CHAPTER 24

Coronaviridae: The Coronaviruses

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Disease: Respiratory illness; possibly infant gastroenteritis.
Etiologic Agents: HCV 229E, OC43; HECV.
Source: Respiratory secretions/fomites; presumably fecal for HECV.
Clinical Manifestations: Upper respiratory illness, coryza, fever; rare lower respiratory tract disease; possible gastroenteritis.
Pathology: Typical viral upper respiratory infection with inclusions.
Laboratory Diagnosis: Indirect fluorescent antibody and enzyme immunoassay on direct respiratory secretions; electron microscopy and immune electron microscopy on stool specimens; hemagglutination inhibition, standard serum neutralization, and enzyme immunoassay serologic tests are best.
Epidemiology: Worldwide.
Treatment: None.
Prevention and Control: None.

Description of Disease

Human coronaviruses (HCV) were discovered in the 1960s during the period of active searching for agents of the common cold. The first evidence that a new group of viruses was involved in human respiratory illness came from Salisbury, England in 1962, when volunteers who were given respiratory secretions developed colds; however, no virus or bacteria could be isolated in the conventional systems then in use (Kendall et al., 1962). Further evidence was obtained, although not recognized at the time, when antibodies to mouse hepatitis viruses (MHV) were found in human sera (Hartley et al., 1964). Shortly thereafter, viruses from the Salisbury study that were distinct from other known respiratory pathogens were grown in human fetal tracheal organ culture, in which viral replication was evidenced by the reduction of ciliary activity in the epithelial cells (Tyrrell and Bynoe, 1965, 1966). Further proof of the involvement of these new viruses in respiratory disease was obtained epidemiologically and by volunteer transmission studies (Bradburne et al., 1967; Tyrrell and Bynoe, 1966).

During this time, another new respiratory virus was recovered in HEK cells from a group of medical students in Chicago (Hamre and Procknow, 1966), and additional viral strains, unrelated to those discussed previously, were isolated in tracheal organ cultures from adults in Washington, D.C. (McIntosh et al., 1967b). The latter were quickly adapted to suckling mouse brain and were shown to be morphologically identical to MHV (McIntosh et al., 1967a). In 1967, all of these new viruses were recognized as morphologically identical to each other and to avian infectious bronchitis viruses, yet distinct from all other known viruses (Almeida and Tyrrell, 1967; Becker et al., 1967; Hamre et al., 1967; McIntosh et al., 1967a; Tyrrell and Almeida, 1967). Because all were characterized by a "corona" of spikes projecting from the membrane, they were termed *coronaviruses*. Thus was established a new and distinct group of viruses, with representative strains already known to infect mice, humans, and chickens (Tyrrell et al., 1968).

Classification of these viruses within the family *Coronaviridae* by the International Committee on Taxonomy of Viruses (ICTV) was made according to similarities in their fine structure as revealed by electron microscopy and, to a lesser extent, their bio-

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logic and antigenic properties (Almeida and Waterson, 1970; Davies and Macnaughton, 1979; Oshiro, 1973; Tyrrell et al., 1975, 1978). The most recent definition of *Coronaviridae* (Third Report of the Coronavirus Study Group, Vertebrate Virus Subcommittee, ICTV) still places primary emphasis on morphology, followed by unique structural, replicative, antigenic, and biologic properties (Siddell et al., 1983a). These unique features are the focus of this chapter, with particular emphasis on the laboratory diagnosis of HCV infections.

The Viruses

Recognized Strains

Of the many coronaviruses putatively associated with human disease, only two are well established in the literature. Progress in identifying new strains has been slow because of their fastidious growth requirements in culture, where culture is possible, and their general physical lability. Furthermore, the occurrence of similar, enveloped-like particles in specimens from nonill control patients has often complicated the establishment of a definite etiology.

The first four HCVs identified—strains B814, 229E, OC43, and 692—were associated with upper respiratory disease (Table 1). Strain B814 (Tyrrell and Bynoe, 1965) was subsequently lost in the laboratory and so cannot be compared with later strains. Strain 229E, isolated from a specimen collected from a patient with upper respiratory infection in January 1962 and selected as the prototype of five strains isolated at that time (Hamre and Procknow, 1966), has been found worldwide in numerous serologic and epidemiologic studies (Bradburne and Somerset, 1972; Cavallaro and Monto, 1970; Hamre and Beem, 1972; Isaacs et al., 1983; Kapikian et al., 1969; Kaye and Dowdle, 1975; Larson et al., 1980; Wenzel et al., 1974). Strain 229E, although usually associated with mild to moderately severe colds in children and adults, can cause tonsillitis, otitis, and pneumonia in young children (Isaacs et al., 1983; McIntosh et al., 1970b, 1974). Currently, strain 229E is considered to represent a subgroup of antigenically related isolates (Macnaughton et al., 1981b; Monto and Lim, 1974; Reed, 1984).

Strain OC43, from a nasopharyngeal wash collected in January 1966, was selected as the prototype of six "IBV-like" strains isolated in fetal tracheal organ culture (McIntosh et al., 1967b). It also has been associated with upper respiratory illness worldwide (Bradburne and Somerset, 1972; Hovi et al., 1979; Isaacs et al., 1983; Kaye et al., 1971; Monto and Lim, 1974; Riski et al., 1974; Wenzel et al., 1974). Strain OC43 has been associated with sporadic cases of bronchitis, pneumonia, and possibly central nervous system disease, pericarditis, pancreatitis, and gastrointestinal disease. However, unlike 229E, these infections can occur in adults as well as children (Isaacs et al., 1983; McIntosh et al., 1970b, 1974; Riski and Hovi, 1980; Wenzel et al., 1974). Like 229E, strain OC43 also has been shown to represent a group of related but heterogeneous strains (Macnaughton et al., 1981b; McIntosh et al., 1967a, 1970b).

Strain 692 was shown to be a coronavirus and unrelated to either 229E or OC43 by immune electron microscopy (Kapikian et al., 1973). It was detected in organ culture harvests of a nasopharyngeal wash collected from a 29-year-old man in January 1966. No further laboratory or epidemiologic work on this virus has been reported.

Tettnang virus was, for a short time, another coronavirus associated with human respiratory disease. Tettnang was isolated in 1978 from the cerebrospinal fluid of a 1-year-old girl with rhinitis, pharyngitis, and mild encephalitis (Malkova et al., 1980). However, the virus was recovered only in suckling mouse brain in single mice on second passage, and exhibited a high degree of cross-reactivity with MHV and HCV OC43 antisera by indirect fluorescent-antibody and complement fixation tests. Also, anti-Tettnang serum, prepared in mice, reacted with MHV and OC43 antigens. Therefore, this isolate appeared to be reactivated MHV or at least heavily contaminated

TABLE 1. Coronaviruses in human respiratory disease

Strain	*Specimen*	*Year*	*Patient*	*Illness*	*Original reference*	*Status*
B814	NS/NW	1960	Youth	Common cold	Tyrrell and Bynoe (1965)	1[a]
229E	NPS	1962	Young adult	Minor URI[b]	Hamre and Procknow (1966)	2
OC43	NPW	1966	Young adult	URI	McIntosh et al. (1967b)	2
692	NPW	1966	29, male	URI	Kapikian et al. (1973)	3

[a] 1 = Strain lost in laboratory; 2 = 229E-like subgroup of strains and OC43-like subgroup of strains are well established virologically and epidemiologically (see text); 3 = definitive studies on viral characterization or etiologic association not yet reported.
[b] URI = Upper respiratory infection.

with MHV. A subsequent study by Bardos et al. (1980) in fact confirmed Tettnang as MHV.

Putative Strains

Many tentative associations have been reported between coronavirus infection and other diseases, such as hepatitis, nephropathy, and multiple sclerosis, and even more attempts have been made to link coronaviruses to necrotizing enterocolitis, gastroenteritis, and other human diarrheal diseases (Table 2). All of these diseases will be discussed in detail. However, it is important to keep in perspective that as of 1988, an etiology has been established only for the common cold coronaviruses HCV 229E and OC43, which have been isolated and thoroughly characterized in the laboratory.

Coronavirus-like particles (CVLPs) were found in sera from a number of patients with chronic active hepatitis who were negative for Australia-SH antigen (Ackermann et al., 1974; Holmes et al., 1970;

TABLE 2. Observations of coronaviruslike particles in humans

				Direct EM[a]		*Culture*[a]		
Country	*Season*	*Age*	*Disease*	*P/T*	*Specimen*	*P/T*	*Serologic confirmation*	*Reference*
England		Adults	Hepatitis	1/2	Serum		Yes	Zuckerman et al. (1970)
United States		Adults	Hepatitis	2/2	Serum			Holmes et al. (1970)
England		Adults	Var. liver	+	Serum			Wright (1972)
Canada		Adults	Hepatitis	11	Serum			Ackerman et al. (1974)
Yugoslavia		All	Nephropathy	7	Kidney		Yes	Apostolov et al. (1975)
Romania		All	Nephropathy	+	Kidney			Georgescu et al. (1978)
United States		33 yr	MS	1	Brain			Tanaka et al. (1976)
England		Young adult	Gastroenteritis	6/9	Stool	0/6		Caul et al. (1975)
	Fall	Young adult	Gastroenteritis	>1	Stool	1/1	Yes	Caul and Clarke (1975)
	Summer	Young adult	Gastroenteritis	Many	Stool			Caul and Clarke (1975)
	Winter	Young adult	Homosexual	8/23	Stool			Riordan et al. (1986)
	Winter	Young adult	AIDS	1/1	Stool			Riordan et al. (1986)
India		Adults	Sprue	14/16	Stool	0/14		Mathan et al. (1975)
		Adults	None	27/29	Stool			Mathan et al. (1975)
		Children	None	10/12	Stool	0/10		Mathan et al. (1975)
Australia	All	Infants	Gastroenteritis	18/94	Stool			Moore et al. (1977)
		All	None	10/65	Stool			Moore et al. (1977)
	All	Children	Gastroenteritis	37/55	Stool			Schnagl et al. (1978)
	Fall	Children	Gastroenteritis	157/537	Stool		Yes	Schnagl et al. (1978, 1986)
	Fall	Children	None	145/226	Stool		Yes	Schnagl et al. (1978, 1986)
France	All	Children	Gastroenteritis	25/190	Stool	4/4		Peigue et al. (1978)
	Summer	Infants	NNEC	30	Stool	1	Yes	Sureau et al. (1980)
	Winter	Infants	NNEC	23/32	Stool	2	Yes	Chany et al. (1982)
	Winter	Infants	Diarrhea	2/12	Stool			Chany et al. (1982)
	Winter	Infants	None	3/47	Stool		No	Chany et al. (1982)
	Winter	Children	Diarrhea	16/19	Stool		Yes	Chany et al. (1982)
	Winter	Adults	None	17/75	Stool		Yes	Chany et al. (1982)
	Summer	Infants	NNEC	8	Stool			Caldera and Badoual (1982)
Germany	Fall, winter, spring	Infants	Diarrhea	15/24	Stool	1	Yes	Maass and Baumeister (1983)
	Fall, winter, spring	Infants	Malaise	5/8	Stool			Maass and Baumeister (1983)
	Fall, winter, spring	Adults	Gastroenteritis	7/116	Stool			Maass and Baumeister (1983)
	Fall, winter, spring	Adults	None	16/265	Stool			Maass and Baumeister (1983)
Italy		Infants	Gastroenteritis	34/208	Stool		Yes	Gerna et al. (1984)
		Infants	None	3/182	Stool			Gerna et al. (1984)
United States	Fall	Infants	Gastroenteritis	32/88	Stool		Yes	Vaucher et al. (1982)
	Winter	Children	Diarrhea	17/38	Stool		Yes	Vaucher et al. (1982)
	Fall, winter	Children	Diarrhea	49/126	Stool			Mortensen et al. (1985)
	Winter	Infants	NNEC	7/15	Stool	2/15	Yes	Resta et al. (1985)
Gabon	Fall, spring	Children	Diarrhea	60/156	Stool			Sitbon (1985)
	Fall, spring	Children	None	75/115	Stool			Sitbon (1985)

[a] EM = electron microscopy; P/T = number of positive specimens over the total number tested, where given; MS = multiple sclerosis; NNEC = neonatal necrotizing enterocolitis.

Wright, 1972; Zuckerman et al., 1970). These particles are occasionally seen in sera from nonill humans and monkeys, suggesting that, at best, coronaviruses are only rarely hepatotrophic in humans (unpublished observations).

In addition to liver disease, coronaviruses may occasionally be involved in human kidney disease. Endemic (Balkan) nephropathy, a slow degenerative kidney disease affecting villagers in close contact with swine herds in Yugoslavia, Bulgaria, and Romania, was first described in the late 1950s, but only recently was associated with porcine coronaviruses. Coronavirus-like particles were seen throughout the nephron in seven patients with endemic nephropathy, and only those families involved in pig husbandry developed the disease (Apostolov et al., 1975). However, attempts to detect swine coronavirus antibodies in the sera of patients with nephropathy using a local HEV antigen were unsuccessful, suggesting that the etiologic agent is unrelated to HEV or its antigenic cousin, transmissible gastroenteritis virus (Georgescu et al., 1978).

Coronavirus-like particles were seen in active lesions in brain tissue from one patient with multiple sclerosis (Tanaka et al., 1976). Later, two coronaviruses (SD and SK) were isolated in suckling mouse brain from the brain tissue of two other patients with multiple sclerosis (Burks et al., 1980). These SD and SK viruses cross-reacted antigenically with OC43, and suitable caution had been exercised regarding the possibility of reactivating latent MHV in the host mice (Gerdes et al., 1981b). Nonetheless, SD and SK viruses were shown to possess 90% of the MHV-A59 RNA genome (Weiss, 1983). Furthermore, neither SK antigen nor OC43 RNA could be detected directly in brain tissue from these patients (Burks et al., 1984; Sorensen et al., 1986). Thus, these isolates are now considered to be adventitious murine contaminants. In an independent study, levels of coronavirus antibodies in patients with multiple sclerosis were identical to those in control groups (Leinikki et al., 1981). No solid evidence currently exists to associate coronaviruses with multiple sclerosis in humans.

