 1.36 g/ml. They appear in negative-contrast electron microscopic preparations to be 70 to 75 nm in diameter and are double shelled with a smooth outer capsid. The class of smaller particles bands at a density of 1.38 g/ml and are 65 nm in diameter. They have a rough surface and are single shelled. Only the smooth, double shelled particle has been found to be infectious (38, 86, 244).

Various rotavirus particle types have also been observed in thin-section electron microscopic studies of intestinal tissue from infected animals and of infected cell cultures (1, 40, 47, 143, 187, 232). Petrie and co-workers (234) have been able to clear up some previous confusion in the literature by demonstrating that the enveloped particles seen within simian SA11 virus-infected cells are not the same as the double shelled particles recovered from CsCl gradients. This finding is consistent with the observation that rotavirus infectivity is resistant to lipid solvents (97). Petrie et al. (234) described four rotavirus particle types: first, a subviral particle which is the altered (uncoated) input virion seen within the lysosomes, and second, an enveloped particle which is a transient form seen in the endoplasmic reticulum. There are, in addition, the two different sized particle types of released virus, the double-shelled particle which is the mature virion and the single-shelled particle which may result from the breakdown of double-shelled particles.

The scheme for SA11 virus maturation proposed by these authors is consistent with morphological studies of HRV (143). It states that infectious virus enters the host cell by endocytosis and is sequestered into lysosomes. Uncoating of the input virions in the lysosome results in the production of a 50-nm subviral particle. Newly synthesized viral RNA and

TABLE 1. Genes and proteins of rotavirus[a]

Genomic dsRNA segment	Mol wt of dsRNA × 10^6 (31)	Designation of final translation product	Mol wt of protein × 10^3	Location	Associated function(s)	Comment(s)
1	2.05	VP1	125 (7, 20, 276)	Inner capsid		
2	1.68	VP2	94 (201)	Inner capsid		VP2 is cleaved by trypsin to produce 88K and 84K segments (95)
3	1.60					Gene 3 is apparently poorly translated both in vivo and in vitro
4	1.60	VP3	88 (201)	Outer capsid (200, 207, 276)	Restriction of growth in cell culture (107, 119, 152); hemagglutinin (152), protease-enhanced plaque formation (150), minor neutralization (124)	VP3 is cleaved by trypsin to produce 60K and 28K fragments (53, 92, 95); McAb (124)
5	0.98	NS53 (201)	53 (7, 201, 276)			
6	0.81	VP6	41 (7, 201, 276)	Inner capsid	Subgroup specificity and the common rotaviral antigen are 2 different antigenic regions on this protein (121)	McAb (66a, 121, 272)
7	0.5	NS34 (201)	34			Genomic segments 7, 8, and 9 are difficult to resolve by PAGE; the order of migration of these genes varies from strain to strain
8	0.5	NS35 (201)	35			The complete sequence of SA11 gene 8 has been determined (31); SA11 NS35 shows 96% homology with the analogous bovine rotavirus protein (78)
9	0.5	VP7 (32)	37	Outer capsid (207)	Major neutralization antigen (7, 12, 88, 124, 152, 171, 204); responsible for serotype specificity (332); probably important in protective immunity (115)	The complete nucleotide sequence of gene 9 has been determined; the deduced amino acid sequence shows that VP7 is 326 amino acids in length with 2 NH_2-terminal hydrophobic regions and a single glycosylation site (32); this glycoprotein shows a great deal of electrophoretic heterogeneity among independent viral stocks (96); McAb (124)
10	0.3	NS29 (201)	20 (80)	Nonstructural (7, 88) or minor virion component (79, 206)	Glycosylated to become a 29K protein; has important role in outer capsid assembly (233, 252, 279)	This coding assignment for genes 10 and 11 is true for SA11 and HRV with "long" electropherotype but the position of these 2 genes is reversed for HRV with the "short" gel pattern (80)
11	0.2	VP9	26	Outer capsid? (201)		

[a] Data are mostly for the best-studied example of rotavirus, the simian SA11 virus. VP, Viral protein; NS, nonstructural; McAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis. Numbers in parentheses are references.

proteins accumulate in the cytoplasm to form viroplasmic inclusions where the viral RNA is packaged into 50-nm core particles. The viral capsid proteins assemble around the core and the particles then leave the endoplasmic reticulum, becoming enveloped in the process. The envelope is later lost as the particles move toward the interior of the endoplasmic reticulum cisternae. Ultrastructural immunocytochemical studies (235) with monospecific antisera to rotavirus proteins indicate that the rotavirus inner capsid proteins (VP2 and VP6) are synthesized throughout the cytoplasm and become concentrated in the viroplasmic inclusions. However, the outer capsid glycoprotein (VP7) is synthesized on ribosomes of the rough endoplasmic reticulum and the outer capsid layer is acquired during virus budding into cisternae of the endoplasmic reticulum. The function of the viral envelope, which is lost in the cisternae, remains unknown. Rotavirus particles do not bud through the plasma membrane but enter the culture medium after the lysis of infected cells.

At least two rotavirus proteins, VP7 (37K) and NS29

(29K) (see Table 1), are glycosylated. Data from studies with tunicamycin (233, 252), a specific inhibitor of N-linked glycosylation, show that in the presence of the antibiotic, production of infectious progeny virus is reduced by as much as 99.9%. Observation (233) of the morphogenesis of SA11 in tunicamycin-treated cells revealed that whereas formation of viroplasmic inclusions and budding of particles into the endoplasmic reticulum occurred normally, 80 to 100% of the particles were enveloped, whereas only about 10% of the particles are enveloped in the absence of tunicamycin. Use of a mutant clone of SA11, clone 28 (96), which is incapable of glycosylating VP7 but glycosylates NS29 in the usual way, has shown that the accumulation of enveloped virus in tunicamycin-treated cells resulted from failure to glycosylate NS29 and was independent of VP7 glycosylation (233). This observation, taken together with the presence of VP7 and NS29 in the portion of the endoplasmic reticulum through which particles bud (235, 279), suggests that the viral envelope plays an important role in assembly of the outer capsid. A proposed function of the 29K protein is that of a scaffolding protein, structurally important during outer capsid assembly but later lost along with the lipid bilayer (233).

Classification of Rotavirus

Until recently confusion has abounded in the literature with regard to the serotypic classification of rotavirus. Before HRV could be cultivated, it was studied primarily by immunological techniques such as solid-phase immunoassays (enzyme-linked immunoassay [EIA] and (radioimmunoassay [RIA]), complement fixation, counterimmunoelectrophoresis, and immune adherence hemagglutination. In addition, a fluorescent focus assay was also used which took advantage of a technique involving the partial replication of HRV in cell culture. In 1978 Belgian investigators described two distinct "serotypes" of HRV which were distinguished first by immune electron microscopy (IEM) and complement fixation (348) and later by EIA (345, 347). At the same time investigators in England defined serotypes of rotavirus by serum neutralization of fluorescent focus formation in cell culture (15, 105). The relationship of the rotavirus types defined by these different tests remained unclear (15, 349) until 1981, when the technique for rescue of non-cultivatable HRV by gene reassortment during mixed infection with temperature-sensitive mutants of cultivatable bovine rotavirus was developed by investigators in Bethesda, Md. (120). Kapikian and co-workers (155), using reassortant HRV in a plaque reduction assay, determined that the cell culture infectivity neutralization antigen and the binding assay (EIA, immune adherence hemagglutination) antigen were completely distinct. These authors suggested that the term serotype be reserved to identify the antigen that reacts with neutralizing antibodies, as is customary for other viruses, and that the term subgroup be used to designate the type specificity previously established by complement fixation, IEM, EIA, and immune adherence hemagglutination. Later work (152) has demonstrated that subgroup specificity is determined by the major inner capsid protein, the product of gene 6, whereas serotype specificity is governed by an outer capsid glycoprotein coded for by gene 8 or 9 (see Table 1 and above, "Mapping of the Rotavirus Genome"). The serotype-specific antigen may be important in protective immunity (115).

Both reassortant and directly cultivated HRVs have been used in the plaque reduction or cytopathic effect neutralization assay to define at least four rotavirus serotypes (128, 261, 310, 329, 330, 332). By the most conservative definition used (329), serotypes are considered to be distinct based on a 20-fold or greater difference between homologous and heterologous antibody titers. According to this scheme the classification of Wa virus, for example, would be serotype 1, subgroup 2, whereas St. Thomas no. 4 virus would be serotype 4, subgroup 2 (Table 2). Although serotyping is currently accomplished only in cell culture, it is likely that monospecific reagents (124, 280, 299) with serotype specificity will be useful in a more convenient EIA type of assay (299). The relationship between serotypes established by plaque neutralization and those established by fluorescent focus neturalization has been reconciled (332).

