

# Coronaviruses and Toroviruses

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## INTRODUCTION

Human coronaviruses (HCVs) are usually thought of in the context of the common cold and, indeed, these viruses are responsible for up to one-third of colds. There is also evidence for coronaviruses being associated with enteric disease in humans. HCVs can grow in some neural cells *in vitro* and have been associated with cases of multiple sclerosis, although the role of the coronavirus in such cases is not clear. The alimentary tract is certainly a site of replication of toroviruses, including those of humans; these viruses superficially resemble coronaviruses, with which they may be confused.

Coronaviruses were first isolated in humans in the mid-1960s (Tyrrell and Bynoe, 1965; Hamre and Procknow, 1966). The original coronavirus strains were passaged in organ cultures of human embryonic trachea or nasal epithelium and in primary human kidney cell cultures. Subsequently several other HCVs have been isolated in organ or tissue cultures. The majority of HCVs studied to date are serologically related to one of two reference strains, 229E and OC43. Molecular analysis has shown that these two viruses differ extensively from each other; they are distinct species of coronavirus, not simply variants of each other.

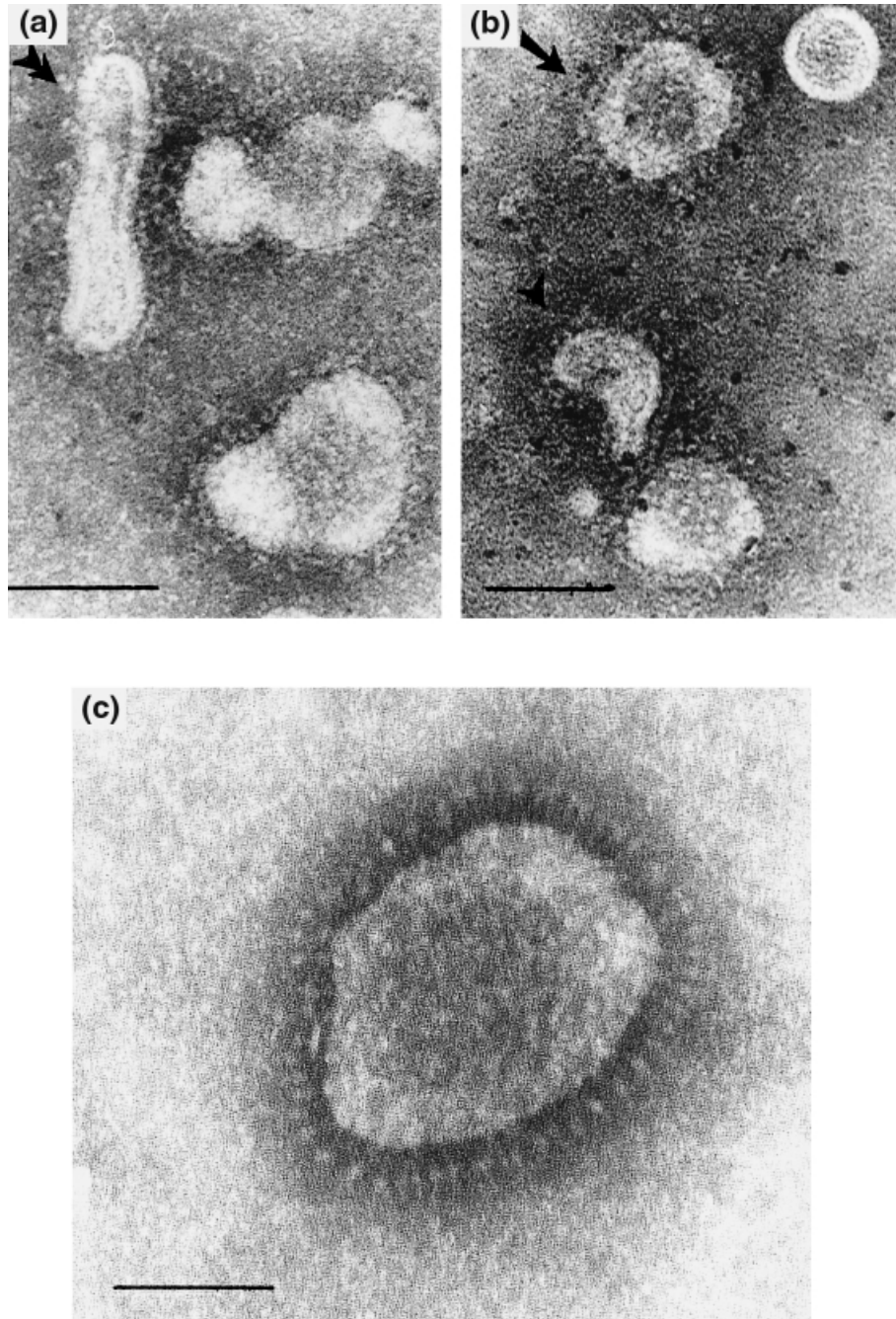
Toroviruses were first isolated from diarrhoeic horse in 1972 (*Berne virus*, BEV) and neonatal calves 1982 (bovine torovirus, BoTV, strain *Breda virus*, BRV) (Weiss and Horzinek, 1987). Subsequently particles resembling these two viruses were observed by electron microscopy in the stools of