Numerous groups have reported an association between CVLPs in stool specimens and human diarrheal disease (reviewed by Macnaughton and Davies, 1981; Resta et al., 1985), but few report an association between the development of serum antibody to CVLPs and recovery from infection (Schnagl et al., 1986). For simplicity, and in anticipation that an etiology will be established, the CVLPs in stool specimens are presently called *human enteric coronaviruses* (HECV). In this respect, there is a parallel with the human enteric adenoviruses that were denoted as a subgroup long before they were actually isolated in the laboratory. Human enteric coronaviruses have been sought and tentatively identified by electron microscopy in various parts of the world. These studies will be summarized here in some detail to draw together common findings and emphasize the need for further research.

The earliest reported outbreaks were in England in 1965 (Weston), 1971 (Bristol), and 1975 (Somerset), in all of which CVLPs were seen in fecal specimens from young adults with gastroenteritis (Caul and Clarke, 1975; Caul et al., 1975). In the Weston outbreak in 23 patients, most had vomiting, 50% had diarrhea, and a few had fever or pharyngitis; CVLPs were seen in six specimens (Caul et al., 1975). In the Bristol outbreak among hospital nurses, a coronavirus was propagated in human fetal intestinal organ culture from a specimen in which large numbers of CVLPs were detected by electron microscopy. This virus, from patient X, could also grow to low titer in primary human embryonic kidney (HEK) cell cultures. Detection in both HEK and the organ cultures was by indirect immunofluorescence with convalescent serum from the patient (Caul and Clarke, 1975). Further ultrastructural studies of Patient X virus in intestinal organ cultures have been reported (Caul and Egglestone, 1977), but the virus has not yet been adapted to more available culture systems. In a later study (Riordan et al., 1986), CVLPs were seen without any clinical illness in 35% of male homosexuals in Manchester.

In Vellore, India, CVLPs were seen in fecal samples from many patients with epidemic tropical sprue (Mathan and Mathan, 1978; Mathan et al., 1975). In one patient with chronic tropical sprue, CVLPs were excreted for 8 months and biopsies of the jejunum revealed coronavirus vesicles. Coronavirus-like particles were also found in fecal specimens from a high percentage of apparently healthy persons in rural areas, but were not found in neonates.

Similar findings were also made in southern and northern Australia. Coronavirus-like particles were found in four relatively closed communities around Adelaide, consisting of Caucasian infants with gastroenteritis, native (Aboriginal) infants with gastroenteritis, healthy Vietnamese refugees, and institutionalized healthy children (Moore et al., 1977). They were also found in many communities of Western Australia, not only among Caucasian and Aborigines children, but also in dogs kept by the Aborigines (Schnagl et al., 1978). In this study, the particles were equally prevalent in children with or without diarrhea. Coronavirus-like particles increased in frequency with increasing age, reaching a high percentage of the adult population. They were rarely associated with symptomatic disease in adults. As in the Indian study, the incidence of CVLPs in stools of nonill persons was higher in rural areas and lower socioeconomic groups. An association between dis-

ease and the occurrence of antibody to CVLPs has been found in Australia, suggesting that CVLPs are infectious agents that may be related to chronic diarrhea in certain Australian populations (Schnagl et al., 1986, 1987).

Human enteric coronaviruses have been detected in Europe since 1978. Peigue et al. (1978) reported finding CVLPs in stool specimens from 25 of 190 children hospitalized with gastroenteritis in the Clermont-Ferrand region of central France over a 1-year period. Laporte's laboratory cultivated coronaviruses from four of these specimens in HRT-18 human rectal tumor adenocarcinoma cells. Growth was monitored by negative-stain electron microscopy (Laporte and Bobulesco, 1981; Sureau et al., 1980), but after 10 passages, the viruses could no longer be detected. They may have become contaminated with NCDCV bovine coronavirus (Patel et al., 1982).

Chany et al. (1982) reported two outbreaks of neonatal necrotizing enterocolitis (NNEC) that occurred in a large hospital in Paris. The first, from March to May 1979, was not studied by electron microscopy and no unusual microbial agents were isolated by routine procedures. In the second, from September 1979 to March 1980, 32 of 58 infants developed NNEC and 23 of these were positive for CVLPs by electron microscopy. Coronavirus was apparently isolated in cell culture from two of these specimens, but further details have not been forthcoming. By including babies born in a second maternity hospital in the same section of Paris, three nonill control infants and two infants with diarrhea were CVLP positive. In addition, 17 adult contacts of the infants with NNEC and 16 children with acute diarrhea in a local nursery were CVLP positive; most were antibody positive as well, suggesting a community-wide outbreak of CVLPs during this period. Serologic response to the CVLPs was documented by immune electron microscopy in 7 of 10 infants with NNEC, whereas none of 15 controls had CVLP antibody (Chany et al., 1982). In a 1981 outbreak of NNEC in one of these hospitals, CVLPs were observed in the stools of eight infants (Caldera and Badoual, 1982).

In a longitudinal study in Munster, West Germany during 1980 and 1981, Maass and Baumeister (1983) found CVLPs in the stools of infants with gastroenteritis and in healthy adults throughout the year, although there was a greater incidence in the winter and spring months. Gerna et al. (1984) reported CVLPs in 34 infants with gastroenteritis and in 3 age-matched controls in Pavia, Italy, and demonstrated a significant bilateral cross-reaction between HCV OC43 and two strains of HECV. The antigenic relatedness was shown in one direction by hemagglutination inhibition and serum neutralization tests with OC43 virus versus sera from sick children, control patients, and mice and guinea pigs immunized with purified HECV, and in the other direction by immune electron microscopy with purified HECV and reference antisera to OC43. Additional relationships between HECV and OC43 were shown in subsequent studies (Battaglia et al., 1987; Gerna et al., 1985).

Coronavirus-like particles have frequently been observed in the western half of the United States. Vaucher et al. (1982) found CVLPs in 49 children, including 32 neonates, with diarrhea or gastroenteritis in Tucson, Arizona. Most of the illnesses occurred during the winter of 1979. Seroconversion, measured by immune electron microscopy, was noted in several children. The CVLPs recovered in this study did not exhibit antigenic cross-reactions with HCV OC43 or 229E (Mortensen et al., 1985; Vaucher et al., 1982). Resta et al. (1985) found CVLPs in seven stool specimens during an outbreak of NNEC in Dallas. Coronaviruses, designated A14 and C14, were isolated in human fetal intestinal organ cultures of two of the specimens and were successfully passaged at least 14 times. Preliminary data have shown that these isolates possess typical coronavirus polypeptides and antigens, and induced a specific serologic response. Further work may show the relationship of these strains to those previously described from other outbreaks of gastrointestinal disease. In a separate study (Rettig and Altshuler, 1985), CVLPs were seen in intestinal contents and within epithelial cells of the ileum in a fatal case of severe enteritis in Oklahoma.

In one study reported from Africa, peaks of CVLPs were detected during the rainy seasons (February to May and October to December) in equatorial Gabon (Sitbon, 1985). In this study, healthy children had a higher incidence of CVLPs (75 of 115 specimens) than did children with diarrhea (60 of 156 specimens). As in earlier studies in India and Australia, the incidence of CVLPs increased with age and in older individuals was clearly unrelated to illness. Coronavirus-like particles were found in diverse population groups in Gabon, as well as in domestic goats and dogs and in captive primates.

Definitive proof of the association between CVLPs and human gastrointestinal disease has been difficult to establish. Coronavirus-like particles have proved extraordinarily difficult to cultivate in cell or organ culture and most of those isolated thus far have been lost during subsequent passage. Also, CVLPs have frequently been observed in the stools of healthy individuals (see Table 2), and similar particles have been detected in the stools of both healthy and diarrheic nonhuman primates and other animals (see Table 3). Thus, the CVLPs in general may just be passenger particles tolerated by the host, with no role in disease. On the other hand, the ubiquity of coronaviruses and their etiology in a variety of well-described nephritic, neurologic, and enteric diseases

TABLE 3. Diversity of disease and organ tropism in animal coronaviruses

Abbreviation	Name	Host	Central nervous system	Eye	Lymphatic	Respiratory	Heart	Abdominal viscera	Gastrointestinal	Genitourinary	Other
IBV	Avian infectious bronchitis virus	Chick				+				+	
TGEV	Transmissible gastroenteritis virus	Pig			+	+			+		
HEV	Hemagglutinating encephalomyelitis virus	Pig	+			+			+		
PECV	CV777 porcine epidemic diarrhea virus	Pig							+		
MHV[a]	Mouse hepatitis virus	Mouse	+		+	+		+	+	+	
FIPV	Feline infectious peritonitis virus	Cat	+	+	+	+		+	+	+	
FECV	Feline enteric coronavirus	Cat			±				+		
SDAV	Sialodacryoadenitis virus	Rat		+	+	+					
RCV	Rat coronavirus	Rat			±	+					
TCV	Bluecomb disease virus	Turkey	±			+		±	+	±	
BCV (or NCDCV)	Neonatal calf diarrhea virus	Cattle							+		
BECV	Bovine enteric coronavirus	Cattle							+		
CCV	Acute enteritis coronavirus 1-71	Dog							+		
CECV	Canine enteric coronavirus	Dog							+		
RbCV	Cardiomyopathy virus	Rabbit		+	+	+	+	±		+	+
RbECV	Rabbit enteric coronavirus	Rabbit							+		
—	Puffinosis virus	Sea birds		+		+					+
EECV	Equine enteric coronavirus	Horse							+		
OECV	Ovine enteric coronavirus	Sheep							+		
SECV	Simian enteric coronavirus	Monkey							+		

[a] Includes serotypes 1, 2, 3, 4 (JHM), A59, and S (including LIVIM), and possibly others less well characterized (Wege et al., 1982).

of animals (see following section) suggests that the list of human diseases in Tables 1 and 2 is far from complete.

Animal Strains

In recent years, many coronaviruses of animals have been implicated in severe or fatal disease, especially in their young (Table 3). These viruses include infectious bronchitis viruses (IBV) (Beaudette and Hudson, 1937; Dawson and Gough, 1971); transmissible gastroenteritis virus (TGEV) of pigs (Doyle and Hutchings, 1946; Kodama et al., 1981); mouse hepatitis viruses (MHV) (Cheever et al., 1949; Rowe et al., 1963); lethal intestinal virus of infant mice (LIVIM) (Kraft, 1962), actually a substrain of MHV-S (Hierholzer et al., 1979); hemagglutinating encephalomyelitis virus (HEV) of pigs (Andries and Pensaert, 1980; Greig et al., 1962); feline infectious peritonitis virus (FIPV) (Pedersen et al., 1981a; Wolfe and Griesemer, 1966); sialodacryoadenitis virus (SDAV) of rats (Bhatt et al., 1977; Hirano et al., 1986; Jonas et al., 1969); rat coronavirus (RCV) (Parker et al., 1970); turkey enteritis (bluecomb disease, TBDV) virus (TCV) (Adams et al., 1970; Panigrahy et al., 1973); neonatal calf diarrhea virus (NCDCV or BCV) (Stair et al., 1972; Tektoff et al., 1983); acute enteritis coronavirus 1-71 (CCV) of dogs (Keenan et al., 1976); rabbit infectious cardiomyopathy virus or Stockholm agent or pleural effusion disease agent (RbCV) (Osterhaus et al., 1982; Small et al., 1979); and puffinosis virus of seabirds (Nuttall and Harrap, 1982). In addition, enteric CVLPs have been reported from many domestic and laboratory animals and may be associated with diarrhea. These animals include cattle (McNulty et al., 1975), horses (Bass and Sharpee, 1975), sheep (Tzipori et al., 1978), dogs (Schnagl and Holmes, 1978), pigs (porcine CV777 epidemic diarrhea virus, PEDV or PECV) (Ducatelle et al., 1981; Pensaert and deBouck, 1978); monkeys (Caul and Egglestone, 1979; Smith et al., 1982), rabbits (contagious diarrheal disease agent, RbECV) (Lapierre et al., 1980; Osterhaus et al., 1982), and cats (Hoshino and Scott, 1980; Pedersen et al., 1981b, 1984; Stoddart et al., 1984).

All of these viruses cause respiratory or enteric disease, or both, and some are also responsible for central nervous system, nephritic, conjunctival, or generalized disease. The animal viruses are, therefore, important as models for HCV disease, not only because their pathogenicity is so diverse, but because their antigenic composition and replication characteristics provide insight into the nature of the HCV. These properties have been the subject of frequent reviews (Bradburne, 1970; Bradburne and Tyrrell, 1971; Estola, 1970; McIntosh, 1974; Monto, 1974; Robb and Bond, 1979; Siddell et al., 1983b; Sturman and Holmes, 1983; Wege et al., 1982).

Physicochemical Properties and Morphology

Despite similarities based on morphology, HCV strains 229E and OC43 differ considerably in their host cell susceptibility, physical stability, and antigenic properties. These viruses are large (diameter, 80 to 200 nm) and pleomorphic, with a buoyant density of about 1.18 g/ml. Their unique morphology is characterized by the presence of clublike projections up to 20-nm long protruding from a lipid-containing envelope. They also possess an internal helical ribonucleoprotein, which has been observed as a long strand 1 to 2 nm in diameter (Caul et al., 1979; Davies et al., 1981; Kennedy and Johnson-Lussenburg, 1976) or as a helix condensed into coillike structures 10 to 20 nm in diameter (Macnaughton et al., 1978).

The HCV genetic material consists of single-stranded RNA with a molecular weight of approximately 6×10^6 daltons, which is the largest for any RNA virus (Macnaughton and Madge, 1978; Tannock and Hierholzer, 1977). An earlier study with OC43 purified from infected suckling mouse brain suggested that its RNA genome was fragile and could readily be disrupted with heat or organic solvents (Tannock and Hierholzer, 1977). However, more recent work with strain 229E grown in cell culture reveals no such lability (Macnaughton and Madge, 1978), and it seems likely that the instability observed for OC43 was associated with extensive nicking of the RNA genome during growth in the suckling mouse brain. Virion RNAs prepared from various animal coronaviruses after growth in cell culture have similarly been shown to be stable (Lai and Stohlman, 1978; Schochetman et al., 1977; Tannock, 1973). Human coronaviral RNA, like that of other coronaviruses, contains polyadenylate residues located at the 3′ terminus, but does not appear to contain an RNA transcriptase in the outer coat of the virion (Macnaughton and Madge, 1978; Tannock and Hierholzer, 1978). Coronaviridae are, therefore, positive-stranded viruses, with their virion RNA having the same polarity as that of their intracellular viral messenger RNAs.