The original two subgroups defined by complement fixation and EIA (345, 347, 348) have been retained, and monoclonal subgroup-specific antibodies have now been described (121, 272). The existence of a third subgroup has recently been proposed (186, 306).

Human and animal rotaviruses have been reported to be in the same realm of the classification scheme for both subgroup and serotype. Almost all mammalian animal rotaviruses studied thus far have been typed as subgroup 1 (121, 155). The exceptions are a porcine and an equine strain which belong to subgroup 2 and murine rotavirus (epizootic diarrhea of infant mice virus), which cannot be classified into either subgroup (121). Two simian rotaviruses (strains SA11 and MMV18006) and a canine rotavirus (CU-1), which are all designated as subgroup 1, have been classified as belonging to serotype 3 (145, 329). In addition, an independent serotyping scheme has been proposed for bovine rotaviruses (219).

Parallel to the development of the subgroup and serotype classification for HRV, there has been an effort to classify these viruses by analysis of the mobility of their virion RNA segments during polyacrylamide gel electrophoresis (91, 154). This technique offered the advantage of circumventing the need for either cultivation of the virus (since dsRNA was extracted directly from clinical specimens) or the development of discriminating standardized typing serum reagents. Electrophoresis also permitted a direct evaluation of all rotavirus genes rather than just a few selected antigens. However, after several years of experience with electrophoretic analysis of the rotavirus genome (9, 31, 90, 184, 192, 242, 246, 284), it is appropriate to ask whether it is possible to classify HRVs by electropherotypes. A recent editorial on this subject (46) concludes that it is unlikely that electrophoretyping will be useful as a form of general classification, but may serve a purpose in some limited situations. A major problem in electropherotype classification is the high variability of electropherotypes. In one report (281) 32 electropherotypes were detected from 149 cases of rotaviral illness. Whereas electrophoretic mobility of genomic RNA reflects many differences among rotatvirus strains, they may not always reflect the changes we are most interested in from an epidemiological point of view. For example, HRVs with identical electropherotypes but different RNA homology by hybridization techniques have been reported (109). In another report, two HRVs of different serotypes had identical electropherotypes (13). It is clear that comigration of equivalent segments of different viruses does not guarantee their identity, nor does the lack of comigration always indicate significant variation. We may speculate that some minor nucleotide changes which do not alter RNA migration may result in either a frame shift or an alteration of the tertiary structure of the protein product, resulting in profound antigenic differences. Conversely, rather large changes in certain areas of the RNA molecule may result in an altered

TABLE 2. Classification of rotavirus[a]

Type of classification	No. of types designated	Viral component involved	Applicable diagnostic tests[b]	Examples		
				Type	Designation	Origin
Serotype	4	Antigenicity of the 37K surface glycoprotein, VP7, the gene 8 or 9 product	Plaque reduction or CPE neutralization (155, 310, 329, 332); fluorescent focus reduction neutralization (15, 105)	1	Wa (332)	Human
				2	DS-1 (332)	Human
				3	SA11 (329)	Simian
				3	CU-1 (145)	Canine
				3	P (329)	Human
				4	St. Thomas #4 (330)	Human
Subgroup	2 (a third subgroup has ben tentatively proposed; 186, 306)	Antigenicity of the 42K core protein VP6, which is the gene 6 product	EIA, CF, CIE, IAHA (121, 272, 345, 347, 348)	1	DS-1 (155)	Human
				1	CU-1 (145)	Canine
				1	SA11 (121)	Simian
				2	Wa (155)	Human
				2	St. Thomas #4 (121)	Human
				2	Gottfried (121)	Porcine
				Not 1 or 2	EDIM (121)[c]	Murine
Electropherotype	Numerous (2 major patterns: "long" and "short")	Migration pattern of genomic RNA in gel electrophoresis (i.e., basically mass and charge of the RNA molecules); migration of genes 10 and 11 determines long or short pattern	PAGE (e.g., 3, 9, 90, 91, 93, 151, 154, 184, 192, 242, 246, 281)	Short	DS-1 (151)	Human
				Long	Wa (151)	Human

[a] Numbers in parentheses are references.
[b] CPE, Cytopathic effect; CF, complement fixation; CIE, counterimmunoelectrophoresis; IAHA, immune adherence hemagglutination; PAGE, polyacrylamide gel electrophoresis.
[c] EDIM, Epizootic diarrhea of infant mice virus.

migration of the genome but remain "silent" with regard to the polypeptide composition.

Although it is now clear that serotype, subgroup, and the long and short electrophoretic pattern modes of classification are all dependent on the contribution of different genes, there is some evidence for linkage of certain rotaviral genes. For example, it has been reported that subgroup 1 HRVs belong to serotype 2 (299) and that the fast or slow migration of genes 10 and 11 in HRVs can be used to distinguish between subgroups 1 and 2. HRVs with the short pattern belong to subgroup 1 and the long pattern is characteristic of HRVs in subgroup 2 (109, 151, 299). These observations do not apply to rotaviruses of animal origin (13) and should be used with some caution until a larger number of HRV strains have been studied. However, subgrouping of HRV is a potential use of electropherotypes. Electropherotypes are also useful in monitoring the spread of virus from one individual to another in a limited outbreak (246). In addition, electropherotypic analysis may be helpful in conjunction with more sophisticated techniques (268, 284) for the study of the evolution of new rotavirus strains through antigenic "shift" and "drift."

Cultivation of HRV

The in vitro propagation to high titer of HRV obtained directly from clinical specimens was first reported in 1981 by Japanese workers (262). They used a combination of techniques that, individually, had been unsuccessful in the past (16). These procedures include the use of roller cultures and the incorporation of trypsin into the culture medium. The success of their procedure has been rapidly confirmed and extended by several other laboratories (16, 185, 311, 332). Representatives of all recognized serotypes and subgroups of HRV have now been cultivated. Isolation rates of 50 to 70% have been reported for EIA-positive clinical stool specimens. Rectal swabs are much less useful for this purpose. Because of the relatively low isolation rates and the frequent need for numerous initial "blind" passages, cultivation at this time is not a viable diagnostic method. It is, however, an invaluable research tool for the study of HRV and may eventually be needed for vaccine production.

Whereas some rotaviruses isolated from animals replicate readily in cell culture, rotaviruses from human feces in the past usually failed to grow directly in cell or organ cultures. On the rare occasions when growth did occur, it was inefficient, inconsistent, and inadequate for viral diagnostic or characterization studies (334). Incomplete viral replication did occur when the virus was centrifuged with a monolayer cell culture since this resulted in the formation of fluorescent antibody-stainable viral antigens in the cell cytoplasm without the production of infectious virus (40, 302). A 1980 report described the efficient serial propagation of a single strain (Wa) of HRV in cell culture after the virus was adapted by undergoing 11 serial passages in gnotobiotic piglets (331). Later, circumvention of the fastidious nature of HRV was accomplished by genetic reassortment during mixed infection with a temperature-sensitive mutant of a cultivatable bovine rotavirus. The gene of the non-cultivatable HRV that restricted growth in vitro was replaced by the corresponding gene from the tissue culture-adapted bovine rotavirus (120). The gene responsible for growth restriction was later identified as gene 4 (119). The product of gene 4 is an 88K outer capsid protein (VP3) which is cleaved by trypsin to produce two fragments of approximately 60K and 28K (53, 92, 95).

Clinical Immunity to HRV

Whereas hyperimmune animal sera clearly distinguish among rotavirus serotypes by neutralization tests, postinfection convalescent human or animal sera are much less specific and are not useful in serotyping rotavirus (94, 115). This observation reflects the heterotypic nature of the immune response to rotaviral infection. The phenomenon is best illustrated by a human volunteer study in which 18

adults were orally administered serotype 1 subgroup 2 HRV (162) and their immune responses evaluated by a number of serological tests. Four individuals experienced diarrheal illness and an additional non-ill volunteer shed rotavirus. Twelve of the 18 subjects (including the 5 who shed rotavirus) developed a fourfold or greater rise in serum antibody (seroconversion) to rotavirus by one or all of the following assays: complement fixation, neutralization, immune adherence hemagglutination, and EIA. Eight volunteers (including the four ill subjects) seroconverted by neutralization assay to the homologous serotype 1 rotavirus. Five of these individuals plus one other subject seroconverted by neutralization assay to the heterotypic serotype 2 rotavirus. Nine volunteers (including the four ill subjects) also showed a fourfold or greater rise in neutralizing serum antibody to the serotypically distinct bovine rotavirus, Nebraska calf diarrhea virus. Seven subjects, including the five who shed virus, seroconverted to both subgroup 1 and 2 rotavirus by subgroup-specific EIA or immune adherence hemagglutination or both. Based on the presence of prechallenge rotavirus serum antibody, all of these volunteers showed previous experience with rotavirus. However, similar heterotypic and heterosubgroup serological responses have also been observed in infants and young children apparently undergoing primary infection with rotavirus (162, 333).