children and adults with diarrhoea (Beards *et al.*, 1984). The human viruses formed immunocomplexes with anti-BEV and BoTV sera. Much less is known about human toroviruses than coronaviruses.

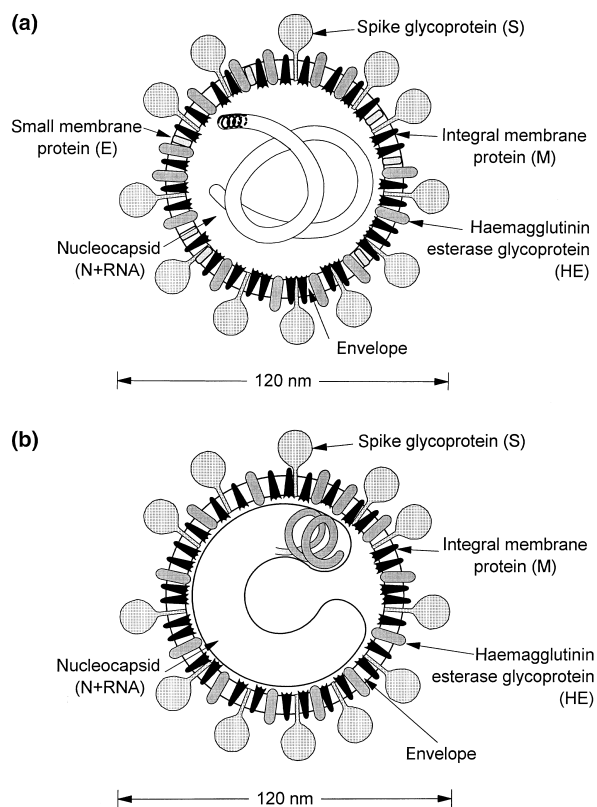
## THE VIRUSES

The coronaviruses, in the single genus *Coronavirus*, were for many years the only members of the family *Coronaviridae*. Recently the genus *Torovirus* was included in this family. Moreover, certain characteristics relating to genome organization, transcription and translation resulted in the creation of the order *Nidovirales* comprising the families *Coronaviridae* and *Arteriviridae* (Cavanagh, 1997; de Vries *et al.*, 1997). No arteriviruses, which are morphologically distinct from coronaviruses and toroviruses, have yet been discovered in humans.

Both coronaviruses and toroviruses comprise RNA-containing viruses of similar size and gross appearance. The coronaviruses are largely associated with respiratory and enteric infections in mammals and birds, while to date the fewer known toroviruses have been associated with diarrhoea (Dales and Anderson, 1995; Koopmans and Horzinek, 1995; Myint, 1995; Siddell, 1995a; Lai and Cavanagh, 1997). The large surface projections of the coronaviruses gave them an appearance reminiscent of a crown, hence the name coronaviruses (Latin *corona*, crown; Figures 10.1 and 10.2). The doughnut shape sometimes seen within torovirus



**Figure 10.1** Electronmicrographs of (a, b) human enteric torovirus showing torus (arrow), crescent (arrow head) and rod (double arrow head), and (c) a human enteric coronavirus. The latter has spikes which are more prominent and readily discernible than those of the torovirus. The shorter, 10 nm, projections on the torovirus particles may be the haemagglutinin-esterase protein (HE). (Bar = 100 nm) (Reprinted from Duckmanton *et al.* (1997) by permission of Academic Press)