The structural protein compositions of HCV OC43 and 229E differ little from those reported for other coronaviruses (Cavanaugh et al., 1986b,c; Garwes and Reynolds, 1981; Hierholzer, 1976; Hierholzer et al., 1972, 1981; Hogue and Brian, 1986; King and Brian, 1982; Macnaughton, 1980, 1981; Robbins et al., 1986; Stern and Sefton, 1982; Stern et al., 1982; Sturman, 1977; Sturman and Holmes, 1977, 1983; Sturman et al., 1980; Sugiyama et al., 1986;

Wege et al., 1979, 1982; Wesley and Woods, 1986). The HCV proteins comprise four major groups of polypeptides, which are summarized in Table 4. The high-molecular-weight peplomeric glycoprotein, P, ranges from 160 to 200 kilodaltons (kDa) in various studies. It constitutes the principal antigen detected by neutralization tests and contains host cell receptors and fusion and cell-mediated immunity (CMI) activities. This glycoprotein is considered to be a dimer of two dissimilar proteins (P1 and P2) weighing 106 and 91 kDa, respectively, which in tetrameric form constitute the peplomer or spike. Monoclonal and polyclonal antibodies to the spike proteins of MHV block virus-mediated cell fusion and neutralize infectivity (Collins et al., 1982; Holmes et al., 1984; Nakanaga et al., 1986). Similar results were found for IBV and TGEV (Cavanagh and Davis, 1986; Cavanagh et al., 1986a; Delmas et al., 1986; Laude et al., 1986; Jimenez et al., 1986). These proteins in avian IBV are not dissociable with 2-mercaptoethanol (Cavanagh, 1983b, 1984) and, therefore, do not appear to be held together by disulfide bonds.

The hemagglutinin protein, H, is a 60- to 66-kDa glycoprotein (the 130-kDa monomer is seen in nonreducing gels) found in the peplomers of HCV OC43 and 229E as well as in hemagglutinating animal coronaviruses. The phosphorylated 47- to 55-kDa nucleoprotein, N, is the core antigen. The family of proteins having molecular weights of approximately 40, 27, 24, and 20 kDa have identical polypeptide structures but varying degrees of glycosylation; they constitute the matrix, M, protein that bridges the double-shelled envelope.

Minor virion polypeptides with molecular weights of 107, 92, and 39 kDa, which had previously been reported (Hierholzer, 1976; Kemp et al., 1984c; Stern et al., 1982; Sturman et al., 1980), are now known from tryptic digest analyses and radioimmunoprecipitation to represent P1, P2, and M1, respectively. The other matrix glycoproteins of HCV, M2, M3, and M4, are thought by analogy with murine and avian coronaviruses to provide a link between the nucleocapsid and envelope and span the lipid bilayer in virion assembly (Rottier et al., 1984; Sturman et al., 1980). Glycosylation by *o*-linked oligosaccharides probably takes place in the Golgi apparatus, and the matrix glycoprotein then influences both the formation of the envelope and budding by the virus into the endoplasmic reticulum (Rottier et al., 1984). The nature of the sugar moieties and the function of coronavirus glycoproteins have been thoroughly reviewed elsewhere (Cavanagh, 1983a,b; Frana et al., 1985; Holmes et al., 1981, 1984; Sturman and Holmes, 1983, 1984, 1985; Sturman et al., 1985).

Strain OC43 readily agglutinates chicken, rat, mouse, vervet monkey, and human group O erythrocytes (Kaye and Dowdle, 1969), a property useful for the detection of antibody by hemagglutination inhibition. Furthermore, attached virus can be readily eluted from erythrocytes by gentle warming, and thus adsorption and elution are an efficient means of partially purifying the virus. The chemical basis for elution is unknown, but does not appear to involve neuraminidase activity (Hierholzer et al., 1972; Pokorny et al., 1975; Sheboldov et al., 1973; Zak-

TABLE 4. Polypeptide composition of the human respiratory coronaviruses

Protein designation[a]						
Functional	*Other*	*Glycosylation*	*Molecular weight*[b] *(kilodaltons)*	*Location*	*Function*	*Assigned mRNA*
P	E2	++	186 106 (P1) 91 (P2)	Peplomer	SN, CF, fusion activity; binds to cell receptors; induces cell-mediated cytotoxicity	3
H	gp65	++	63	Peplomer	Hemagglutinin	?
N	N	−−	50	Internal core	Ribonucleoprotein, phosphorylated	7
M	E1	++	40 (M1) 25 27 (M2) 24 (M3) 20 (M4)	Envelope	Matrix or transmembrane protein	6

[a] Functional designation (P = peplomer; H = hemagglutinin; N = nucleoprotein; M = matrix) taken from Hogue and Brian (1986); other designations (e.g., envelope 2 and 1) taken from Sturman and Holmes (1977) and Lai (1987).
[b] P exists predominantly in the 186-kDa form in either reducing or nonreducing gels, and therefore P1 and P2 are "minor"; H is found predominantly in hemagglutinating coronaviruses; and exists as a 130-kDa dimer in nonreducing gels or as the 63-kDa monomer in reducing gels; N may also be seen as a 160- to 165-kDa trimer or aggregate in nonreducing gels; M constitutes a family of identical polypeptides with varying degrees of glycosylation, with M1 being a minor component and M2–4 present in approximately equal amounts. Molecular weights are the means of published reports on HCV (Hierholzer, 1976; Hierholzer et al., 1972; Hogue and Brian, 1986; Hogue et al., 1984; Kemp et al., 1984c; Macnaughton, 1980; Schmidt and Kenny, 1982).

stelskaya et al., 1972a). Human coronavirus OC43 also effects a nonspecific or "false" hemadsorption with rat or mouse erythrocytes when the virus is grown in BSC-1, MK, HEK, or WI38 cell cultures, a property of potential usefulness in neutralization tests (Bucknall et al., 1972a; Kapikian et al., 1972). The nonspecific HAd is due to the presence of a high-density lipoprotein inhibitor in fetal calf serum (Bucknall et al., 1972a).

Human coronavirus 229E does not hemagglutinate, even when grown or concentrated to high infectivity titers (Hierholzer, 1976) or after treatment with trypsin, as is required for IBV hemagglutination (unpublished observations). Like many other nonhemagglutinating viruses, however, it does adhere to tannic acid-treated sheep red blood cells to make an indirect hemagglutination antigen that is the basis of a convenient IHA serologic test (Kaye et al., 1972). Data on the viral stability for HCV OC43 and 229E were variable; OC43 is more stable than 229E both at 33°C and 37°C and at low pH, but both viruses have the same ultraviolet inactivation rates (Bucknall et al., 1972b).

Replication

Several reviews on the molecular features of coronavirus replication have been derived mainly from studies on animal coronaviruses, especially the mouse hepatitis viruses (Lai, 1987; Siddell, 1983; Sturman and Holmes, 1983). Replication begins with viropexis similar to that in a number of other viral groups, and has been demonstrated for HCV 229E infecting diploid fibroblast cells (Patterson and Macnaughton, 1981).

The principal unique features of coronavirus replication are summarized in Fig. 1. Human coronaviral RNA is large and polyadenylated, and there is no evidence of an RNA polymerase (transcriptase) within the virion outer coat (Macnaughton and Madge, 1978; Tannock and Hierholzer, 1978). The isolated RNAs of other coronaviruses have, in addition, been shown to be infectious (Schochetman et al., 1977; Wege et al., 1978) and to possess a 5′ methylated cap structure (Budzilowicz et al., 1985; Lai et al., 1982). Therefore, the polarity of viral RNA replication is the same as that of intracellular viral mRNAs. Replication occurs within the cytoplasm, where up to seven intracellular viral RNA species can be detected. All have common 3′ terminal ends of varying size, all copied from the same negative-stranded template to produce a so-called 3′ coterminal "nested set." The nested-set scheme allows the independent synthesis of each intracellular RNA, except for the largest RNA, without a processing step from a large precursor molecule. This type of RNA transcription is unique to coronaviruses and involves RNA-dependent RNA polymerase(s), which are coded for by the virion RNA (Brown et al., 1986; Dennis and Brian, 1981; Kapke and Brian, 1986; Lai, 1987; Lai et al., 1981; Leibowitz et al., 1982a; Mahy et al., 1983; Sawicki and Sawicki, 1986; Stern and Kennedy, 1980). Brayton et al. (1982) noted two separate polymerase functions, one occurring at 1 h and the other at 6 h postinfection. The early polymerase may be responsible for the synthe-

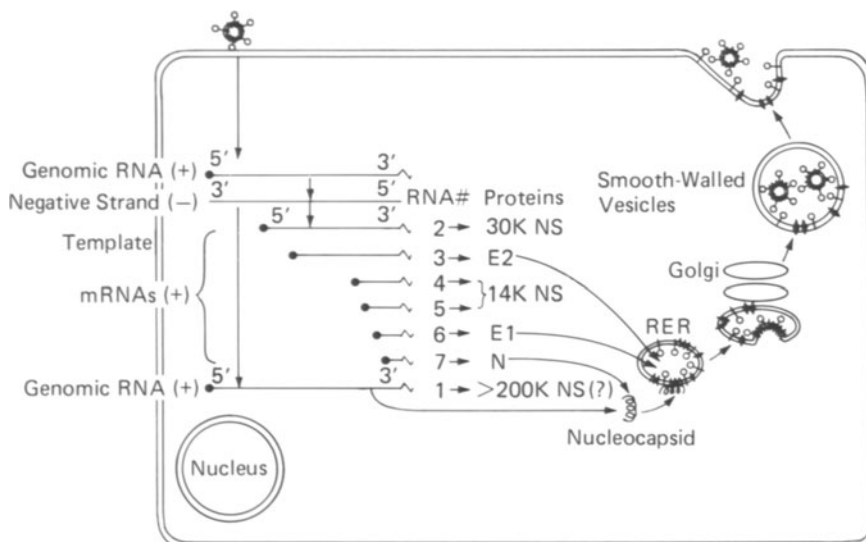

FIG. 1. Nested-set hypothesis for coronavirus RNA replication (Sturman and Holmes, 1983). (Reprinted by permission of the author and Academic Press, Inc.)

sis of the negative-stranded template and later for the synthesis of the positive-stranded RNAs. Oligonucleotide mapping studies have revealed the presence of common sequences at the 5′ termini of most MHV A59 RNAs, that are translocated from genomic RNA and thus constitute a leader sequence (Lai and Stohlman, 1978; Lai, 1987).

The role of the cell nucleus in replication is uncertain. Hierholzer (1976) showed that replication of HCV 229E in human embryonic lung fibroblast cells was susceptible to actinomycin D (AMD), a study later confirmed with 229E grown in L132 cells (Kennedy and Johnson-Lussenburg, 1978). An earlier report indicated that replication of IBV required nuclear function and host transcriptional factors (Evans and Simpson, 1980). However, more recent studies indicate that yields of MHV strains A59 or JHM are unaffected by treatment of cells with AMD or the DNA inhibitor α-amanatin, and that growth can take place in enucleated cells (Brayton et al., 1981; Mahy et al., 1983; Wilhelmsen et al., 1981). This area therefore remains controversial.

Studies of viral-specific proteins present in infected cells have proved difficult because there is little inhibition of cell macromolecular synthesis during infection. However, the translation products of several subgenomic RNAs of MHV have been determined after cell-free synthesis in *Xenopus* oocytes or rabbit reticulocytes. The largest RNA (RNA 1) is believed to code for RNA-dependent RNA polymerase(s), RNA 2 for a 35K nonstructural protein, RNA 3 for the peplomeric surface glycoprotein, RNA 4 for a 14K nonstructural protein, RNA 5 for a 10K nonstructural protein, RNA 6 for the M or transmembrane protein, and RNA 7 for the nucleocapsid protein (Jacobs et al., 1986; Leibowitz et al., 1982b; Rottier et al., 1981; Siddell, 1983; Skinner and Siddell, 1985; Skinner et al., 1985; Stern and Sefton, 1984). RNA 1 is translated initially as a large 250K polypeptide, which is subsequently proteolytically cleaved into 220 and 28K polypeptides (Denison and Perlman, 1986). Such studies have not been conducted with HCV, but a similar mechanism of replication is assumed to exist.

Assembly and Maturation

Unlike most enveloped RNA viruses, which mature by budding at the cell membrane, coronaviruses bud into the lumen of the rough endoplasmic reticulum, where assembly of monomeric glycoproteins and nucleoproteins occurs (Becker et al., 1967; Bucknall et al., 1972a; Hamre et al., 1967; Holmes et al., 1984; Oshiro et al., 1971). During this time, the peplomer protein is glycosylated as the glycoproteins migrate to the Golgi apparatus (see Fig. 1). The assembled particles then appear in single-walled vesicles that migrate to the cell membrane, where they are probably released by exocytosis (Sturman and Holmes, 1983).

During replication of HCV 229E in human embryonic lung fibroblasts, roughly circular electronlucent structures with dense limiting membranes, sometimes empty but frequently containing netlike beaded strands, can be seen in the cytoplasm (Kemp et al., 1984b). These structures vary in size from 300 to 900 nm in diameter and appear approximately 6 h postinfection (i.e., at the initiation of viral protein synthesis, but before virion assembly). Formation of these electronlucent structures was not inhibited by treatment of infected cells with an inhibitor of glycosylation (tunicamycin) or of glycoprotein transport (monensin). These structures, which were noted in suckling mouse brain infected with HCV OC43 and a number of other coronaviruses, appear to be an early feature of replication for the *Coronaviridae* (Kemp et al., 1984a,b).

Antigenic Composition

Soon after the discovery of coronaviruses, significant antigenic relationships among strains from different animal species became apparent, along with significant antigenic divergence among strains within the same animal species. Antigenic variation has been noted within strains of IBV, as measured by neutralization, IFA, and enzyme immunoassay tests (Dawson and Gough, 1971; Snyder and Marquardt, 1984), in strains of TGEV differentiated by monoclonal antibodies (Laude et al., 1986), and in strains of HCV 229E and OC43 distinguished by enzyme immunoassays (Macnaughton et al., 1981b; Reed, 1984). Antigenic divergence among MHV strains, first noted by the marked variations in pathogenicity, has recently been measured by complement fixation, neutralization, and radial hemolysis tests (Hierholzer et al., 1979) and by plaque reduction tests and radioimmunoprecipitation (Childs et al., 1983; Dalziel et al., 1986; Fleming et al., 1983, 1986).