In the volunteer study, the presence of preinoculation serum immunofluorescent antibody to the challenge strain or high levels of neutralizing antibody to either serotype 1 or 2 HRV correlated with resistance to diarrheal illness. However, the relationship of preexisting local neutralizing activity in the intestinal fluid of volunteers could not be associated with protection. Development of both homotypic and heterotypic intestinal fluid neutralizing activity was observed in inoculated volunteers. There is an increase after naturally occurring infection in rotavirus-specific immunoglobulin A (IgA) in both the serum (269) and the gut (69), but these specimens have not been analyzed by type-specific tests.

Two volunteers who had developed diarrhea after initial inoculation were rechallenged with the same inoculum 19 months later. Neither subject developed diarrhea, although one experienced constitutional and gastrointestinal symptoms (162). In a prospective longitudinal study of young Australian children (17), neonates were monitored for the first 14 days of life for rotavirus excretion and were then kept under serological surveillance for 3 years. It was found that symptoms associated with the first postneonatal rotavirus infection were significantly less frequent and less severe in the children who had experienced neonatal infection than in those who had not. Whereas neonatal rotavirus infection by one strain of rotavirus did not confer immunity against reinfection by other strains, it did provide protection against the development of clinically severe disease during reinfection. By contrast, in a study of an isolated tribe of South American Indians, when a rotavirus of a serotype to which none of the tribe had been exposed was introduced, people of all ages developed symptoms (190).

Heterotypic serological responses and cross-protection have also been observed in animal studies. Calves that were experimentally infected with bovine rotavirus developed heterotypic neutralizing antibody to serotype 1 HRV and were later protected during challenge with serotype 1 HRV (333, 335). In one study, however, where gnotobiotic piglets were "vaccinated" by oral administration of culture-adapted representatives of four rotavirus serotypes, only the homoserotypic vaccines afforded protection from either symptoms or virus shedding after challenge with a virulent porcine strain (serotype 3) of rotavirus (115). Another animal study in which the findings apparently differ from the human volunteers demonstrated that in lambs local antibody to rotavirus in the lumen of the gut rather than serum antibody is of primary importance in protection from rotavirus illness (278). Children rarely have severe rotavirus gastroenteritis more than once, but sequential infections by different subgroups or serotypes of HRV have been observed (17, 112, 247, 271, 345).

There is a general clinical impression that breast-fed babies have less diarrhea than formula-fed infants. However, as reviewed by Cushing and Anderson (67), specific studies to investigate this phenomenon have yielded contradictory results. Many such studies are hard to interpret because of difficulties encountered by the investigators in controlling for sanitary and socioeconomic conditions, poor definition of the amount and duration of breast feeding, and the use of supplementation, as well as the retrospective nature of some studies. Both breast- and bottle-fed infants develop rotavirus diarrhea (18); however, in some studies breast-fed babies were observed to excrete rotavirus significantly less frequently and in lower titers than formula-fed infants (52, 209). In a recent longitudinal study in northern Canada, breast feeding was not observed to provide any protection from either rotavirus infection or symptomatic illness (132). Although in vitro studies suggest that several factors in breast milk may be important in protecting infants from rotavirus infections, it has been difficult to demonstrate the efficacy of these factors in vivo. Virus-specific secretory IgA antibody is commonly detected in breast milk by EIA (344) or neutralization (300) and may persist in high titer for 6 to 9 months postpartum, with occasional significant titer rises which are likely associated with subclinical rotavirus infection of the mother during lactation (63). However, the presence of rotavirus-specific EIA and neutralizing antibody in ingested breast milk could not be associated with protection by one group of investigators (303, 304) but was found to correlate with protection in another study (209). Serotype specificity of milk antibody has not been studied to date, but lacteal antibody has been shown to clump rotavirus in stool specimens (301). Trypsin-inhibitory activity of milk was associated with protection in one study (209) but not in another (304). Evidence from animal studies indicates that protection depends on the level of the challenge dose, and infection occurs when the rotavirus infectious titer exceeds the colostral neutralizing antibody capacity (56, 98, 188, 277); this may be an explanation for the discrepancy among the studies involving humans (304).

Besides its role in protection, the humoral immune response apparently also plays a role in recovery from rotavirus infection. In two immunodeficient patients, one with X-linked agammaglobulinemia and the other with severe combined immunodeficiency, chronic symptomatic rotavirus infection with viral excretion lasting for more than 6 weeks has been observed (263). In another study, the titers of IgG rotavirus antibody in convalescent serum specimens were found to be significantly lower in patients with severe or prolonged rotavirus infection than in specimens from subjects with mild or moderate disease (239). In a study of rotavirus-infected adults (275), both symptomatic and asymptomatic subjects had similar rotavirus-specific EIA antibody rises after infection, but asymptomatic infection was associated with development of simian rotavirus neutralizing antibody which was not seen in ill individuals.

The role of cell-mediated immunity in either protection or recovery from rotavirus needs to be investigated. It seems to

us that the murine system would be an ideal one with which to begin investigation of cell-mediated immunity since a great deal of knowledge about mouse immunity and appropriate test reagents already exist. The parameters of murine rotavirus infection, pathogenesis, and the pathology involved have been well studied, and suitable assay procedures have been described (55, 191, 240, 248, 270, 322). A further advantage of the murine system is that rotavirus disease in mice has a high morbidity and low mortality and a large number of inbred animals can be used. With the recent apparent success in the propagation in cell culture of the epizootic diarrhea of infant mice virus, the murine rotavirus (121), the murine model should become available for cell-mediated immunity studies.

Pathogenesis and Pathophysiology

The average incubation period for rotavirus infection in children as well as in experimentally inoculated adults is 1 to 3 days (68, 162, 213). The onset of illness is characterized by severe watery diarrhea, vomiting, and low-grade fever. Vomiting is a particularly common feature of rotavirus diarrhea in children compared with the occurrence of this symptom in gastroenteritis due to other causes (245). The mean duration of rotavirus illness is 5 to 8 days (174). The concentration of rotavirus in feces reaches its maximum titer shortly after the onset of illness and diminishes gradually until day 9 or 10 (222, 314). The disease is usually self-limited, but symptomatic relapses can occur (73, 291).

Common clinical features of rotaviral illness are isotonic dehydration and compensated metabolic acidosis. Dehydration occurs in 40 to 83% of cases. The level of dehydration is usually <5% but can be greater in severe cases (73, 140, 245). In fatal cases of rotavirus disease many of the children have been 10 to 20% dehydrated (42). Fatal rotavirus infection with prominent dehydration has also been reported in geriatric patients with severe underlying disease (199). An increased concentration of sodium and chloride but low concentrations of sugar are evident in stools of rotavirus patients (291). The stools are usually liquid and without blood, fecal leukocytes are uncommon, and mucus is sometimes seen (140, 245). Pale fatty stools are significantly associated with rotaviral diarrhea, suggesting that the virus infection can result in impedence of both the digestion of fat and the pigmentation of feces (296). In Japan, rotavirus infection is often associated with white stools (174). The major pathophysiological mechanism for diarrhea in rotavirus patients appears to be decreased absorption of salt and water related to selective infection of the absorptive intestinal villus cells, resulting in net fluid secretion. There is a secondary contribution of carbohydrate malabsorption, resulting in osmotic diarrhea (due to the presence of undigested carbohydrates in the gut), and metabolic acidosis is due to the bacterial fermentation of these malabsorbed carbohydrates (253).

Symptomatic illness occurs most commonly in children 6 months to 3 years in age. Neonates may excrete virus but are usually asymptomatic (18, 52). Adults typically tend to have asymptomatic or mild disease (318), but severe symptoms in adults have also been reported (81). Four of 18 adult volunteers challenged orally with HRV developed diarrheal illness, whereas 12 developed serological evidence of infection (162).

Symptoms of upper respiratory tract infection have been reported to be associated with rotavirus illness (116, 189, 245). However, since the seasonalities of respiratory tract infections and rotavirus overlap, and since rotavirus itself has never been demonstrated to infect the respiratory tract (162, 189), the association may be coincidental. Several studies, however, have reported a rapid person-to-person spread of rotavirus infection, which in the absence of a common source may be more consistent with a respiratory than a fecal-oral route of spread (113, 118, 133).