**Figure 10.2** Virions of (a) a coronavirus and (b) a torovirus. The HE protein is possessed by HCV-OC43 but not by HCV-229E. Similarly, an HE protein is possessed by some, though not all, toroviruses. (Reproduced from Cavanagh, D. *et al.* (1994) *Archives of Virology*, **135**, 228–239, with permission of Springer-Verlag, Vienna)

particles resulted in the use of the root ‘toro’, from the Latin *torus*, the lowest convex moulding in the base of a column (Figures 10.1 and 10.2). Coronaviruses such as HCV and *Avian infectious bronchitis virus* (IBV) of chickens are most notable for growth in respiratory tissues, although IBV can infect kidney, oviduct and enteric tissues and some HCV strains infect enteric tissues. Other coronaviruses such as *Canine coronavirus* (CCV), *Bovine coronavirus* (BCV), turkey coronavirus (TCV), some strains of *Feline coronavirus* (FCV), *Porcine transmissible gastroenteritis virus* (TGEV) and *Porcine epidemic diarrhoea virus* (PEDV) replicate predominantly in enteric tissues but also replicate to a lesser extent, and probably initially, in the respiratory tract. Some strains of FCV infect macrophages and cause infectious peritonitis, hence the

name *Feline infectious peritonitis virus* (FIPV). *Murine hepatitis virus* (MHV, murine coronavirus) strains, in addition to infecting one or more of respiratory, enteric and hepatic tissues, replicate in cells of the central nervous system, causing encephalitis and demyelination (Dales and Anderson, 1995; Houtman and Fleming, 1996). *Porcine haemagglutinating encephalomyelitis virus* (HEV) also selectively infect neuronal tissue. Coronaviruses have been assigned to one of groups 1, 2 and 3 (Table 10.1) on the basis of amino acid sequences, supported to some extent by antigenic analyses.

## Structure

Coronaviruses are pleomorphic, ether-labile, enveloped viruses with diameters ranging from 60 to 220 nm and have a buoyant density in sucrose of  $1.15\text{--}1.18\text{ ml}^{-1}$  (as do toroviruses). The particles are often described as having club-shaped surface projections or spikes (S) of up to 20 nm in length, although the shape varies among the species. Particles may be seen with incomplete coronas of spikes.

Toroviruses are 100–140 nm in diameter and are variously described as being doughnut-, disc-, kidney- or rod-shaped, depending on the angle of view. The large spikes of BEV and BoTV measure approximately 17–20 nm in length and are not unlike those of some coronaviruses in appearance (Weiss *et al.*, 1983). However, many particles of BEV, which had been prepared for electron microscopy were devoid of the 20 nm S protein, the virions having a smooth surface (Weiss *et al.*, 1983). Similarly, electron microscopy of BoTV has revealed few of the 20 nm spikes but rather an intact fringe of smaller spikes, some 7–9 nm in length (Woode *et al.*, 1982; Cornelissen *et al.*, 1997). The toroviruses recently isolated from human faeces had an intact fringe of 10 nm spikes (Duckmanton *et al.*, 1997), which resembled those seen on human toroviruses by Beards and colleagues (Beards *et al.*, 1984). The latter reported observing the 20 nm spikes only rarely. It is possible that the 10 nm spikes observed by Duckmanton and colleagues are haemagglutinin-esterase (HE) protein. It has recently been shown that BoTV, in contrast to BEV (which has an incomplete HE gene, a pseudogene) has an HE protein which forms a fringe of spikes approximate-

**Table 10.1** Coronavirus groups

Group 1	Group 2	Group 3
HCV-229E	HCV-OC43	IBV
CCV	BCV	TCV
FCV (FIPV)	HEV	
PEDV	MHV	
TGEV		

ly 6 nm in length (Cornelissen *et al.*, 1997). The toroviruses described by Duckmanton and colleagues did cause haemagglutination (Duckmanton *et al.*, 1997).

Both genera have genomes which are non-segmented, single-stranded RNA of approximately 30 000 nucleotides and positive polarity (de Vries *et al.*, 1997). Species of each genus produce five or more subgenomic mRNAs which form a 3'-terminal nested set. The coronaviruses also have a common 5'-terminal leader sequence derived from the 5' end of the genome; this has not been observed for toroviruses.

Both genera contain, in addition to S, a smaller integral membrane protein (M), largely embedded in the virus envelope, and a nucleocapsid protein (N), which surrounds the genome (Figure 10.2). Virions of coronaviruses also possess another membrane-associated protein, E (for envelope; also referred to as sM), comprising about 80–110 amino acids. A function has not yet been assigned to this protein, although it is essential for virus particle formation. Human coronaviruses of the OC43, but not 229E, group contain an additional protein, the HE protein, which forms a 5 nm layer of surface projections. As described above, BoTV has an HE protein which is absent from BEV, as this virus has an incomplete HE gene (Cornelissen *et al.*, 1997). The BoTV HE protein has approximately 30% amino acid sequence identity with that of coronaviruses and *Influenza C virus*.