The antigenic relationships among viruses from different species emphasize the importance of studies of animal coronaviruses for an understanding of human disease. In particular, it is important to know whether the same strain can infect more than one animal species or whether the coronaviruses as a group actually share major antigenic determinants. Several examples of cross-species infections have been noted, all under experimental rather than natural conditions. In one study, newborn pigs were infected with TGEV, FIPV, and CCV (Woods et al., 1981). In others, cats were infected with TGEV (Reynolds and Garwes, 1979) and rodents were infected with BCV (Akashi et al., 1981). In all of these studies, the infected animals developed typical clini-

cal disease, gut pathology, and serum antibody responses, suggesting that infection in multiple species can occur.

There is little question that the coronaviruses may share at least one major antigenic determinant, which is probably located on the peplomers. The original observation of Hartley et al. (1964) that antibodies in human sera (now known to be anti-OC43) reacted with MHV has been confirmed by a number of independent studies (Leinikki et al., 1981; McIntosh et al., 1969, 1970b). Also, animal hyperimmune antisera against HCV OC43 and various MHV strains have been shown to contain high levels of cross-reactive antibodies in both directions (Bradburne, 1970; Gerdes et al., 1981a,b; Hierholzer et al., 1979; Hogue et al., 1984; Macnaughton, 1981). The cross-reaction between OC43 and MHV-3 is variable with different isolates of OC43 virus (Macnaughton et al., 1981b).

Human convalescent sera and animal hyperimmune antisera have also been shown to react with BCV of cattle (Cereda et al., 1986; Gerna et al., 1981, 1982; Hogue et al., 1984; Kaye et al., 1975; Storz and Rott, 1981), HEV of pigs (Georgescu et al., 1978; Kaye et al., 1977), and the IBV and puffinosis strains of birds (Macnaughton, 1981; McIntosh et al., 1969; Miller and Yates, 1968; Nuttall and Harrap, 1982). All of these relationships have been determined with HCV OC43, are high titered, and, for the most part, are reciprocal.

Studies with HCV 229E have demonstrated bilateral cross-reactions with TGEV and FIPV (Pedersen et al., 1978), with rabbit cardiomyopathy virus (RbCV) (Small et al., 1979), and with MHV-3 (Hasony and Macnaughton, 1982). However, the cross-reaction between 229E and MHV-3 is a false one, apparently caused by the adherence of bovine serum components from culture media; the cross-reaction is removed by absorption of antisera with bovine serum or by growing antigen in serum-free cultures (Kraaijeveld et al., 1980a).

No antigenic relationship has been demonstrated between HCV OC43 and 229E (McIntosh, 1974; Robb and Bond, 1979; Schmidt, 1984; Schmidt and Kenny, 1981). Furthermore, each serotype represents a group of antigenically similar strains (Macnaughton et al., 1981b; Reed, 1984). The more recently described CVLPs seen in infant and adult stool specimens, or HECV, may well represent more than one serotype, at least one of which cross-reacts with HCV OC43 (Gerna et al., 1984; Macnaughton and Davies, 1981; Schnagl et al., 1986).

In addition to important bilateral relationships between the HCV and mammalian and avian coronaviruses, many antigenic relationships exist among the animal viruses themselves. The TGEV of pigs cross-reacts with the CCV of dogs (Garwes and Reynolds, 1981; Horzinek et al., 1982; Norman et al., 1970), the FIPV of cats (Horzinek and Osterhaus, 1979; Pedersen et al., 1981b, 1984; Reynolds et al., 1977), and others (Horzinek et al., 1982; Pedersen et al., 1978; Pike and Garwes, 1979). The HEV of pigs cross-reacts with the BCV of cattle (Sato et al., 1980). The swine PECV (CV777) is antigenically related to several other coronaviruses (Pensaert et al., 1981). RbECV cross-reacts both with IBV and TGEV (Descoteaux et al., 1985). Many cross-reactions have been shown among and between the canine and feline coronaviruses (Evermann et al., 1981; Horzinek et al., 1982; Pedersen et al., 1978, 1981a,b, 1984; Tupper et al., 1987). The puffinosis coronavirus is related to MHV, RCV, SDAV, and HEV in addition to OC43 as noted above (Nuttall and Harrap, 1982); FIPV is antigenically related to FECV (Pedersen et al., 1981b, 1984); and RCV cross-reacts with SDAV (Bhatt et al., 1977). Many of these relationships have been reviewed in detail elsewhere (Bradburne, 1970; Bradburne and Tyrrell, 1971; McIntosh, 1974; Robb and Bond, 1979; Siddell et al., 1983b; Sturman and Holmes, 1983; Wege et al., 1982).

A phylogenetic tree summarizing these relationships has been constructed for the better-studied coronaviruses (Fig. 2) (Brian et al., 1984). Starting from an ancestor that will probably never be identified, the coronaviruses formed two major branches in avians and two major branches in mammals, each with several subbranches. These antigenic lineages were determined by reciprocal serum neutralization, IFA, enzyme immunoassay, and radioimmunoprecipitation tests carried out by different laboratories and summarized by Wege et al. (1982) and Brian et al. (1984). Undoubtedly, more relationships will be documented as additional strains are compared in reciprocal serologic tests. Altogether, the intricate picture of antigenic-relatedness among the human and animal coronaviruses will remain a major focus of research because of its impact on the biology and pathogenesis of these viruses.

The serologic cross-reactions observed among coronaviruses are related to antigenic similarities on the spike or peplomer component. Three to six virion antigens have been detected in studies with HCV grown in cell culture, and three have been found in OC43 grown in suckling mouse brain. No soluble antigens have been found in cell culture supernatant fluids. The 186-kDa glycoprotein of the human strains is the predominant protein in the peplomer (Table 4) and contains the hemagglutinating, complement-fixing, and neutralizing activities (Hierholzer, 1976; Hierholzer et al., 1972; Kaye et al., 1970; Macnaughton et al., 1981a; Pokorny et al., 1975; Schmidt, 1984; Schmidt and Kenny, 1981, 1982; Yaseen and Johnson-Lussenburg, 1981). Macnaughton

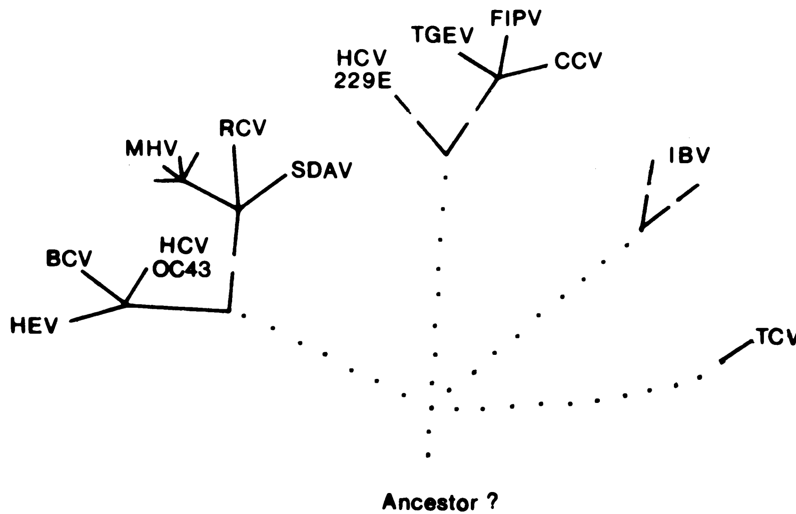

FIG. 2. Phylogenetic tree showing antigenic interrelationships among the human and animal coronaviruses (Brian et al., 1984). (Reprinted by permission of the author and publisher from Proceedings of the 4th International Symposium on Neonatal Diarrhea, October 3–5, 1983, University of Saskatchewan, p. 100–115, Veterinary Infectious Disease Organisation, Saskatchewan, Canada.)

et al. (1981a) showed that human volunteers experimentally infected with HCV 229E produced most antibody to the P antigen and little antibody to N or M antigens, as determined by antigen-specific enzyme immunoassays. This finding was consistent with P antibody having neutralizing and protective functions. Most naturally acquired human antibody to HCV infection also is to the P antigen (Schmidt, 1984). The same observations have been made with many animal coronaviruses, in which hemagglutinating and neutralizing activities were shown to reside in the largest glycoprotein, which was predominant in the intact peplomer (Cavanagh et al., 1984; Jimenez et al., 1986; King et al., 1985). Direct evidence in support of these findings came from bromelain-treated HCV, in which totally despiked particles were devoid of biologic activity (Hierholzer, 1976; Hierholzer et al., 1972; Schmidt and Kenny, 1982), and from monoclonal antibodies to the large glycoprotein of TGEV, which were shown to fully neutralize the virus (Jimenez et al., 1986).

The other major antigens are the N or ribonucleoprotein core and M or transmembrane glycoprotein, both of which are detectable by immunoelectrophoresis and immunodiffusion tests (Schmidt, 1984; Yaseen and Johnson-Lussenburg, 1981). Antibodies to the N and M antigens are detectable in human convalescent sera and probably react to some extent in complement-fixation tests, but they are not involved in hemagglutination inhibition and serum neutralization tests (Schmidt, 1984). Furthermore, the human antibody response to the P, N, and M antigens of 229E and OC43 is type-specific; that is, no cross-reactions were observed between the viruses by antigen-specific enzyme immunoassay, immunoelectrophoresis, or complement fixation tests.

Genetics

Because of technical difficulties in growing human coronaviruses to workable titers in cell culture, genetic studies have generally been precluded. Persistent infections with HCV 229E have been demonstrated in a variety of cell cultures, in particular L132 cells derived from human embryonic lung (but containing HeLa markers), and their relevance to human infection has been widely discussed (Chaloner-Larsson and Johnson-Lussenburg, 1981; Sturman and Holmes, 1983). Persistent and latent infections of MHV in mice are well known to cause sweeping epidemics of encephalomyelitis, hepatitis, and gastroenteritis in laboratory mouse colonies (Lamontagne and Dupuy, 1984; Robb and Bond, 1979) and equally well known as the source of contaminating viruses recovered after inoculation of mice with unrelated materials (Bardos et al., 1980; Weiss, 1983).

In addition to latency, MHV in mice has been subjected to critical analyses of the genetic variation found within the MHV subgroup of strains. The MHV strain 4 (JHM) is highly neurovirulent, strains 2, 3, and A59 are slightly neurovirulent, and strain 1 is not neurovirulent at all. Using T_1-RNAse-resistant oligonucleotide mapping, Wege et al. (1981) found that the nucleotide sequence of JHM differed significantly from that of the other four strains. Lai et al. (1983) found that the oligonucleotide fingerprints of MHV strains causing bone marrow necrosis and diarrhea were similar to those of the A59 strain, but were distinct from the fingerprints of other MHV strains, and suggested that this sequence divergence occurred naturally during persistent infection. Furthermore, mutations in the E2 peplomer antigen selected by monoclonal antibodies reveal that certain

epitopes on E2 are the site of neurovirulence in MHV-4 (Dalziel et al., 1986). "Virulence groups" have also been shown with TGEV (Kodama et al., 1981), but as yet no similar data are available for HCV.

Some temperature-sensitive mutants of MHV-A59 and MHV-JHM have been made and used to study different aspects of the replication of the murine coronaviruses (Robb and Bond, 1979). Leibowitz et al. (1982a) found seven overlapping complementation groups in their genetic analysis of 37 temperature-sensitive (*ts*) mutants of JHM. Plaque-size variants and *ts* mutants of A59 were recovered from persistently infected Balb/c-3T3 cells (Holmes and Behnke, 1981). Also using *ts* mutants of A59, Makino et al. (1986) and Keck et al. (1988) showed that the genomes of different MHV strains could recombine at a high frequency, with five distinct recombination sites identified between the parent strains.

Reactions to Physical and Chemical Agents

A study by Kaye et al. (1970) showed that HCV OC43 could be inactivated by heating at 56°C for 30 min, a procedure that did not reduce its hemagglutinin activity. Treatment with trypsin, ether, Tween-80, sodium desoxycholate, sodium lauryl sulfate, *b*-propriolactone, and Triton X-100 also abolished infectivity and caused a reduction in hemagglutinin activity according to the time of incubation (Kaye et al., 1970; Schmidt and Kenny, 1981; Zakstelskaya et al., 1972a). Bucknall et al. (1972b) studied the kinetics of inactivation of strain 229E and a strain of OC43 that had been adapted to monkey kidney cells by continuous passage. They showed that in the presence of 0.2% bovine plasma albumin, strain 229E was more labile. When the rate of ultraviolet inactivation for either virus was determined in the presence of 2% fetal calf serum, multihit infectivity inactivation kinetics were observed due to the capacity of the virions to clump. However, in the presence of 0.2% bovine plasma albumin, one-hit kinetics similar to those obtained with influenza A were obtained.

Human coronavirus 229E is similar to OC43 in its sensitivity to lipid solvents and detergents (Schmidt and Kenny, 1981; Yaseen and Johnson-Lussenburg, 1981). Hierholzer (1976) showed that treatment of strain 229E with chloroform (5% for 10 min), heat (50°C for 1 h at pH 7.0 in the presence or absence of 1 M $MgCl_2$), or acid (pH 3, 4 h, 23°C) completely abolished infectivity. In the same study, depending on the multiplicity of infection, peak viral titers were obtained by 22 to 24 h postinfection during growth in human embryonic lung diploid fibroblast (HELF) cultures at 35°C. Thereafter, a precipitous decline in infectivity (4 to 5 $\log_{10}$ $TCID_{50}$) occurred, which coincided with the development of an extensive cytopathic effect (CPE). Similar growth curves have been reported in L132 and HeLa cells (Bradburne, 1972; Bradburne and Tyrrell, 1969; Chaloner-Larsson and Johnson-Lussenburg, 1981), in WI38 cells (Hamre et al., 1967), and in FT and RD cells (Schmidt et al., 1979), except that the decline in infectious viral titers that peaked by 1 day of growth was less when lower incubation temperatures (namely, 33°C) were used. Strain 229E is therefore a relatively difficult virus to passage, which may explain why few diagnostic laboratories report its isolation in cell culture.