Rotavirus was proposed as an etiological agent of intussusception based on the detection of the virus by electron microscopy in the stools of 11 of 30 such patients (177). No controls were run in this study as it was assumed that rotavirus shedding in nondiarrheal children is rare. A recent study indicates that this assumption may not be valid (17). In any case, two later prospective studies and one retrospective study could not associate rotavirus with intussusception (218, 226). Two case reports present patients with central nervous system disease (one with Reye's syndrome and the other with encephalitis) with concurrent rotavirus infections, but the etiological role of rotavirus in these cases was not established (256). Rotavirus was associated with 5 of 11 cases during an outbreak of sudden infant death syndrome occurring in Baltimore, Md. However, stool specimens from 11 cases of sudden infant death syndrome in Boston, Mass., were rotavirus negative (340). The possibility that rotavirus may trigger some cases of sudden infant death syndrome needs to be further investigated. A review of the literature on an etiological role of rotavirus in inflammatory bowel disease indicates a lack of an association (25). In one report, neonatal necrotizing enterocolitis was associated with rotavirus based upon the positivity of stools by the commercial Rotazyme (Abbott Laboratories, North Chicago, Ill.) EIA test (249). This report must be interpreted with caution, however, since Rotazyme has been reported to produce many false-positive results in stool specimens collected from neonates (180).

Epidemiology

Prevalence studies indicate rapid acquisition by young children of binding (EIA-type) serum antibodies to rotavirus worldwide during the ages of 6 to 24 months. The great majority of adults and children older than 2 years possess such antibodies (20, 28, 83, 159, 229). Thus, serological surveys have served to indicate the ubiquitous nature of rotavirus. Serum antibody may be acquired after symptomatic or asymptomatic infection (20, 162), and it is unknown how long detectable antibody persists after infection or whether it has a protective role. The highest incidence of rotavirus illness is found in patients 6 to 24 months of age (34, 159), with the virus being responsible for about half of the hospitalized cases of acute diarrheal illness in this age group (34, 159).

Rotavirus infection can occur in neonates, but as a rule newborn babies experience asymptomatic infection or very mild illness (17, 41, 52, 220). However, in one study at a special care baby unit in London (71) neonatal rotavirus infection was associated with severe gastrointestinal symptoms. Of 76 babies found to excrete rotavirus in this unit, 32 developed diarrheal illness, of which 12 were severely ill. A high incidence of neonates experiencing gastroenteritis with fecal excretion of rotavirus was also observed at a Texas hospital (305). These two reports of symptomatic neonatal rotavirus infection (71, 305) should, however, be interpreted with caution since both groups of investigators used the commercial EIA test, Rotazyme, which, as noted above, has been reported to be unreliable for neonatal stool specimens (180).

Adults are less likely to become infected with rotavirus than children and infected adults are also more frequently asymptomatic than children. In a large prospective family study (318) the attack rate in children was 32% compared with 17% for adults. Seventy percent of infected children and 40% of infected adults were symptomatic. When 18 selected adult volunteers were experimentally inoculated with rotavirus, 67% became infected but only 22% were ill (162). In a major rotavirus epidemic in an isolated Pacific island group involving almost 3,500 cases, the illness attack rate was 40% in infants, 62% in young children, and 12% in adults (113).

Rotavirus infections often occur in adults who are in close contact with young children (130, 159, 172); however, they have also been reported in the absence of any obvious contact with children among adult travelers, military personnel, patients on a cardiology ward, and the institutionalized elderly (57, 82, 84, 85, 136, 144, 168, 199, 251, 315, 316). Whereas symptoms of rotavirus infection in adults tend to be mild (130, 172), severe symptoms were reported in geriatric patients (199) and in apparently normal young adults in Thailand (81).

In general, however, children are the major source of rotavirus in the community (87, 130). The virus is usually spread from person to person by the fecal-oral route (326). Increased family size is reportedly a significant risk factor for acquiring rotavirus infection (132). This is also consistent with the strict seasonality observed for rotavirus infection in the winter in temperate climates (34, 132, 159, 176). Increased indoor crowding may facilitate an airborne mode of transmission which is probably not strictly respiratory but may be due instead to environmental contamination by fecally shed rotavirus (132). Low indoor relative humidity has also been proposed as being important in enhancement of rotavirus transmission (33). Indoor climate conditions are of particular significance in temperate climates since very young infants spend most of their time indoors and show a seasonal pattern of infection (34). The seasonal waves of rotavirus infection in the Washington, D.C., area were observed over an 8-year period to be more regular and predictable than infections with any other pediatric pathogen studied (34). The seasonal effect on rotavirus infection is not fully explained at this time, and there seem to be exceptions to this effect. Rotavirus detection was extremely low in the summer months in Washington, D.C. (34), and northern Japan (176), but the virus was found during the summer in about 20% of patients with diarrhea in London, England (39), and Melbourne, Australia (68). Reports from tropical areas are inconsistent. No rotavirus seasonal variation was observed in Venezuela (74) and Ecuador (287). HRV detection increased during the dry season in Costa Rica (140), India (198), and Bangladesh (21). In Nigeria (231) low relative humidity was associated with increased rotavirus detection, but relative humidity was found to be irrelevant in northern Japan (176).

Nosocomial spread of rotavirus may be facilitated by asymptomatic or mildly ill infected staff, many of whom may be young parents (102); rotavirus can also spread between patients by contact with the hands of uninfected attendants (257). Community-wide epidemics of rotavirus diarrhea have been associated with fecally contaminated drinking water (144a, 193, 285) but common source outbreaks of infection, thus far, appear to be the exception, not the rule.

Epidemiological studies of rotavirus subgroups indicate that infections with the two major subgroups occur simultaneously in a population and that the proportion of each subgroup appears to remain constant over a period of years (111, 306). In most geographic locations, illness due to subgroup 2 has been found to be about four times more prevalent than that due to subgroup 1 (306, 345). In addition, a small fraction of rotaviruses that have been detected have recently been classified into a third subgroup (186, 306). In 1978, Yolken et al. reported that most children living in the Washington, D.C., area acquire antibody to both subgroups by the time they are 2 years old. The significance of this finding must now be reinterpreted in light of the observation of heterosubgroup serological responses in infants and young children (162, 333; see above). The heterosubgroup serum antibody response represents an alternative to the originally proposed explanation that infections with both subgroups occur with equal frequency but that subgroup 2 produces more illness. The clinical symptoms associated with both subgroups appear to be similar (345).

The epidemiology of rotavirus serotypes has not been studied in detail, but it is already clear that rotaviruses from around the world fall into a limited number of serotypes (at this time four or five) (299, 332).

Control, Treatment, and Prevention of HRV Infection

Young children are the major source of rotavirus in the community. Avoidance of close contact with them greatly reduces the risk of acquiring infection (87, 130), especially in a crowded family setting. Children excrete rotavirus in very high titers, with 10^{11} virus particles per g of feces not being unusual (102). Rotavirus in feces may survive for days or weeks on environmental surfaces (215) at ordinary temperatures and at high or low relative humidity (but not at intermediate levels of humidity). Some disinfectants, in particular 95% ethanol and chlorinated phenolic compounds, are effective against rotavirus in feces, but skin disinfectants such as sodium hypochlorite, chlorhexidine gluconate, and providone-iodine are ineffective. Formaldehyde is somewhat useful in inactivating rotavirus (292). However, as Flewett has recently pointed out (102), wiping a surface with disinfectant may reduce infectious virus by a millonfold but is of little use if 10,000 million particles are present.

Nosocomial outbreaks of rotavirus infection have been controlled successfully only if the affected ward was closed and staff movement restricted for 10 days (57, 102). Less drastic control measures have not proven effective (71).

Fluid replacement is an important supportive measure in the treatment of rotavirus diarrhea. Patients with severe dehydration, particularly very young, elderly, or debilitated individuals, require either parenteral fluid replacement or oral rehydration with a glucose-electrolytes formula (100, 259). Avoidance for 1 or 2 days of lactose-containing milk or formulas in non-breast-fed infants with moderate to severe diarrhea has also been recommended (100). Specific treatment orally with human gammaglobulin containing rotavirus antibodies was found to modify the course of infection and disease caused by rotavirus in low-birth-weight babies by delaying excretion of rotavirus and reducing the duration and quantity of virus excretion (10). Diarrhea was found to be clinically less severe in babies receiving gammaglobulin compared with a placebo group, but any effect of the treatment on the risk of infection could not be assessed in this study. In animal experiments several antiviral drugs have shown some initial promise for the specific treatment of rotavirus infection (274).