Pathogenesis, Pathophysiology, and Pathology

In early morphogenetic studies with OC43, the human respiratory coronaviruses were thought to have a clear tropism for ciliated epithelial cells because of their effect on these cells in tracheal organ cultures and their failure to grow in standard cell monolayers. Their cell tropism and their growth at 33°C indicated that they replicate in the surface layer of the upper respiratory tract. In in vivo studies, 229E and OC43 established superficial infection in nasal tissue within 3 or 4 days after inoculation of human volunteers with nasal drops; the infection was documented by cytoplasmic immunofluorescence in the cells obtained by nasal wash and serologic evaluation (McIntosh et al., 1978). Other volunteer studies have shown production of high titers of B814 virus (Tyrrell and Bynoe, 1965), 229E virus (Callow, 1985; Kraaijeveld et al., 1980b; Larson et al., 1980), and OC43 virus (Larson et al., 1980) in nasal secretions within 2 to 5 days of inoculation by intranasal drops. These volunteers also produced measurable antibody responses to whole virus (Bradburne and Somerset, 1972; Kraaijeveld et al., 1980b; Macnaughton et al., 1981b; Reed, 1984) and to the P, N, and M antigenic components (Macnaughton et al., 1981a). In addition, a specific IgA humoral antibody response was shown in nasal secretions from 229E-infected volunteers (Callow, 1985).

In HECV infection, CVLPs were abundant in ileum epithelial cells obtained at autopsy from an infant with enteritis (Rettig and Altshuler, 1985). Another report described biopsies of jejunal tissue obtained during a case of chronic tropical sprue, in which the cytopathologic findings consisted of viral-filled vesicles similar to coronavirus vesicles found in experimentally infected animals (Mathan and Mathan, 1978). These vesicles are one of the prominent features of intestinal coronavirus infection in birds and mammals (Bridger et al., 1978; Ducatelle and Hoorens 1984; Haelterman, 1972; Hierholzer et al.,

1979; Mebus et al., 1973; Mengeling et al., 1972; Pomeroy et al., 1978).

The pathologic descriptions of coronaviral infections in animals are extensive because coronaviruses infect such a diversity of organ systems in these species (see Table 3). Coronaviral pathogenesis has been particularly well studied in laboratory, domestic, and food-source animals because of the great economic impact of these infections. (Boyle et al., 1987; Dalziel et al., 1986; Ducatelle and Hoorens, 1984; Fishman et al., 1985; Fleming et al., 1986; Keljo et al., 1987; Nguyen et al., 1986; Pearson and Mims, 1985; Reynolds et al., 1985; Robb and Bond, 1979; Siddell et al., 1983; Sorensen et al., 1984; Van Berlo et al., 1986; Wege et al., 1982; Wilson et al., 1986).

Clinical Features

Mode of Transmission

HCV is transmitted by the respiratory route via aerosols, droplets, and probably fomites. As discussed in the previous section, experimentally induced infection in volunteers was achieved by inoculating B814, 229E, and OC43 strains into the nose in the form of filtered drops. HECV is presumably transmitted by the fecal-oral route. Animal coronaviruses are infectious by the fecal-oral route and probably also by the respiratory route, except for the enteric coronaviruses (CVLPs). Because of the difficulty in recovering HCV or HECV in cell culture, no definitive transmission data are available. However, the epidemiologic behavior of HCV in the few outbreaks studied suggests that HCV is transmitted by aerosol and by large droplet, thus allowing rapid spread in the community (Cavallaro and Monto, 1970; Kaye and Dowdle, 1975; Macnaughton, 1982; Monto and Lim, 1974).

Animal reservoirs or vectors do not appear to have a role in HCV or HECV transmission because most coronaviruses are species-specific in their natural environments. The laboratory-induced infections of certain animals with coronaviruses from unrelated animal species are noted earlier in this chapter.

Incubation and Infectious Periods

The incubation period of coronavirus colds has been studied in volunteers and ranges from 2 to 4 days. Virus is shed from the time symptoms begin and for 1 to 4 days afterward. In one study, some volunteers shed 229E virus longer than 5 days, which appeared to be correlated with decreased nasal IgA production (Callow, 1985). In another study, some children with recurrent upper and lower respiratory illness appeared to shed 229E or OC43 for up to several months (Macnaughton et al., 1983). HECV is apparently excreted in the stool for several months at a time, with little correlation with illness (Chany et al., 1982; Gerna et al., 1984; Maass and Baumeister, 1983; Mathan et al., 1975; Moore et al., 1977; Mortensen et al., 1985; Schnagl et al., 1986; Sitbon, 1985; Vaucher et al., 1982).

Symptoms, Signs, and Clinical Course

Human coronavirus is associated with upper respiratory illness and common colds of mild to moderately severe intensity like those typical of rhinoviruses. Experimentally induced B814 colds were of similar intensity as those caused by 229E in human volunteers, and the same appears to be true in natural infections (Bradburne and Somerset, 1972; McIntosh et al., 1978; Tyrrell and Bynoe, 1965). Studies of human volunteers, peaks of cases during community outbreaks, and serodiagnosed individual infections, all showed coryza, rhinitis, and profuse watery nasal discharge as the prominent findings (Bradburne and Somerset, 1972; Hendley et al., 1972; Kraaijeveld et al., 1980b; McIntosh et al., 1978; Reed, 1984). In serologic studies, approximately 30% of patients are asymptomatic; the symptomatic patients report coryza (as much as 100%), sore throat or pharyngitis, cough or wheezing, headache, fever, cervical adenitis, and gastrointestinal symptoms (abdominal pain or diarrhea), in decreasing order (Cavallaro and Monto, 1970; Kapikian et al., 1969; Kaye and Dowdle, 1975; Larson et al., 1980). Adults reported more headache than children. Otherwise, the prevalence of symptoms was the same, regardless of age.

For 229E, the incidence of lower respiratory illness (including croup, bronchitis, and pneumonia) ranges from very rare in infants to 50% in preschool children to scattered in adults (Isaacs et al., 1983; McIntosh et al., 1970a,b, 1974; Wenzel et al., 1974). Tonsillitis and otitis media also have been reported occasionally. Persons with OC43-related illnesses have considerably more cough and sore throat and a higher incidence of lower respiratory symptoms than do those with 229E infections (Kaye et al., 1971; McIntosh et al., 1974; Riski and Hovi, 1980; Wenzel et al., 1974). As with 229E, headache, fever, diarrhea, and other symptoms are reported occasionally.

In volunteers, HCV colds last from 6 to 8 days and occasionally as long as 20 days, with generally no symptoms other than coryza and sore throat. Therefore, the symptoms reported in the serologic studies cited above must be viewed with caution; these studies are limited by the serologic methods used and by the lack of evidence that dual viral infections might be causing the additional symptoms.

Human enteric coronavirus appears to be related to human gastrointestinal disease, but not to respira-

tory or central nervous system disease. It has been associated with gastroenteritis, diarrhea, and necrotizing enterocolitis (see Table 2), and in some studies with vomiting and fever. However, because of the extraordinary difficulties in identifying CVLPs as HECV, developing specific serologic assays, or cultivating the virus, any association of coronavirus with human enteritis must remain an open possibility at best.

Complications

Complications from the human respiratory coronaviruses have not been proved. This finding is significant because they have been actively sought based on animal disease studies. The serologic studies that implicate HCV, especially OC43, in scattered cases of pneumonia, encephalitis, gastroenteritis, and the like have not been confirmed by isolation or antigen detection and are subject to other interpretations.

Diagnosis

Despite recent advances in enzyme immunoassay and other technologies, the laboratory diagnosis of HCV or HECV infections is still developing. This predicament arises from the general inability of laboratories to work with coronaviruses and their lessened interest due to the mild nature of proven human coronaviral disease. Isolation of HCV or HECV from either respiratory or enteric specimens is almost never accomplished and, therefore, is rarely attempted. The time-honored serologic tests are not very sensitive, although they have been used to generate a base of epidemiologic data. The procedures reported for direct detection of virus and viral antigens and for viral isolation and serology are described here in detail (Table 5).

Specimen Collection

As with any virus, the type of specimen and the manner of collection and storage depend on the laboratory methods anticipated. Nasal swabs are the easiest specimens to collect for respiratory viruses and are also the best specimens for the respiratory coronaviruses. Comparative data in one study on direct detection by enzyme immunoassay (EIA) showed that 34% of nasal swabs, 18% of throat swabs, and 18% of nasopharyngeal aspirates were positive for HCV, mostly 229E (Macnaughton et al., 1983). For nasal swabs, urogenital calginate swabs are inserted

TABLE 5. Laboratory diagnosis of human coronavirus infections

Concept	*Test method*	*Nasal specimens (HCV)*[a]				*Stool HECV*
		B814	*229E*	*OC43*	*692*	
Direct viral detection						
	Electron (EM) and immunoelectron (IEM) microscopy				–	+
Direct antigen detection						
	Indirect fluorescent antibody (IFA)		+	+		
	Enzyme immunoassay (EIA)		+	+		
Viral isolation in organ cultures						
	Identified by cilia, interference, EM/IEM	+	+	+	+	+
	Identified by IFA, SRH, immunoblot					+
	Identified by CF, SN		+			
Viral isolation in cell cultures						
	Identified by EM/IEM, CPE, CF, SN	+	+			
Serology (IgG)						
	Immunoelectron microscopy (IEM)					+
	Serum neutralization (SN)		+	+		
	Complement fixation (CF)		+	+		
	Hemagglutination inhibition (HI)			+		
	Indirect hemagglutination (IHA)		+			
	Single radial hemolysis (SRH or HIG)		+	+		
	Indirect fluorescent antibody (IFA)		+	+		+
	Immune-adherence hemagglutination (IAHA)		+			
	Solid-phase radioimmunoassay (RIA)			+		
	Immunoperoxidase assay (IPA)		+	+		
	Enzyme immunoassay (EIA or ELISA)		+	+		

[a] HCV = Human coronavirus; HECV = human enteric coronavirus.

into the nasal passages, gently rotated to absorb mucus and cells, and then vigorously twirled into 2 ml of transport medium (such as tryptose phosphate broth with 0.5% gelatin, veal heart infusion broth, or Trypticase soy broth [BBL Microbiology Systems, Cockeysville, Md.]), with or without antibiotics. Throat swabs can be obtained with cotton-tipped wooden applicators in the usual fashion. Nasopharyngeal aspirates are collected with a neonatal mucus extractor and mucus trap to which transport medium is added (Isaacs et al., 1983). Because of the lability of coronaviruses, specimens for indirect fluorescent antibody (IFA) tests or culture should be placed on wet ice and transported to the laboratory for immediate testing; specimens for other procedures may be frozen on dry ice and stored in ultracold (less than −60°C) freezers until testing.

Nasal washings may be preferred for organ culture (Larson et al., 1980) and are clearly preferred for IFA to obtain a suitable number of intact epithelial cells. Nasal washings can be obtained by washing the nasal cavity with 10 to 15 ml of normal saline and then mixing the wash with an equal volume of nutrient broth, or by instilling up to 10 ml of PBS or normal saline in the nostrils and collecting the expelled fluids (Kapikian et al., 1973; McIntosh et al., 1978). Specimens should be collected within 2 days of onset of symptoms. Fluids are kept cold and quickly processed for IFA by low-speed centrifugation (1,000 × *g*, 4°C, 10 min), dropping the cells resuspended in phosphate-buffered saline (PBS) onto slides, and fixing in cold acetone in usual fashion.

Nasal swabs are the preferred specimen for isolation of HCV in organ or cell cultures, although nasopharyngeal aspirates or washings have been used. Nasal swabs and washes stored at −60°C were the source of B814 and OC43 (McIntosh et al., 1967b; Tyrrell and Bynoe, 1965). Nasal washes yielded several strains of 229E (Larson et al., 1980; Reed, 1984). As described previously, all specimens for viral isolation must be collected early in the illness, kept cold between collection and storage, and stored at low temperatures. For processing for viral isolation, the specimens are thawed, treated with antibiotics, spun lightly to remove cell debris and bacteria, and inoculated onto the organ cultures or cell monolayers. Inoculum volumes of 0.2 to 0.5 ml per tube are used and adsorbed onto the cells for 1 h at ambient temperature before a fortified maintenance medium is added. Cultures are best incubated at 33°C on roller or rocker platforms. Under optimal conditions, freshly collected specimens should be inoculated as soon as possible to reduce loss during storage; however, "bedside inoculation" has not been reported for HCV, probably because the patients are rarely hospitalized.

Stool specimens for the direct detection of HECV or for viral isolation must be collected within 2 days of onset of abdominal symptoms. Specimens are handled as described previously between collection and processing. For processing, a 10 to 20% suspension is made in distilled water, PBS, Eagle minimal essential medium (MEM), or similar medium, shaken vigorously, and clarified by intermediate-speed centrifugation (2,000 to 4,000 × *g*, 4°C, 30 min). The extract may be used as is for electron or immunoelectron microscopy or can be treated with antibiotics for attempts at culture (Maass and Baumeister, 1983; Sitbon, 1985; Vaucher et al., 1982).

Serum specimens for serologic assays are collected during the acute and convalescent phases of illness in usual fashion. A 3-week interval between serum collections is preferred to assure peak levels of convalescent antibody.

No special safety precautions are required for HCV procedures. Coronaviruses are Class II agents, causing minimal disease, and thus only common-sense safeguards are indicated. Clinical specimens of unknown etiology, however, should routinely be processed under a laminar flow hood by persons wearing gloves and mask and being careful to avoid autoinoculation via aerosolized droplets or finger-to-eye or mouth transmission. Serum specimens should be presumed to contain hepatitis B virus, so that appropriate safety precautions for this very stable pathogen will be taken automatically.

Direct Viral Detection

Coronaviruses have been consistently sought by electron microscopic (EM) observation of stool specimens or tissue sections and by immune electron microscopy (IEM) of specimens reacted with antibody. Both are direct tests, involving no culture or excessive manipulations. Successful EM and IEM have resulted both from the large size of the virion, which enables a threshold of 4 or 5 logs of virus to be visible, and from the large numbers of particles often present in HECV-positive stools or in tissue sections containing virus-packed vesicles.