It is clear at the present time that indications for rotavirus vaccination exist in both developed and developing countries. In developed nations rotavirus is responsible for about 50% of pediatric hospitalizations for acute diarrheal disease. In developing regions, an even greater need exists since in these areas the estimated annual death toll attributable to rotavirus may be 1 million or more (312). Although young children are the obvious candidates for vaccination, it may be desirable to vaccinate adults in special circumstances. These may include the institutionalized elderly, travelers, and medical personnel in pediatric units to decrease rotavirus transmission, and perhaps pregnant or nursing mothers to induce immunity in their offspring.

It is, however, likely that before vaccines can be developed we need to know more about the nature of rotavirus protective immunity in humans; i.e., we must decide what type of immune responses we need to induce and to which viral antigens these immune responses should be directed to achieve protection. Initial vaccines will probably be cell culture adapted, attenuated HRV, or attenuated animal rotaviruses (101, 161) or reassortants between human and animal rotaviruses. An attenuated, cell culture-adapted bovine rotavirus "vaccine" was recently administered to adults as well as young children and was found to be safe and to possess some immunogenic properties. This live, oral vaccine administered to 2 year olds did not produce gastrointestinal or constitutional symptoms nor did it result in virus shedding. One oral dose induced seroconversion to the homologous virus in 88% of children seronegative by EIA and in 68% of children seronegative by the neutralization test (312).

Diagnosis

A rapid diagnosis of rotavirus illness provides the physician with useful information for formulating a prognosis and may prevent the unnecessary use of antibiotic therapy and prolonged hospitalization. It also allows for the timely institution of appropriate infection control measures and for the gathering of epidemiological and clinical information which is vital to vaccine development.

Although cultivation of HRV (see above) has now been achieved, the relatively low isolation rates and the frequent need for initial "blind" passages preclude cultivation as a viable diagnostic method at the present time. The characteristic morphology of rotavirus is readily recognized in negatively stained stool preparations under the electron microscope. Indeed, electron microscopic studies resulted in the original identification of rotavirus (19). This technique has been used extensively for rotavirus diagnosis and remains the standard by which other assays must be judged. However, the use of electron microscopy is limited by the requirement for highly specialized equipment and personnel as well as by difficulties in handling large numbers of specimens.

The diagnostic test of choice for most situations is the solid-phase immunoassay. It offers the possibility of same-day or next-day diagnosis and can be performed on a large scale with routinely available equipment and personnel. In this type of assay the stool specimen to be tested is exposed to a solid phase which has been coated with antirotavirus antibody. After an absorption period and washing, in the direct test a labeled virus-specific detection antibody is used to demonstrate the presence of rotavirus bound to the solid phase. In the indirect test, an unlabeled antirotavirus serum (prepared in a species other than the origin of the serum in the first step) is added followed by a labeled antiglobulin. Details of the test procedures and the preparations of diagnostic reagents have been reviewed (22, 65, 163, 337, 342).

In the original design of the solid-phase immunoassay for rotavirus (153, 338), the reactivity of the test specimens was compared with the reactivity of a panel of known negative stool samples. A confirmatory test was recommended for determining the status of borderline or weakly positive specimens. The performance of an extra confirmatory test, consisting of exogenous blocking (neutralization) of sample reactivity with high-titered antirotavirus serum, was thought to be unnecessary for routine use with each specimen tested. However, it became apparent after several years of experience with the test that the frequency of nonspecific reactions was a serious problem (35, 180, 341, 343). Nonspecific reactions to goat serum were first recognized in stools from patients living in certain geographic areas, especially the Far East, and were postulated to be related to patient diets. Other stools contained nonspecific antiglobulin activity related to copral IgM antibody present in the specimen. To circumvent problems of non-specificity, investigators have used various additions to stool specimens, including normal serum, reducing agent, chelating agent, protease inhibitors, and pH neutralization (137, 141, 146, 212, 341). Some nonspecific reactions could be eliminated by these measures. Experience at Children's Hospital in Washington, D.C., showed (35) that, of 5,626 fecal specimens tested, 1,344 gave positive results with the indirect EIA test, which could not be confirmed by electron microscopy, blocking EIA, confirmatory EIA, or a combination of these methods. Thus, 73% of 1,834 presumptively positive EIA tests were in fact not positive. False-positive reactions were especially common with specimens from hospitalized infants in a tertiary care nursery. It was suggested that nonspecific binding of antibody to intestinal bacteria or their products such as staphylococcal protein A may have been the basis of some of the false-positive reactions.

Based on this extensive study it is apparent that measures such as the addition of reducing agent are inadequate because they avert only some kinds of nonspecific reactions. Reliance on a panel of known negative stools as controls is also inadequate. Furthermore, all potential positive reactions must be confirmed, not just borderline specimens. As in the system originally used for Norwalk virus antigen detection (129), the reaction of each stool specimen in the microtiter plate well coated with rotavirus-positive (post-immunization) serum is compared with the reaction of the same specimen in a control well coated with rotavirus-negative (preimmunization) serum (163, 342). When a confirmatory test of this type was included in the indirect EIA at Children's Hospital in Washington, D.C. (35), false-positive results were not encountered in 400 consecutive tests.

The dangers of not using a confirmatory test have again been pointed out more recently with regard to the unreliability of the commercial Rotazyme EIA test for testing neonatal specimens (180). A large number of specimens have been tested by the Rotazyme assay in several laboratories (14, 48, 137, 146, 250) with favorable results as to the sensitivity of the test. The Rotazyme test, which is based on the use of anti-simian rotavirus serum (62), appears to be somewhat less sensitive in detecting HRV than most electron microscopic or indirect EIA assays. Despite the fact that the manufacturer (Abbott Laboratories) has not provided a confirmatory test, problems of non-specificity have only been reported with neonatal specimens. Apparently, the direct test design and special formulations of sample diluent and wash solutions are sufficient to eliminate many false-

positive results. However, with neonatal specimens, only 4 (7%) of 61 Rotazyme-positive stools were also positive by confirmatory tests such as electron microscopy, confirmatory EIA, and RNA dot hybridization (180). Laboratories may elect to use an exogenous confirmatory blocking test with any high-titered antirotavirus serum in conjunction with the Rotazyme test. Recent experience in our laboratory indicates that replacement of the polyclonal coating antibody with rotavirus-specific monoclonal antibody improves the sensitivity and specificity of the rotavirus immunoassay (66a). We have, however, not yet tested neonatal specimens. Monoclonal antibodies should be useful for development of serotype- and subgroup-specific EIA tests (see above).

Other techniques that have been used to detect rotavirus antigen have mostly proven to be less sensitive than the EIA. They include counterimmunoelectrophoresis (307), complement fixation (156), immune adherence hemagglutination (155), and other agglutination techniques (134, 258, 273). Direct detection of rotaviral nucleic acid in silver-stained polyacrylamide gels has also been utilized (139). The dot hybridization assay is a more specific test for rotavirus RNA. This assay is based on the in situ hybridization of labeled single-stranded RNA probes (obtained by in vitro transcription of rotavirus particles) to heat-denatured rotavirus RNA derived from test stools and immobilized on nitrocellulose membranes (110). This recently described test appears to be highly sensitive and specific and will be useful for epidemiological studies of rotavirus, particularly if probes for specific viral genes are used.

Pararotaviruses

Recently, a new viral agent termed pararotavirus has been observed in stool specimens from an Australian (243), a French (225), and two Bulgarian (75) infants. Pararotaviruses are morphologically indistinguishable from conventional rotaviruses, but they are antigenically distinct in that they lack the group-specific common antigen shared by all previously recognized rotaviruses from both mammalian and avian hosts. Pararotaviruses contain 11 segments of dsRNA which, however, yield a unique and characteristic electrophoretic migration pattern. Their lack of the group-specific antigen explains the negative result produced by pararotavirus in currently used rotavirus immunoassays. Based on available reports, human shedding of pararotavirus appears to be a rare event. However, we must keep in mind that, for a specimen to be recognized as containing pararotavirus, it must have been analyzed by a combination of tests including electron microscopy, immunoassay, and RNA gel electrophoresis. It is important to include the last test because antigenically distinct rotaviruses that produce conventional RNA patterns may exist (75). The medical importance of pararotaviruses as a cause of diarrheal illness cannot be assessed at this time. Antigenically distinct rotaviruses have also been observed in the feces of piglets and chickens (30, 37, 210, 254). The alternative term, "group B rotaviruses," has also been proposed to describe these agents (75).

NORWALK VIRUS

In 1972, a 27-nm particle was visualized (160) by IEM examination of an infectious stool filtrate derived from an outbreak of gastroenteritis which had occurred 4 years earlier in Norwalk, Ohio. This was the discovery of the first recognized human gastroenteritis virus of medical importance, the Norwalk agent. Its infectivity and pathogenicity were demonstrated by serial passage of the virus in adult volunteers (76, 327). The Norwalk particle is the prototype and best-studied member of a group of morphologically similar but immunologically mostly unrelated small round viruses (see "Small Round Virus Particles") which are commonly seen in human stool by electron microscopy. The medical importance and etiological roles of these Norwalk-like viruses are currently not established.

Norwalk virus has not been propagated in vitro in any cell or organ culture system. Administration of the virus to laboratory animals including a number of primate species has not resulted in the production of illness (326). Inoculated chimpanzees were, however, found to shed virus and demonstrate a Norwalk-specific immune response (328).

When Norwalk virus-containing fecal filtrate is administered orally to human volunteers, about 50% of inoculated individuals become ill (27). Diarrhea or vomiting or both may be seen among symptomatic volunteers who receive identical Norwalk inocula. No prolonged illness or long-term sequelae have been observed in Norwalk-inoculated subjects (27, 327). Much of what is known today about Norwalk virus has depended upon reagents gathered as a result of volunteer studies.

Biological Characteristics

The Norwalk virus is a 27-nm-diameter, nonenveloped, round particle of unclear substructure. It has a buoyant density in cesium chloride of 1.36 to 1.41 g/cm^3. Its infectivity remains stable after exposure to ether, acid, and heat. Based on similarities of stability, size, and density with the DNA-containing parvoviruses, Norwalk virus has been termed "parvovirus-like" (27, 76, 157). However, one report (122) indicates that the Norwalk virus particle, concentrated from feces, contains a single 66,000-molecular-weight protein which is characteristic of the RNA-containing caliciviruses (see "Caliciviruses"). A definitive classification of Norwalk virus cannot be made without determination of its nucleic acid. This, however, is likely to require the laboratory propagation of the virus, since it is shed in human stool in relatively low titer (126).

Diagnosis

Due to the small size and amorphous surface of Norwalk virus, IEM is required to directly visualize and identify the agent in stool samples. This procedure involves mixing a stool specimen with a drop of serum containing virus-specific antibody, concentrating the mixture, and observing the clumped virus-antibody complexes with an electron microscope. The degree of clumping can also be used to rate the amount of antibody present in an unknown serum. IEM is cumbersome and impractical to perform on large numbers of specimens and requires specialized equipment and personnel.

The development of an RIA for the detection of Norwalk virus in stool as well as for the measurement of serum antibody to the virus resulted in major advances in the study of this agent (22, 129). The availability of the RIA permitted the rapid testing of large numbers of specimens required for epidemiological studies. The RIA test procedure, however, requires the use of carefully defined fecal and serum samples obtained from experimentally infected volunteers and is therefore limited to a few research laboratories that possess small amounts of these valuable reagents of human origin. Norwalk virus has not been purified sufficiently to permit preparation of hyperimmune animal serum reagents usable for diagnostic purposes. Development of diagnostic reagents

that would circumvent the current reliance on volunteer materials is highly desirable. The resulting wider availability of Norwalk virus testing would permit a greatly expanded epidemiological surveillance and evaluation of disease outbreaks by public health authorities and would allow for the study of Norwalk virus by additional research laboratories. In addition, RIAs similar to that developed for Norwalk virus are greatly needed for the other Norwalk-like viruses.

Epidemiology

Seroepidemiological studies indicate that infection with Norwalk virus occurs worldwide (20, 26, 64, 123, 129, 158). In the United States approximately two-thirds of adults possess serum antibody to Norwalk virus. Serum antibody is uncommon during childhood but is acquired rapidly during late adolescence (26, 158). These findings correlate with the lack of association of Norwalk virus with severe gastroenteritis of infancy in the United States (36, 236). However, in less-developed nations serum antibody to Norwalk virus commonly appears during childhood (64, 123) and may produce some mild illness in the younger age group (20). Norwalk virus, in contradistinction to rotavirus, is currently regarded as primarily a pathogen of older children and 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 Norwalk virus (164). This high percentage suggests that, even though a large variety of viruses are observed in stool, only a few viral serotypes cause most outbreaks of gastroenteritis. Outbreaks associated with Norwalk virus have occurred in the following settings: in recreational camps, on cruise ships, in regions with contaminated drinking or swimming water, in schools and nursing homes, and in association with ingestion of raw shellfish or other uncooked foods such as salads or cake frosting handled in an unsanitary manner (117, 125, 131, 164–167, 221, 295, 321; J. N. Kuritsky, M. T. Osterholm, H. B. Greenberg, J. A. Korlath, J. R. Godes, C. W. Hedberg, J. C. Forfang, and A. Z. Kapikian, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 23rd, abstr. no. 419, p. 159, 1983; K. E. White, M. T. Osterholm, J. A. Korlath, J. A. Mariotti, J. N. Kuritsky, D. Lawrence, and Investigative Team, Program Abstr. Intersci. Conf. Antimicrob. Agents. Chemother. 23rd, abstr. no. 361, p. 149, 1983). Some of the outbreaks associated with contaminated food or municipal drinking water have involved thousands of individuals (165, 221; Kuritsky et al., 23rd ICAAC, abstr. no. 419). A small percentage (3 to 15%) of travelers diarrhea has also been associated with Norwalk virus (82, 168, 251).

It is clear from volunteer studies (27, 327), as well as from epidemiological reports, that Norwalk virus is transmitted by the fecal-oral route. About half of ill volunteers shed detectable virus in the feces during the first 72 h after the onset of illness (297). Respiratory symptoms have not been associated with Norwalk illness, and throat washings from ill volunteers were noninfectious when administered to a few subjects (76). The strikingly rapid secondary spread of Norwalk virus infection may involve airborne transmission via aerosolized virus-containing vomitus (127).

Pathogenesis

A characteristic though not unique histological lesion of the mucosa of the proximal small intestine has been observed during the first 2 weeks after inoculation of volunteers with Norwalk virus (267). This transient lesion has occurred in all ill subjects examined and in a few asymptomatic volunteers who developed seroconversion to the virus. The virus has not been detected within involved mucosal cells, perhaps because of its small size and patchy distribution. The gastric and colonic mucosa remain histologically normal during Norwalk illness (2, 211, 319). Malabsorption of fat and xylose occur during illness and may persist for at least 1 week even though clinical symptoms last for only 1 or 2 days (27).

Immune Response and Clinical Immunity

Our understanding of the immune response and clinical immunity associated with Norwalk virus infection is derived primarily from human volunteer studies. The striking pattern of clinical immunity seen in these volunteers fails to fit immunological concepts usually associated with common viral illness (26, 230). Only about half of unselected subjects inoculated with Norwalk virus become ill. The rise in serum antibody seen after Norwalk illness appears to be a marker for infection in susceptible individuals but lacks a protective role. Norwalk illness commonly occurs in the presence of serum antibody. By contrast, volunteers who resist illness usually have low to absent levels of serum antibody both before and after exposure to the virus. Intestinal antibody levels appear to follow the same pattern as serum antibody (26, 126). Paradoxically, then, the presence of preexisting Norwalk virus-specific antibody and the ability to generate it constitute risk factors for this illness.

When a group of 12 volunteers was inoculated with Norwalk virus and then rechallenged 27 to 42 months later, precisely the same 6 volunteers who became ill on the initial challenge became ill again on rechallenge (230). Significant antibody rises, usually from base lines of preexisting antibody, occurred with each illness. Those six volunteers who were clinically well on the initial challenge remained unaffected on rechallenge. Antibody responses and prechallenge antibody were for the most part absent in these non-ill individuals. Interestingly, short-term resistance to Norwalk illness was observed in that most previously ill volunteers remained well when rechallenged 4 to 14 weeks later (26, 27, 126, 230).

The reason for the pattern of clinical immunity to Norwalk virus is unknown. Speculation has centered on the genetic control of Norwalk virus resistance or susceptibility, perhaps on the level of intestinal receptor sites. A limited number of inoculated volunteers have been studied for the histocompatibility loci A, B, and D, but no correlation with resistance or susceptibility to Norwalk illness has been found. An alternative explanation for the pattern of clinical immunity is that repetitive exposures to the virus are needed to generate eventual immune response as well as concomitant illness. According to this hypothesis, the resistant adult volunteers are not "primed" due to fewer naturally occurring previous exposures to the virus than susceptible subjects. The volunteer studies indicate that immunity to Norwalk virus is not long lasting and that bouts of illness throughout life would seem possible.