Direct EM of clarified 10 to 20% stool extracts or of gently homogenized organ culture tissue fragments is conducted by negative staining (see Table 2). The technique is variable, but in general consists of staining with 1.6 to 3% potassium phosphotungstic acid (PTA) at pH 6, 6.5, or 7 and placing approximately 10 μl on 300- or 400-mesh carbon–Formvar-coated copper grids (Chany et al., 1982; Hierholzer et al., 1979; Maass and Baumeister, 1983; Mortensen et al., 1985; Tyrrell and Almeida, 1967; Vaucher et al., 1982). Grids can be loaded either by the droplet method or by the agar diffusion pseudoreplica technique. Additional variations include ammonium molybdate as stain (Schnagl et al., 1978) and carbon–collodion-coated copper grids with 4% PTA, pH 6.5 (Sitbon, 1985).

Alternatively, the HECV in clarified stool extracts can be pelleted by ultracentrifugation (50,000 $\times g$, 2 h), transferred to grids, and negatively stained with 1.5% PTA, pH 6.5 (Caul and Egglestone, 1977). Virus in tissue culture harvests and in organ culture supernatants can be examined after concentration in the same way. In one study, 229E-infected organ culture supernatants were clarified and the virus pelleted at 100,000 $\times g$ for 1 h, resuspended in PBS, and applied to parlodion–carbon grids for negative staining (Larson et al., 1980).

In addition, HCV and HECV can be directly sought by thin-section EM on organ culture explants and infected tissues. In one study, sections were prepared for EM by fixing with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and then postfixing for 1 h with 1% osmium tetroxide in the same buffer (Caul and Egglestone, 1977). The tissues were then dehydrated in a graded ethanol series and embedded in araldite for thin sectioning. The ultrathin sections were finally stained with uranyl acetate and lead citrate.

Immune electron microscopy enhances EM by aggregating viral particles with specific antibody. For HCV organ culture harvests, IEM was conducted by mixing 0.1 ml of harvest with 0.1 ml of a 1 : 20 convalescent serum dilution, incubating at ambient temperature, and pelleting the virus–antibody complex at 15,000 $\times g$ for 2 h. The pellet was resuspended in 0.1 ml of distilled water and stained with 3% PTA, pH 7.2, on 400-mesh carbon–Formvar-coated grids (Kapikian et al., 1973). For HECV stool extracts, IEM has been carried out by mixing 1 drop of extract with 1 drop of heat-inactivated (56°C, 30 min) convalescent serum at low dilutions (1 : 1 to 1 : 10) in PBS, usually at 23°C for 1 h. Grids are prepared and stained as for direct EM (Mortensen et al., 1985; Resta et al., 1985; Schnagl et al., 1986; Vaucher et al., 1982). Alternatively, HECV was sought by IEM after the virus–antibody complex was concentrated by ultracentrifugation (Chany et al., 1982; Maass and Baumeister, 1983). A generally applicable IEM technique is to incubate the virus with a 1 : 15 dilution of heat-inactivated antibody at 37°C for 2 h, followed by overnight incubation at 4°C and examination by pseudoreplica technique. The viral sample is dropped onto a 2% agarose block and allowed to air-dry, Formvar is added to the surface, and the film is floated off into the stain (2.5% PTA, pH 6.5) and onto a 400-mesh copper grid. Serum controls for IEM should be human or animal antisera to other agents.

Direct Antigen Detection

Detection of coronavirus directly in respiratory specimens has been conducted by indirect fluorescent-antibody (IFA) and enzyme immunoassay (EIA or ELISA). In the IFA study (McIntosh et al., 1978), nasal washings or NPS specimens were subjected to low-speed centrifugation, and the epithelial cells resuspended in a small volume of PBS for dropping onto glass slides. The slides were air-dried, fixed with cold acetone, and reacted with OC43 and 229E rabbit antisera that had been absorbed with normal host cells. After suitable incubation and washing, fluorescein-conjugated anti-rabbit globulin was added as the indicator. The slides were again washed and then read in usual fashion. Proper controls, such as cultures of other common respiratory viruses and normal host cells, showed that the coronavirus sera were specific. For both viruses, nasal epithelial cells exhibited bright green, particulate fluorescence in the cytoplasm. Some cross-reaction was observed between OC43 virus and 229E antiserum, but was not judged to be a significant problem.

In the EIA study (Macnaughton et al., 1983), purified HCV 229E and HECV CV-Paris (used as a cross-reactive antigen for OC43) were prepared to format the test system and evaluate type-specific rabbit antisera. Nasal specimens were collected and immediately frozen on dry ice for transport to the laboratory, where they were stored at −70°C until tested. For the EIA, specimens were diluted in carbonate buffer and adsorbed to polystyrene plates in 0.2 ml volumes by overnight incubation at ambient temperature. The plates were washed four times in a PBS/0.05% Tween 20/0.02% azide buffer, and 0.2 ml of type-specific rabbit antisera were added to respective wells. After a 4 h incubation at ambient temperature, the plates were again washed four times, and alkaline phosphatase-conjugated, anti-rabbit IgG was added at 0.2 ml per well. The plates were incubated overnight at ambient temperature, washed as before, reacted with 0.2 ml of substrate (0.1% *p*-nitrophenylphosphate in 10% diethanolamine buffer/ 0.02% azide/0.01% $MgCl_2$, pH 9.8), and finally read for absorbance at 405 nm. A 1 : 200 dilution of nasal swab and a 1 : 20 dilution of rabbit antiserum provided the best combination for both viral types. This direct detection EIA was subsequently applied to a prospective epidemiologic study with good results (Isaacs et al., 1983), and will probably by very useful in future studies because of the increased popularity and sensitivity of EIA.

Direct Viral Nucleic Acid Detection

Methods for the direct detection of viral nucleic acids have been described recently for some viruses and mycoplasmas and may become a major tool in rapid diagnosis. Biotinylated RNA probes, in particular, may be able to detect small amounts of virus in clinical specimens. To date, however, these procedures have not been applied to HCV or HECV.

TABLE 6. Growth of human coronavirus and human enteric coronavirus in tissue and cell cultures

Cell type	HCV				HECV
	B814	229E	OC43	692	
For direct isolation					
Human embryonic nasal, tracheal organ culture	+	+	+	+	
Human embryonic intestinal organ culture					+
Primary, secondary human embryonic kidney (HEK)	–	+	–	–	–
Human embryonic lung diploid fibroblast (WI38)	–	+	–	–	–
Continuous human embryonic lung diploid fibroblast (MRC-c)		+			
Continuous human embryonic lung epithelium (LI32)	+	+			–
Human embryonic intestine diploid fibroblast (MA177)		+		–	–
For adaptation					
Suckling mouse brain (SMB)	–	–	+	–	–
Human embryonic lung diploid fibroblast (WI38, HEL, WD, RU-1, MRC-5, HELF, MA-321)	–	+	±	–	–
Human embryonic intestine diploid fibroblast (MA177)	–	+	–	–	–
Continuous human embryonic lung diploid fibroblast (MRC-c)		+	+		
Continuous human embryonic lung epithelium (LI32)	+	+	–	–	–
Human fetal tonsil diploid fibroblast (FT)	+	+	+		–
Human embryonal heteroploid rhabdomyosarcoma (RD)	+	+	+		–
Primary rhesus, African green monkey kidney (MK)	–	–	+	–	–
Continuous green monkey kidney epithelioid (BSC-1)	–	±	+		–
Rhesus monkey kidney epithelial line (LLC-MK_2)			±		
Human peritoneal macrophages		+			

[a] HCV = Human coronavirus; HECV = human enteric coronavirus.

Viral Isolation, Adaptation to Cell Cultures, and Identification

Only one of the human coronaviruses—either the respiratory HCV or the gastrointestinal HECV—can be propagated in cell cultures in the usual fashion (Table 6). As discussed previously, the HCV 229E group of strains can be isolated and serially passaged in human cell cultures and has therefore been used in genetic and biophysical studies. The other HCVs have been isolated only in organ cultures; some have been adapted to cell monolayers with varying degrees of success. Many attempts to isolate the HECV have resulted in putative growth, at least for a few subpassages, in human embryonic intestinal organ culture. It is clear, then, that standard viral isolation procedures are not as relevant to HCV and HECV studies as are more specialized procedures.

Originally HCV B814 was isolated in organ cultures prepared from the tracheas of 14- to 22-week human embryos (Tyrrell and Bynoe, 1965). Tissue fragments were planted in plastic dishes, ciliated side up, and immersed in medium 199. The cultures were incubated at 33°C with daily changes of medium. The explants were inoculated with 0.3 ml of nasal specimens, dripped onto the ciliary surface, and maintained for up to 10 days. Viral replication was seen by three methods: cessation of ciliary activity; viral interference tests with Sendai, echo-11, or parainfluenza-3 challenge viruses; and production of colds in human volunteers. Growth was consistently observed in human tracheal organ cultures, but not in ferret tracheal organ cultures (Tyrrell and Bynoe, 1965). Subsequently, organ cultures were gently homogenized and examined by negative-stain EM for evidence of viral growth (Tyrrell and Almeida, 1967). B814 was later isolated from nasal washes directly in L132 (continuous human embryonic lung epithelial) cells, but with only minimal cytopathology (Bradburne and Tyrrell, 1969). The B814 from L132 cells was adapted to human fetal tonsil diploid fibroblast (FT) and human embryonal heteroploid rhabdomyosarcoma (RD) cells and to plaque assay in these cells (Schmidt et al., 1979).

Organ cultures were also used to isolate six strains of HCV (OC16, 37, 38, 43, 44, and 48) in a study of adult upper respiratory infection in 1966 (McIntosh et al., 1967b). Tracheas were obtained from 5- to 9-month fetuses and stored in cold Hanks balanced salt solution with 10% fetal calf serum for 2 to 48 h. Then, the tracheas were cut into 2- to 3-mm squares and placed in petri dishes with Leibovitz medium–0.2% BSA. After inoculating with 0.2 ml of a nasopharyngeal wash specimen, cultures were incubated at 33°C on a rocker platform with daily changes of medium. Viral replication was evidenced by ces-

sation of ciliary movement and by negative-stain EM. These isolates did not grow in conventional cell cultures, but two of them, OC38 and OC43, were readily adapted to suckling mouse brain (SMB) (McIntosh et al., 1967a). Adaptation occurred on the first passage at 11 to 15 days after intracranial inoculation; encephalitic symptoms developed after progressively shorter times until all mice died within 60 h postinfection.

Subsequently, OC38 and OC43 were adapted to low-level growth in both rhesus and vervet primary monkey kidney (MK) cell monolayers (Bruckova et al., 1970; McIntosh et al., 1970b). Adaptation to rhesus MK was slightly faster from SMB-grown virus (two passages) than from organ culture-grown virus (three passages). The CPE observed was focal and somewhat syncytial, with gradual spread throughout the cell monolayer. From African green MK cells, OC43 was further adapted to MA-321 cells (Gerna et al., 1980). From rhesus or vervet MK cells, both OC38 and OC43 were further adapted to BSC-1 (continuous African green monkey kidney epithelioid) cells. In BSC-1, the CPE appeared on first passage and was less syncytial, but still involved the entire monolayer by 10 to 14 days after inoculation. All cultures were maintained under a fortified medium at 33°C on roller drums. From BSC-1 cultures, OC38 and OC43 were further adapted to FT and RD cells with production of definitive CPE and high infectivity titers (Schmidt and Kenny, 1982; Schmidt et al., 1979). In addition, OC43 was adapted from BSC-1 to LLC-MK_2, a rhesus monkey kidney epithelial line (Monto and Rhodes, 1977).

Other strains of OC43-like virus (GI, HO, RO) were isolated in tracheal organ cultures and in human volunteers and subsequently passed in both organ culture and MRC-c continuous human embryonic lung diploid fibroblast line (Larson et al., 1980). These are the most recent isolations of OC43 virus reported.

Plaque assays for OC38 and OC43 virus have been carried out in FT and RD cells (Schmidt and Kenny, 1981, 1982; Schmidt et al., 1979). Virus has also been identified in the cultures by EM, CPE, IFA, neutralization tests, and complement fixation.

Strain 229E was first isolated in secondary human embryonic kidney (HEK) cells and adapted to human embryonic lung diploid fibroblast cells (WD, HEL, WI38). The cytopathic effect was described as slow and stringy after 6 to 10 days of incubation at 33°C on roller drums (Hamre and Procknow, 1966; Hamre et al., 1967). Other strains of 229E have been isolated in MA177 human embryonic intestine diploid fibroblast cell cultures (Kapikian et al., 1969); in WI38, L132, and primary HEK cells and human embryonic tracheal organ cultures (Bradburne, 1969, 1972; Bradburne and Tyrrell, 1969); in WI38 cells (McIntosh et al., 1974); in human embryonic nasal organ culture (Larson et al., 1980); and in MRC-c cell culture (Reed, 1984). All isolations were made under fortified medium, such as Eagle MEM, Leibovitz, or medium 199, with 2% fetal calf serum, in roller cultures at 33°C. The 229E-like strains isolated by Larson et al. (1980) in organ culture or in volunteers (strains AD, PA, PR, TO, KI) were passed both in organ culture and in MRC-c cells for further study.

Adaptation to other cells has been easily accomplished. Human coronavirus 229E was adapted from WI38 cultures to FT and RD cells (Schmidt and Kenny, 1982; Schmidt et al., 1979); from nasal organ cultures to MRC-c and MRC-5 cells (Larson et al., 1980; Reed, 1984); and from MRC-c cells to human macrophage cells (Patterson and Macnaughton, 1982). Again, incubation at 33°C in roller cultures appeared to be critical.

Plaque assays for 229E have been successful in a number of systems, which have been useful for viral replication studies. Plaque production was reported in various human embryonic lung diploid fibroblast cells (Hamre et al., 1967; Macnaughton et al., 1980), in L132 cells (Bradburne, 1972; Bradburne and Tyrrell, 1969; Chaloner-Larsson and Johnson-Lussenburg, 1981), and in FT and RD cells (Schmidt and Kenny, 1981, 1982; Schmidt et al., 1979). Details of cell concentrations, overlay mediums, incubation times, and stains used for visualization are given in the reports cited.

Virus can be identified in cell culture by EM, CF, and IFA (Hamre et al., 1967; Kapikian et al., 1969; Macnaughton et al., 1980), by plaque reduction neutralization assays (Bradburne, 1972; Bradburne and Tyrrell, 1969; Macnaughton et al., 1980; Schmidt et al., 1979), and by fluorescent focus assay (Macnaughton et al., 1980). In peritoneal macrophage culture, 229E was identified by an infectious center assay read by IFA (Patterson and Macnaughton, 1982). Cytopathic effect was also a valuable indicator of viral growth in most cell systems. As described in the section Antigenic Composition, coronaviruses do not produce excess proteins or soluble antigens during culture, which might amplify an assay.

Strain 692 replicated in human embryonic tracheal organ culture, but without inhibiting ciliary motion, was seen by IEM of the culture sediment when mixed with the patient's convalescent serum (Kapikian et al., 1973). This virus could not be grown in cell cultures used successfully for 229E.

The HECV has been propagated to a limited extent in human embryonic intestinal organ culture (Caul and Clarke, 1975; Caul and Egglestone, 1977; Resta et al., 1985). As with HCV in organ cultures, growth in virus was evidenced by destruction of the villous epithelium and by EM or IEM of organ culture fluids and cell sediments. Intestinal organ cul-

tures are prepared from small (2 × 2 mm) pieces of small intestine from 5- to 8-month fetuses and are maintained under enriched medium, such as Leibovitz L-15 with 0.4% BSA, at pH 6.5 to 6.8. Rocker cultures at 35 to 37°C were optimal. Trypsin at a final concentration of 5 μg/ml appeared to enhance infectivity (Resta et al., 1985). Identification of virus was accomplished by EM, IEM, and IFA (Caul and Clarke, 1975; Resta et al., 1985) and by single radial hemolysis, immunoblot, and Western blot (Resta et al., 1985).

Antibody Assays

Serologic tests have provided all of the clinical and epidemiologic information about HCV. These range from immunodiffusion (ID), immunoelectrophoresis (IE), IEM, and IFA for simple detection of antibody (Schmidt, 1984), to broadly applicable neutralization and CF tests, to highly sensitive EIA tests in various formats. All of these tests presumably measure IgG antibody; no early or IgM serologies have been reported for HCV or HECV. Because the peplomers of HCV interact with animal erythrocytes, HI tests for OC43 and IHA tests for 229E are readily available. Most serologic tests for HCV require the intact virion as antigen because the coronaviruses do not produce soluble antigens during their replication cycle. The various serologic tests used for HCV diagnosis are outlined in Table 5 and described in detail below. For all serologic tests, serum specimens, generally at a 1 : 4 starting dilution, are routinely heat-inactivated at 56°C for 30 min.

Immune Electron Microscopy

Antibodies to HCV and HECV have been detected in patient sera by IEM, as have viruses in stool specimens. Serologic IEM aims to detect the presence, not the titer, of an antibody that can bind to virus and produce small clumps of viral particles. The procedure involves mixing a 1 : 30 to 1 : 100 dilution of "antigen," incubating for a minimum of 2 h, and then examining under the electron microscope. The antigen may consist of stool suspension, tissue homogenate, or cell or organ culture supernatant. Stool extracts are prepared by making an approximating 10% suspension in veal heart infusion broth, PBS, or similar medium, shaking vigorously with glass beads, and then clarifying by centrifugation at 5,000 × g for 30 min (Chany et al., 1982; Gerna et al., 1984; Maass and Baumeister, 1983; Mortensen et al., 1985; Schnagl et al., 1986; Vaucher et al., 1982). This force will not bring down coronaviruses (Kaye et al., 1970). Tissue homogenates are prepared by gently grinding with mortar and pestle or glass homogenizers and clarifying as above. Cell and organ culture supernatant fluids (the maintenance medium bathing the cells during viral culture) are used as antigen without dilution, after clarifying by centrifugation (Kapikian et al., 1973; Resta et al., 1985). The serum–antigen mixtures are incubated for at least 1 h at ambient temperature; in our laboratory, we prefer 2 h at 37°C and overnight at 4°C. Pseudoreplica grids are then prepared, negatively stained with PTA, and examined by EM as described previously.

Serum Neutralization

The standard serum neutralization (SN) test has been common to all studies of human and animal coronaviruses, both to identify isolates and to measure specific antibody response in the host species and heterotypic antibody responses in other species. In coronaviral research, as with so many other viral groups, the SN test is still regarded as the "gold standard" to which other tests are compared.

For 229E, conventional SN and plaque-reduction SN tests have been used. Antibody titers of 1 : 8 to 1 : 64 were considered positive titers in tube cultures of WI38, HEL, MA177, and RU-1 fibroblast cells (Cavallaro and Monto, 1970; Hamre and Procknow, 1966; Kapikian et al., 1969; Kaye et al., 1972; Monto and Lim, 1974; Monto and Rhodes, 1977). These tests used an infectious antigen that was freeze–thawed once and clarified by light centrifugation. After titration for viral endpoint, 30 to 300 $TCID_{50}$s of the virus per 0.1 ml were mixed with an equal volume of serial twofold dilutions of serum and incubated for 1 h at ambient temperature. The mixtures were then inoculated at 0.2 ml per tube, adsorbed at 35°C for 1 h, and overlaid with 1 ml of a fortified maintenance medium. The tests were read for inhibition of CPE after 4 to 10 days of incubation at 33°C on a roller drum. A positive titer was any value of 1 : 8 or higher; a seroconversion was a fourfold or greater increase in titer between the acute- and convalescent-phase sera.

A similar macro-SN test in HEK cells was described by Miyazaki et al. (1971). In that study, 32 $TCID_{50}$s of virus were mixed with serial serum dilutions and adsorbed onto the cell monolayers for 2 h. After maintenance medium was added, the cultures were incubated at 33°C on roller drums and read at 7 days.

Micro-SN tests for 229E antibody have been described with WI38, MRC-c, FT, and C-16 cells in standard 96-well, flat-bottomed microtiter plates (Callow, 1985; Gerna et al., 1978; Kraaijeveld et al., 1980b; Reed, 1984; Schmidt, 1984; Schmidt and Kenny, 1981). In a typical test in FT cells (Schmidt and Kenny, 1981), 300 $TCID_{50}$s of virus in 0.025 ml were mixed with 0.025 ml of twofold serum dilutions and incubated at ambient temperature for 30 min.

Then, 16,000 FT cells in 0.05 ml of MEM–5% fetal calf serum growth medium were added per well and the plates incubated at 33°C in a 2.5% CO_2 atmosphere. The plates were read for CPE and cell staining with crystal violet when the viral controls and back-titration showed 100 $TCID_{50}$s. In this and similar micro-SN tests, the tests are readable by 5 days and titers of 1:8 to 1:128 are commonly obtained.

Serum neutralization tests for OC43 antibodies were conducted in suckling mouse brain at first, using as antigen a 10% SMB suspension in PBS or Veronal-buffered diluent, clarified at 600 × *g* for 20 min (Kaye and Dowdle, 1969). After adaptation to cell culture, macro-SN tests were carried out in BSC-1 cell monolayers and read by hemadsorption after 4 days of incubation on rollers at 33°C (Monto and Rhodes, 1977). Micro-SN tests in FT cells were as described previously (Schmidt, 1984; Schmidt and Kenny, 1981). In all three systems, titers of 1:4 to 1:128 were commonly obtained.

Plaque-reduction SN tests have been used for serologic studies with 229E and OC43. For 229E, tests were conducted with L132 cells and endpoints read as 90% reduction in plaques (Bradburne and Somerset, 1972; Bradburne and Tyrrell, 1969). For OC43, tests were carried out with MA-321 cells and serum endpoints read by an immunoperoxidase assay (Gerna et al., 1980). Serum titers of 1:20 to 1:2560 were obtained in the OC43 test.

Complement Fixation

As for other viral antibodies, the microtiter complement fixation (CF) test for coronaviruses is highly reproducible, but it is not sensitive (Kaye et al., 1969). It was used extensively for earlier epidemiologic studies because both 229E and OC43 virus were includable in one test, often along with many other antigens in the so-called respiratory battery (Kapikian et al., 1969; Tyrrell and Bynoe, 1965). The antibody measured by CF appears to be relatively short-lived, which further reduces the sensitivity of the test (Cavallaro and Monto, 1970).

In most studies, the antigen for 229E was a crude, clarified fibroblast cell harvest, prepared by three freeze–thaw cycles of WI38, HELF, or RU-1 cultures after 3 days of incubation followed by low-speed centrifugation (Cavallaro and Monto, 1970; Gerna et al., 1978; Hendley et al., 1972; Kaye et al., 1972; McIntosh et al., 1970a,b, 1978; Monto and Lim, 1974; Monto and Rhodes, 1977). In other studies, antigens were prepared in L132 cells (McIntosh et al., 1974), sometimes followed by purification and concentration by Sepharose 4B chromatography (Bradburne and Somerset, 1972), or were prepared as purified virus from RD cell cultures (Schmidt, 1984; Schmidt and Kenny, 1981). Serum titers of 1:8 to 1:64 were considered positive titers (Gerna et al., 1978; Hamre and Procknow, 1966; Kapikian et al., 1969; Schmidt and Kenny, 1981; Wenzel et al., 1974).

The antigen for OC43 was a 10 to 20% SMB suspension in veronal-buffered diluent, clarified at 1000 × *g* for 20 min (Hierholzer and Tannock, 1977; Hierholzer et al., 1979; Hovi et al., 1979; Kaye and Dowdle, 1969; Kaye et al., 1971; McIntosh et al., 1970a,b, 1978; Monto and Lim, 1974; Monto and Rhodes, 1977; Riski and Hovi, 1980). Alternatively, Sepharose-purified virus from SMB or virus purified from RD cells has been used in other studies (Bradburne and Somerset, 1972; Schmidt, 1984; Schmidt and Kenny, 1981). Serum titers of 1:8 to 1:128 were considered positive for past exposure to OC43 virus.

Hemagglutination

After OC43 was adapted to SMB—the key to success with this virus—Kaye and Dowdle (1969) showed that virus grown to high titer in SMB could agglutinate human, vervet monkey, chicken, rat, and mouse erythrocytes. This HA activity was different from that of myxoviruses because elution did not occur, a finding later confirmed by the failure to detect neuraminidase (Hierholzer et al., 1972; Zakstelskaya et al., 1972a). The property of hemagglutination was immediately utilized in hemagglutination inhibition (HI) tests for serum antibody (Kaye and Dowdle, 1969). The HI test was practical, high-titered antigen was easily prepared as a clarified 10% SMB suspension in PBS, and HI was more sensitive than CF or SN in detecting seroconversions (Kaye and Dowdle, 1969; Kaye et al., 1971; Monto and Lim, 1974).

The standardized microtiter HA/HI procedure calls for treatment of serum by heat inactivation only, utilization of 4 HA units of antigen/0.025 ml and 0.5% chicken red blood cell suspension, and incubation of the test at ambient temperature (Hierholzer et al., 1969). Virtually all OC43 HI tests reported have used the SMB antigen with chicken erythrocytes (Bradburne and Somerset, 1972; Hendley et al., 1972; Hierholzer and Tannock, 1977; Hovi et al., 1979; Kaye and Dowdle, 1969; McIntosh et al., 1974; Monto and Lim, 1974; Monto and Rhodes, 1977; Reed, 1984; Riski and Estola, 1974; Riski and Hovi, 1980; Wenzel et al., 1974; Zakstelskaya et al., 1972b). Serum titers commonly run from 1:10 to 1:640, but probably only titers above 1:20 are positive (Gerna et al., 1980; Hendley et al., 1972; Hierholzer and Tannock, 1977; Kaye and Dowdle, 1969; Riski and Estola, 1974). In one study, OC43 HA antigen was prepared as purified, concentrated virus from RD cells and gave serum HI titers of 1:8 to 1:64 (Schmidt and Kenny, 1981). Some-

times, human serum specimens have been found to contain an inhibitor (possibly the same high-density lipoprotein factor responsible for false hemadsorption, as described in the section Physicochemical Properties and Morphology) which can be removed by treating the serum with phospholipase C (Gerna et al., 1980). In some systems, this treatment has been necessary to avoid false-positive titers in HI tests and in plaque-reduction SN tests (Gerna et al., 1980; Hovi et al., 1979).

Indirect Hemagglutination

Human coronavirus 229E grown in cell cultures does not hemagglutinate under any condition and has not been adaptable to SMB despite repeated attempts by many laboratories. However, 229E can sensitize glutaraldehyde-fixed, tannic-acid-treated sheep erythrocytes to form the basis of an indirect hemagglutination (IHA) test (Kaye et al., 1972). The IHA antibodies range from 1 : 10 to 1 : 5120 and are significantly higher than the CF or SN antibodies found in the same sera (Gerna et al., 1978; Hierholzer and Tannock, 1977; Kaye and Dowdle, 1975; Kaye et al., 1972).

Single Radial Hemolysis

The single radial hemolysis (SRH) test was developed for coronaviruses because it had broad applicability in many types of studies, much like the CF test (Hierholzer and Tannock, 1977). For the SRH test, sheep erythrocytes were washed and stabilized with 0.0073% glutaraldehyde. Then, to use the binding properties of the chromic cation, a 25% erythrocyte suspension was mixed with a high concentration of purified virus in the presence of 0.0016% aged chromic chloride. The reaction was stopped with phosphate–saline, and finally the treated, rewashed cells were mixed with complement and agarose at 45°C to prepare a gel on a microscope slide. The final mix consisted of 1% agarose, 0.1% azide, 5% reconstituted complement, and 0.82% treated cells. Wells 2 mm in diameter were loaded with 5 μl of serum dilution, incubated overnight at 4°C for diffusion of antibody and fixation of complement, and then incubated for 1 day at 37°C for development of zones of hemolysis. The diameter of the hemolytic zone was linearly related to antibody concentration as determined by CF, SN, HI, and IHA serologic tests (Hierholzer and Tannock, 1977). In a separate study, antibody to HECV in infant sera that was quantitated by SRH also appeared to correlate with antibody detected by IEM and Western blot analyses (Resta et al., 1985).

A similar hemolysis-in-gel test was described by Riski et al. (1977). This test also correlated well with HI titers in serosurveys (Hovi et al., 1979; Riski and Hovi, 1980).