The serum IgM response to Norwalk virus of inoculated volunteers has been investigated (66). An IgM response has occurred in subjects who became ill, whether or not prechallenge total serum antibody was present. The peak IgM response occurred at about 2 weeks after illness but IgM was detectable in lower titers for up to 21 weeks after infection. On long-term rechallenge, volunteers who were previously ill and had produced IgM antibody again became ill, and a secondary IgM response, greater than the first, was seen. Non-ill, challenged volunteers, as well as previously ill

volunteers on short-term rechallenge, usually failed to generate an IgM response, whether or not they had an IgG response. It seems clear that virus-specific IgM is not necessarily indicative of primary infection with Norwalk virus inasmuch as reinfection produces an enhancement of the IgM response. Furthermore, Norwalk-specific IgM responses appear not to be associated with subclinical illness. IgM testing has yet to be reported for naturally occurring Norwalk virus disease outbreaks.

Prevention and Control

Inasmuch as Norwalk infections are often associated with contaminated water, a useful step in infection control would be a readily available test which was sensitive enough to detect the virus in environmental specimens. The currently available RIA test is not sufficiently sensitive for this purpose, and, as discussed above, it is restricted to a few laboratories.

Based on our knowledge of the clinical immunity to Norwalk virus, prospects for immunoprophylaxis are not encouraging at this time. Since ill individuals can redevelop disease 2.5 to 3.5 years later upon rechallenge with identical inocula, the use of a vaccine for producing long-lasting immunity seems improbable. Perhaps short-term immunity could be produced by vaccination of individuals at high risk of short-term exposure, such as travelers. However, the technology for producing a vaccine for Norwalk virus is undeveloped, because the agent has yet to be cultivated in vitro or even to be purified extensively from feces.

ENTERIC ADENOVIRUSES

A unique subgroup of adenoviruses, termed enteric or fastidious adenoviruses, is emerging as a cause of diarrheal illness in infants and young children, although the epidemiological importance of these viruses has yet to be fully defined. The enteric adenoviruses differ from the well-characterized 39 conventional serotypes of adenoviruses which are readily propagated in standard tissue cultures such as human embryonic kidney cells; many of these cultivatable serotypes are well known as etiological agents in a wide range of human diseases, including respiratory tract infections and conjunctivitis. Large-scale epidemiological studies failed to establish conclusively an etiological role for these cultivatable adenoviruses in infantile gastroenteritis because these viral strains were also present in the feces of many asymptomatic children (149, 216, 336, 346). During the 1970s, non-cultivatable adenoviruses were demonstrated by electron microscopy in the stools of children with diarrhea (103, 238, 266). A potential medical importance of these adenoviruses in diarrheal illness began to emerge when several investigators pointed out that strains which were visualized by electron microscopy but which could not be cultivated in human embryonic kidney cells were more highly associated with gastroenteritis than were adenoviruses which could be readily grown in vitro (36, 197, 237). It was also recognized that the fastidious adenoviruses purified from the stools of children with acute gastroenteritis make up distinct types of adenovirus determined both by antigenic and DNA restriction endonuclease banding techniques (114, 148). Based on these techniques, two new serotypes of adenovirus have been characterized, designated types 40 and 41, also representing two new subgroups, F and G, which have been isolated from cases of infantile gastroenteritis (72, 309, 320).

The enteric adenoviruses have been recognized as being, after rotavirus, the second most commonly identified agent in stools of infants and young children with viral gastroenteritis (34, 169, 309, 313, 339) in various parts of the world. Enteric adenoviruses may also be found in asymptomatic children, but less frequently. In a prospective study of 410 Swedish children with acute gastroenteritis a viral agent was identified in 57% (309). Rotavirus was the major agent found in 43% of patients, whereas adenovirus was found in 13% of ill children and 1.5% of controls. (In a U.S. study, 3.9% of ill children versus 0.6% of controls shed enteric adenovirus [36].) Seventy percent of adenovirus strains were identified as enteric adenoviruses in the Swedish study. Whereas patients with enteric adenovirus infections tended (71%) to seroconvert by the hemagglutination inhibition test, seroconversion was not seen in patients shedding conventional adenoviruses. Infection with enteric adenoviruses occurred early in life, with 85% of patients being less than 3 years old. Diarrhea was the main symptom, lasting for an average of 9 days. Fever and vomiting was mild, with a mean duration of 2 days. Respiratory symptoms occurred in 20% of the cases in the Swedish study and were seen even more frequently in the United States (339). Infection with enteric adenoviruses in Sweden showed two small seasonal peaks in summer and late winter but no seasonality was noted in the United States (34) or Finland (313).

Both group-specific (anti-adenovirus type 2 hexon antigen) (4, 135) and type-specific (types 40 and 41) (148, 309) solid-phase immunoassays have been developed for the detection of enteric adenoviruses in stool specimens. When human embryonic kidney cells are infected with enteric adenoviruses, a transient cytopathic effect which cannot be passaged may sometimes be seen (72, 197), and detection of intracellular adenovirus antigens by immunofluorescence has been reported (114, 237, 238). Some strains of fastidious adenoviruses have reportedly produced cytopathic effect over a small number of passages in Chang conjunctiva cells (72, 170). The enteric adenoviruses, however, have been grown efficiently in Graham 293 cells, an adenovirus type 5-transformed human embryonic kidney cell line (290). The abortive infection of untransformed cells by enteric adenoviruses is due to an early replicative block restricting viral DNA synthesis (289). Early enteric adenovirus functions are, however, expressed in untransformed cells as evidenced by fluorescent antibody-stainable viral antigen and complementation of adeno-associated virus (289). It has been postulated that the adenovirus-transformed cell line enables growth of enteric adenoviruses by providing early viral functions. According to these authors (289) enteric adenoviruses can be functionally defined as those adenoviruses that grow in transformed 293 cells but not in untransformed parental human embryonic kidney cells. It is interesting to speculate whether perhaps the enteric adenoviruses are able to grow to high titer in a child's gastrointestinal tract due to either a concurrent infection with conventional adenoviruses (possibly responsible for the observed respiratory symptoms) or the presence of latent adenoviruses which may be shed in stool for months after a respiratory infection. It has also been suggested that some normal human cells may express early adenovirus sequences (289).

CALICIVIRUSES

The name calicivirus is derived from the characteristic cup-shaped (chalice) indentations observed by electron microscopy on the surface of negatively stained virions. The caliciviruses, whose recognized members include the vesicular exanthema of swine virus, feline calicivirus, and San

Miguel sea lion virus, are about 31 to 35 nm in size, contain single-stranded RNA as their genome, and were formerly classified as a genus of the family Picornaviridae. With the recognition of their distinct morphology (sometimes described as a Star of David appearance), a single major polypeptide composition, and differing genome strategy, they have been reclassified as a separate family, Caliciviridae (264).

Caliciviruses of human origin were first reported to be observed by electron microscopy in stools of children with gastroenteritis in 1976 (104, 195). Since that time continually accumulating evidence has suggested the etiological role of this virus in human diarrheal illness. The agents of human origin are termed calicivirus based upon their observed morphology, size, and buoyant density, although the latter two properties are shared by several groups of viruses. These viruses, like several other agents of viral gastroenteritis, have not been cultivated in vitro. They are not known to cross-react immunologically with animal caliciviruses. Although the classical caliciviruses of animals are readily propagated in culture and do not produce gastrointestinal disease, non-cultivatable calicivirus-like particles have been observed in association with diarrhea in calves and pigs (254, 324).

Community and nosocomial outbreaks, as well as sporadic cases of gastroenteritis associated with human calicivirus, have been reported from Japan (49, 50, 227, 255, 288), England (58–61), Canada (282), and Scandinavia (173). Seroconversions and rises in calicivirus-specific IgM antibody have been demonstrated in ill subjects by IEM (50). Even though caliciviruses are sometimes seen in asymptomatic individuals, this finding is not inconsistent with subclinical infection. Calicivirus particles were not found in any of seven stool specimens obtained by chance 2 to 10 days before a diarrheal outbreak in a Japanese orphanage. However, the virus was detected in 18 (95%) of 19 fecal samples collected within 4 days after the outbreak. These included three samples from asymptomatic infants who nonetheless seroconverted (50).

Laboratory studies with human calicivirus have relied until recently on electron microscopy. With the recent development of a microtiter solid-phase RIA for the detection of human calicivirus in stools (223), additional information with regard to the significance of this agent in human disease should be more readily forthcoming.