Indirect Fluorescent Antibody Test

Monto and Rhodes (1977) described serologic indirect fluorescent-antibody (IFA) test for 229E and OC43 antibodies. For 229E, WI38 cells were grown on Leighton tube cover slips and infected with 229E by adsorption of virus to monolayers for 2 h at 37°C. Maintenance medium (MEM–2% fetal calf serum) was added and the cultures incubated at 34°C for 2 to 3 days. Then, the cover slips were rinsed, fixed in acetone, and stored at 4°C until tested. For OC43, LLC-MK_2 cells were grown on Leighton cover slips and infected with BSC-1 adapted virus at a high multiplicity. Virus was adsorbed for 2 h at 37°C, maintenance medium (medium 199–1% horse serum) was added, and the cultures were incubated at 34°C for 14 to 16 days, when CPE appeared. The cover slips were then washed and fixed as described above.

For the IFA test, dilutions of human sera were incubated with the cover slips for 1 h at 37°C. The cover slips were washed well, incubated with fluorescein-conjugated anti-human globulin, rewashed, and then counterstained with Eriochrome black. The IFA test was specific for both viruses and was most likely to be positive if the patient's seroconversion was detectable by more than one other test (Monto and Rhodes, 1977).

Immune-Adherence Hemagglutination

An immune-adherence hemagglutination (IAHA) test was described by Gerna et al. (1978) as a sensitive test for 229E antibodies. Human serum dilutions were added in 0.025-ml amounts to microtiter V plates. Human coronavirus 229E antigen, prepared in RU-1 or WI38 cells, was added in 0.025 ml amounts and incubated at 37°C for 1 h. Then, 0.025 ml of an optimal dilution of complement, determined as for the CF test, was added and the plates were incubated at 37°C for 40 min, followed by 0.025 ml of 0.3% dithiothreitol in 0.04 M EDTA–Veronal buffer. Finally, 0.025 ml of 0.4% human O erythrocytes were added and the plates were incubated at ambient temperature for 1 h. Hemagglutination patterns were read as nonagglutinated cells (negative) to agglutinated cells (positive). The diluent throughout the test was gelatin–Veronal buffer as used in CF. The IAHA test performed in this manner appeared to be more sensitive than IHA, SN, or CF. Positive titers ranged from 1 : 16 to 1 : 512 (Gerna et al., 1978).

Radioimmunoassay

A solid-phase radioimmunoassay (RIA) for OC43 antibodies has been described by Hovi et al. (1979). The OC43 virus, diluted in PBS, was adsorbed onto polystyrene beads, 6.4 mm in diameter and at a concentration of 6 μg per bead, during overnight incubation at ambient temperature. After air-drying, the

beads were reacted with serum dilutions at 37°C for 1 h. The beads were then washed twice, ^{125}I-labeled anti-human IgG was added for another hour at 37°C, and the beads were finally washed and assayed in a gamma counter. The RIA test was decidedly more sensitive than SRH, HI, or CF (Hovi et al., 1979).

Immunoperoxidase Assay

Gerna et al. (1979, 1980) described an immunoperoxidase assay (IPA) for OC43 antibodies that was useful in reading plaque assays. The IPA test was conducted on microcultures of primary African green monkey kidney cells or of human embryonic lung diploid fibroblast cells (MA-321). The cell cultures were grown under a fortified medium, but without serum. The cells were fixed with absolute ethanol when the monolayers showed 50% infected cells. Dilutions of human acute- and convalescent-phase sera were added to the fixed cells and incubated at 37°C for 60 min. The cultures were washed three times in PBS and then reacted with peroxidase-conjugated anti-human IgG. The test was developed histochemically. Optimal dilutions of the reagents, including those for the histochemical detection of the peroxidase, were predetermined by titrations of reference mouse immune ascitic fluid to OC43, followed by peroxidase-conjugated sheep anti-mouse IgG serum. In the serologic IPA, positive titers ranged from 1 : 40 to 1 : 640, similar to those obtained by HI (Gerna et al., 1980).

Enzyme Immunoassay

The enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) is the most sensitive and versatile test described to date for HCV. In the procedure described by Kraaijeveld et al. (1980b) for HCV 229E, the viral antigen was grown in MRC-c cells and the test carried out in flat-bottomed, polystyrene microtiter plates. The plates were prewashed four times with Dulbecco PBS, and the wells were then coated with 0.2 ml of optimal antigen dilution in 0.1 M carbonate buffer, pH 9.6, during overnight incubation at ambient temperature. The plates were washed four times with PBST (PBS–0.05% Tween 20–0.02% azide), shaken dry, and used without further treatment. For the EIA test, 0.2 ml of serum dilutions in PBST were added to wells in duplicate and incubated 4 h at ambient temperature. The plates were again washed four times with PBST and shaken dry. Then, 0.2 ml of conjugate (anti-human IgG–alkaline phosphatase) was added per well and incubated overnight at ambient temperature. After another washing series, 0.2 ml of substrate (0.1% sodium *p*-nitrophenyl phosphate–0.01% magnesium chloride–0.02% sodium azide, in 10% diethanolamine buffer, pH 9.8) was added per well. The reaction was developed for 30 min and was then stopped with 0.05 ml of 3 M NaOH per well. Absorbance values were read at 405 nm.

The EIA test for 229E was more sensitive than a micro-SN test in MRC-c cells for detecting infections in volunteers and in patients with common colds (Kraaijeveld et al., 1980b; Macnaughton et al., 1981a, b). The same procedure was applied to OC43 using a 10% SMB antigen (Macnaughton et al., 1981b) or a cross-reacting CV-Paris antigen grown in HRT-18 cells (Macnaughton, 1982). In these studies, the EIA was used to document type-specific infection with 229E and OC43, to confirm the distinction between these two viruses, and to measure the frequency of HCV infections in populations (Macnaughton, 1982; Macnaughton et al., 1981a, b). Other EIA tests have been described using purified, concentrated 229E and OC43 viruses grown in RD cells (Schmidt, 1984; Schmidt et al., 1986) or clarified 229E virus grown in C-16 cells (Callow, 1985). The choice of antigen may be decided by each laboratory, but EIA is the test of choice for HCV serology because it can measure antibody levels to both coronaviruses at the same time and with high sensitivity.

Interpretation of Serologic Data and Limitations of Tests

Serologic tests such as IEM, ID, and IE are useful only to detect the presence or absence of HCV or HECV antibody because the sera must be used at low dilutions (1 : 1 to 1 : 15). Other tests (e.g., IFA, CF, SN, and HI) do quantitate the antibody and may be easily performed, but are not sensitive for low levels of antibody. Still others (e.g., plaque-reduction SN, SRH, and EIA) are more complex, but are much more sensitive for serodiagnosis and serosurveys.

Proper controls must be built into all serologic tests. These include known positive and negative sera to check the specificity of the antigen, known positive and negative antigen or cell culture controls to check the sensitivity of the test, and reagent blanks to allow calculation of background values. When the test is performed properly and all controls give the expected values, a serodiagnosis is possible by demonstrating a fourfold or greater rise in antibody titer between the acute- and convalescent-phase sera. It is critical that both sera in a pair be tested at the same time to obviate the day-to-day variation found in any test system.

Due to the ubiquity and reinfection rate of HCV and HECV, paired sera are required for serodiagnosis. The acute-phase serum should be drawn within 7 days of onset of symptoms. The convalescent-phase serum should be drawn approximately 3 to 4 weeks after onset so that peak titers by any test procedure are realized. This is true for all of the serologic tests

described above. The high reinfection rate observed with coronaviruses implies that homotypic or heterotypic anamnestic antibody responses might confuse the serodiagnosis of a patient's illness. This possibility does exist, but as yet data are insufficient to evaluate the extent of the problem.

Serosurveys can be carried out on single serum specimens to measure the prevalence of antibody in various populations. These single titers will indicate past (or current) infection, but cannot be interpreted beyond this finding. The relatively low serum titers obtained with the tests used for HCV might suggest that specific HCV antibodies are not long-lived or that HCV does not elicit a dramatic serum antibody response at all. These questions warrant further research, particularly with regard to reinfection throughout life.

The antibody titer of a serum is recorded as the highest serum dilution that inhibits the property or effect of the antigen tested, when the antigen back-titration confirms that a standard dose was employed in the test. The property is different for each test, such as infectious viral dose, fixing complement, agglutinating red blood cells, cytoplasmic fluorescence, or optical density readings. Accordingly, the definition of serum endpoint dilution (titer) is an integral part of and is unique to each test. Definitions of titers and of acceptable background values are found in the test descriptions cited.

The choice of which serologic test or tests to perform is often dependent on what is most efficient for the laboratory. It is obviously preferable to use available tests, if they will suffice, rather than setting up new ones. The other factor in choosing a test is sensitivity. For HCV 229E, the order of sensitivity (from most to least sensitive) is EIA, IAHA, SRH, micro-SN, IHA, tube SN, CF, IFA, IE, ID, and IEM. For HCV OC43, the order of sensitivity is EIA, RIA, plaque-reduction SN, SRH, IPA, micro-SN, HI, tube SN, CF, IFA, IE, ID, and IEM. Results from any test procedure should be interpreted in consideration of the patient's clinical history (i.e., to make a serodiagnosis, the results should be used in conjunction with information available from clinical evaluation, other diagnostic tests, and the epidemiology of the virus).

Epidemiology and Natural History

The respiratory HCV 229E and OC43 are considered from various studies to account for 5 to 35% of infections of the upper respiratory tract and are therefore a major cause of common colds worldwide. Significant levels of specific antibody have been found in all age groups in the United States (Hamre and Beem, 1972; Hendley et al., 1972; Kaye and Dowdle, 1975; Kaye et al., 1971; McIntosh et al., 1970a,b; Monto and Lim, 1974; Schmidt et al., 1986); Japan (Miyazaki et al., 1971); England (Bradburne and Somerset, 1972; Isaacs et al., 1983; Macnaughton, 1982); Russia (Zakstelskaya et al., 1972b); Finland (Hovi et al., 1979; Riski and Hovi, 1974) Brazil, and Italy (Gerna et al., 1978). Antibody prevalence for both HCV types ranges from 6 to 37% in the less than 1-year-old age group, to 54 to 80% in the 1- to 5-year-old age group, to 100% of persons infected after age 5. Between ages 21 and 50, only 85% of persons tested had antibody, suggesting that fewer reinfections occurred during adulthood.

Seasonal and annual fluctuations in HCV outbreaks were noted in many studies. All documented epidemics of HCV-related upper respiratory illnesses occurred during the winter and spring seasons. Outbreaks with both 229E and OC43 follow a 2- to 3-year cycle, with winter-season peaks of infections occurring annually (Kaye and Dowdle, 1975; Kaye et al., 1971; McIntosh, 1974; Monto, 1974). In one study in a childrens' home, outbreaks of 229E-related illness alternated with those caused by OC43; this observation, however, could have been by chance (Kaye and Dowdle, 1975).

The rate of asymptomatic HCV infection appears to be high, as one might assume from the generally mild nature of HCV-related colds. In one study in which this factor could be measured, 55% of adults infected with 229E, as determined by appropriate serologic tests, reported no symptoms at all (Cavallaro and Monto, 1970). In the volunteer studies described previously, approximately 30% of persons given intranasal drops with 229E or OC43 failed to develop any symptoms, although the virus could generally be demonstrated in their nasal washings and increases in antibody found in their sera. Approximately 50% of children infected with 229E or OC43, as documented by seroconversions, did not have symptoms of respiratory illness (Kaye and Dowdle, 1975; Kaye et al., 1971).

The nonprotective effects of serum antibodies and the apparently high reinfection rates of HCV 229E and OC43 are related to the asymptomatic infection rate (Monto, 1974). Preexisting HI antibody to OC43 was found in one-third of the children who seroconverted to OC43, and this antibody did not appear to play a role in modifying the severity of subsequent OC43-related illness (Kaye et al., 1971). In an identical manner, preexisting IHA antibody was found in one-third of children with 229E seroconversions, and this antibody had no obvious ameliorating effect on the respiratory illness caused by the virus (Kaye and Dowdle, 1975).

Hamre and Beem (1972) showed that the frequency of increases in SN titer to 229E was inversely proportional to preexisting levels of SN antibody,

suggesting that this antibody possessed some protective effect. However, in other studies, up to 81% of adults infected with OC43 possessed prior SN antibody (Monto and Lim, 1974), and preexisting SN, CF, HI, or EIA antibodies to 229E or OC43 failed to protect children or adults against homotypic reinfection (Callow, 1985; Cavallaro and Monto, 1970; Hendley et al., 1972; Isaacs et al., 1983; Macnaughton, 1982; Reed, 1984; Schmidt et al., 1986). In fact, it was not uncommon to find two distinct HCV infections in adults within a single year, even in the presence of detectable levels of antibody. Thus, the relationship of circulating SN or other antibodies to modification of HCV infection is not clear at present. In addition, because of the frequency of reinfections by both viruses throughout life, it has not been possible to determine the natural persistence of the different antibodies elicited by HCV infection.

Because HCV infections involve the surface of the upper respiratory tract, it is likely that secretory IgA antibody is important in protection. Callow (1985) has shown that locally produced IgA does indeed protect from 229E infection and also shortens the period of virus shedding. However, this antibody is short-lived and probably has little effect on the natural spread of the respiratory coronavirus in communities or families.

The epidemiology of the HECV is a fertile field. Because the CVLPs have been found in many animal species in addition to humans, and are not clearly related to any disease, little is known beyond their observation by EM and IEM. There are no diagnostic or serologic tests that are specific for HECV and no HECV strains available with which to construct serologic assays.

Prevention and Control

At this time, control of HCV infections is neither possible nor necessary. The respiratory HCVs cause only mild to moderately severe colds in volunteers; the more severe illness seen in a low percentage of patients by serologic tests has not been confirmed by more direct antigen or isolation tests. More serotypes possibly exist, and the importance of HCV 692 has yet to be defined. Furthermore, the frequency of re-infection observed with these agents is high and indicates that IgG antibody is not protective. Environmental control of infection to minimize the spread of virus by droplets and fomites may be useful, as it would for any respiratory virus. However, such control has rarely been practical or possible in school or home settings.

In a study of prophylactic control of HCV (Turner et al., 1986), 55 volunteers were given recombinant interferon intranasally for 15 days and exposed directly to HCV by intranasal inoculation on the 8th day. In the placebo group, 73% of the volunteers developed colds, compared with 41% in the interferon-treated group. The interferon also reduced the severity of the cold symptoms and shortened the duration of the colds.

The HECV probably will have the greatest need for control once its role in infant gastroenteritis is ascertained. At present, the HECV requires proof of existence, relevance to disease, and comprehensive virology before its role as a pathogen in diarrhea can even be assessed.

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