The epidemiological and clinical features reported for human calicivirus appear to be similar to those of rotavirus. Antibody to calicivirus is rapidly acquired between 6 and 24 months of age, and 90% of older children and adults have serum antibody (255). Symptomatic illness most often occurs in younger children but not in neonates. One outbreak of illness has also been reported among residents of a home for the elderly (61). Duration of calicivirus illness is similar to that of rotavirus as are the reported symptoms; however, symptoms are usually somewhat milder with calicivirus (58).

It has been suggested that Norwalk virus may be a calicivirus, based on its polypeptide composition and small size (122). No studies have been reported, however, on any potential antigenic relatedness between human calicivirus and Norwalk virus. The reported sizes of these two viruses differ slightly, with calicivirus being approximately 31 to 35 nm in diameter and Norwalk virus being 27 nm. These measurements have been made by different investigators, and careful comparative electron microscopic studies are needed. It should also be noted that antibody prevalence levels rise at a much earlier age for calicivirus than for Norwalk virus in populations residing in developed, nontropical areas.

ASTROVIRUSES

Small round virus-like particles, 29 to 30 nm in size, have been observed in the feces of infants with mild gastroenteritis and are named astroviruses based on their characteristic six-pointed-star morphology (196). Experimental inoculation (182) of eight adult volunteers with an astrovirus-containing fecal filtrate obtained from a child with mild gastroenteritis resulted in development of vomiting and diarrhea by one subject, who concurrently shed large amounts of virus. Nine other volunteers were given a fecal filtrate derived from the volunteer with gastroenteritis, and astrovirus shedding subsequently occurred in two of them, without, however, diarrhea or vomiting. Virus-specific antibody rises were noted in 13 of 16 inoculated volunteers. It appears that the astrovirus strain studied causes a transmissible infection that is of low pathogenicity for adults.

A seroepidemiological study indicates that in a British population over 70% of individuals have acquired antibody to astrovirus by 3 to 4 years of age (181). Several pediatric outbreaks of gastroenteritis have been associated with astrovirus (8, 175, 183). In a recent report from Japan (175), 43 (54.2%) of 84 children in a kindergarten were ill during an outbreak of gastroenteritis. Three acute-phase stool specimens were examined by electron microscopy and astrovirus was found in two of them. In addition, an IEM-determined virus-specific antibody rise was demonstrated in all of six paired sera obtained from ill children. Paired sera from individuals infected with Norwalk virus, calicivirus, and Otofuke agent (see "Small Round Virus Particles") failed to show a serological response to astrovirus by IEM (175).

Although astrovirus has not been propagated serially in vitro, immunofluorescence-stainable viral antigens have been noted in inoculated primary human embryonic kidney cells (182).

CORONAVIRUSES

Coronaviruses (208) are medium-sized, lipid-containing, enveloped viruses with an RNA genome which are recognized as a distinct genus. They possess a distinctive morphology in that they appear as round, oval, or moderately pleomorphic particles with widely spaced club-shaped projections in negatively stained electron microscopic preparations. The overall diameter of the particles, including projections, is 80 to 180 nm, and the projections themselves are 18 to 20 nm long. Coronaviruses are recognized pathogens in many animal species and include agents which cause hepatitis and encephalitis in mice, bronchitis in chickens, and severe gastroenteritis in neonatal calves, piglets, and dogs. Human coronaviruses, which have been cultivated most successfully in tracheal organ culture, produce a mild upper respiratory infection with characteristics of the common cold.

In the past 8 years a number of investigators have observed coronavirus-like particles (CVLPs) in stool specimens from adults and children with gastroenteritis as well as from healthy subjects (reviewed in references 142 and 194). Fecal CVLPs have been reported from various parts of the world, including Europe (45, 54, 205, 286), Australia (265), India (203), and South America (202). The association of CVLPs with gastroenteritis cannot be made at this time since in most reports virus was observed in a similar proportion of ill and non-ill individuals. For example, in one British study (54) fecal CVLPs were seen in specimens from 15 (4.2%) of

355 adults with diarrhea and from 5 (5.2%) of 96 adults without diarrhea. Similar results are reported from Germany (205) and India (203), but in Australia (265) CVLPs were seen more frequently in neonates with gastroenteritis than in controls. Further confounding the association of CVLPs with disease is the frequent observation of individuals who chronically excrete the particles over a period of months or years (54, 203, 217). One group of workers has reported that CVLPs were shed in the feces of a high proportion of ill babies during an outbreak of severe necrotizing enterocolitis in a maternity unit in France (286). This agent, in contrast to other enteric CVLPs of human origin, was readily propagated in cell culture. However, this unique observation awaits confirmation, since no structural details of the original agent or immunological or biophysical characterization of the cultivated isolate have been published and since the possibility of culture contamination by a coronavirus of bovine origin has been raised (194). In another study (44), 1 of 15 CVLP-containing fecal specimens showed some evidence of viral replication upon inoculation of primary human embryonic kidney monolayers and human fetal intestinal organ cultures, but the virus could not be further passaged.

In summary, the presence of CVLPs in human feces has not been conclusively associated with gastroenteritis, and characterization of the particles as coronavirus-like depends entirely on morphological criteria.

SMALL ROUND VIRUS PARTICLES

In recent years, electron microscopic examination of human diarrheal stool specimens has revealed a variety of noncytopathic small round virus-like particles (SRVs). The first recognized SRV, described in 1972 (160), was the 27-nm Norwalk virus (see above). The etiological role of Norwalk virus in human gastroenteritis is well established. Other SRVs of similar size, i.e., 25 to 35 nm, have been observed, some of which possess a distinctive morphology and others of which lack this distinctiveness. The astroviruses and calicivirus-like particles are examples of agents with a characteristic definable structure and have already been discussed above.

The SRVs in the 25- to 35-nm size range without distinctive morphology have been referred to as Norwalk-like or parvovirus-like viruses. Although an attempt has been made to subdivide this group based on electron microscopic substructure (43), there is no general agreement on this. This group of agents, in general, shares properties of morphology, density, and derivation from epidemics or family outbreaks of gastroenteritis. They are immunologically distinguishable from hepatitis A virus. Agents belonging to this group have usually been named by the geographic location of the outbreak or the presumed source of contagion. Examples of Norwalk-like viruses include Hawaii (298), W-Ditchling (5), Cockle (6), Parramatta (51), Marin County (228), and Snow Mountain (77). Other unnamed amorphous SRVs in this size class have also been frequently observed in association with gastroenteritis (e.g., 179, 214, 241). By IEM testing, the Norwalk, Hawaii, and W-Ditchling agents are recognized as being serologically distinct, and the Marin County and Snow Mountain agents show antigenic differences from one another and from Hawaii agent and Norwalk virus. Thus, based on IEM testing, there is some evidence to suggest four or five antigenically distinct agents: Norwalk, Hawaii, Marin County, Snow Mountain, and perhaps W-Ditchling. However, this evidence is based mostly on IEM studies, using convalescent human sera, and definitive antigenic analysis is currently lacking. The antigenic relationships of the other agents in the Norwalk-like virus group are not known. The transmissible infectious and pathogenic nature of Norwalk, Hawaii, W-Ditchling, and Snow Mountain agents has been demonstrated by human volunteer studies, some of which have also shown a lack of cross-protection between Norwalk and Hawaii agents in short-term rechallenge studies (327). The medical importance of the Norwalk-like viruses, with the exception of Norwalk virus itself, remains to be established.

The suggestion has been made (241) that a group of slightly larger SRVs (33 to 40 nm), which have been associated with diarrhea in several areas of the world, may actually represent a single group. They have been variously termed minireovirus (214), minirotavirus (282), Otofuke (294), and Sapporo (178) agents. As a group these agents share similar size range and some common morphological features. The Otofuke agent has produced naturally occurring disease in several outbreaks affecting children and adults. By IEM testing, it is distinct from Norwalk virus, W-Ditchling, and calicivirus-like particles (293). SRVs of all classes were recently observed in fecal samples from infants with diarrhea who were admitted to a New York State hospital over a 2-year period (241).

Within a single outbreak SRVs are usually described as being consistent in size and appearance, but there is often variation among outbreaks. Some of this variation may be due to extraneous factors such as specimen storage, electron microscopic technique including microscope calibration, presence of copral antibody (224), and, of course, the interpretation of the investigator. Development of immunoassays for SRVs, continued search for ways to propagate them in vitro, and frequent exchange of materials among laboratories would be helpful in delineating the role in human disease of these frequently observed but poorly understood agents.

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