The reader is referred to Brian *et al.* (1995), Cavanagh (1995), Laude and Masters (1995), Rotter (1995), Siddell (1995b), Snijder and Horzinek (1995), Cornelissen *et al.* (1997) and Lai and Cavanagh (1997) for comprehensive reviews, including references to gene sequences, of coronavirus and torovirus proteins.

## Sequence of HCV and Torovirus Proteins

Several HCV genes have been cloned and sequenced. The heavily glycosylated S glycoprotein of HCV-229E comprises 1173 amino acids. Whereas the S proteins of some coronaviruses, e.g. IBV, are efficiently cleaved into two subunits, S1 and S2, the S of HCV-229E grown in human embryonic lung cells is not cleaved. In accord with this, sequencing shows that the HCV-229E S protein lacks a highly basic sequence such as occurs in IBV at the S1–S2 cleavage site. Most of the S of HCV-OC43 grown in a human rectal tumour cell line is not cleaved but addition of trypsin cleaves S into S1 and S2. This indicates that the extent to which the S of HCV-OC43 is cleaved in various cell types depends in part on the presence in the cells of proteases which cleave adjacent to a basic S1–S2 connecting peptide, a situation which also applies to MHV. The S protein of HCV-OC43 is appreciably larger, at 1353 amino acids, than that of HCV-229E.

The S protein of Berne torovirus comprises 1581 amino acids and, like S of HCV strains, has an  $M_r$  of about 200 kDa when glycosylated. It has a highly basic S1–S2 connecting peptide and occurs as two subunits in virions.

The M proteins of HCV-229E, OC-43 and BEV torovirus comprise 225, 230 and 233 amino acids, respectively, of which probably only 10%, associated with one N-linked oligosaccharide in the case of HCV-229E and potentially several O-linked oligosaccharides for HCV-OC43, protude at the virion surface.

The different sizes of the N proteins of the 229E (389 amino acids) and OC43 (448 residues) strains serve to emphasize that these two viruses are not simply variants of each other. The N protein of 229E is very similar in size to that of TGEV, with 46% amino acid identity within the first third of the protein, while that of OC43 is identical in size to that of BCV and shares 97.5% identity overall. The N protein of Berne torovirus is much smaller than that of any coronavirus, comprising only 160 amino acids.

Strain OC43, in common with other coronaviruses in group 1 (Table 10.1) has an HE glycoprotein which has 95% amino acid identity with the HE of BCV strain G95. Remarkably, the coronavirus and torovirus HE proteins have 30%

sequence identity with the haemagglutinin-esterase-fusion (HEF) protein of *Influenza C virus*. Two functions have been assigned to coronavirus HE protein, receptor-binding and receptor-destroying (see below). The human toroviruses of Duckmanton *et al.* (1997) agglutinated rabbit and human erythrocytes. Coronavirus 229E lacks HE, in common with other group 2 and group 3 members (Table 10.1), and BEV also lacks HE.

### Antigenic Structure

Virus-neutralizing (VN) antibodies are induced by both the S and HE proteins of coronaviruses (reviewed by Cavanagh, 1995), and some antibodies against the M protein neutralize virus in the presence of complement. Monoclonal antibodies have shown that neutralizing and haemagglutination-inhibiting antibodies are induced by the torovirus S protein.

Epitope mapping of coronaviruses has been done mostly for S and has shown that VN epitopes are formed largely by the amino-terminal S1 half of the protein, which correlates with the much greater amino acid sequence variation in S1 than in S2 (Cavanagh, 1995). Removal of glycans from S of IBV greatly diminished the binding of VN monoclonal antibodies. The S protein has been shown to be a major inducer of protective immunity, although the other structural proteins, particularly the nucleocapsid protein, contribute to the induction of protection. In the case of FIPV it is the immune response to S which exacerbates the disease. It is thought that FIPV attached to anti-S antibody remains infectious and is more readily taken up by macrophages (antibody-dependent enhancement), in which FIPV replicates, than virus without antibody (de Groot and Horzinek, 1995).

Coronaviruses have been placed into one of three groups (Table 10.1). Species have higher amino acid identities with viruses within their group than with those in the other groups. Previously the groups had been referred to as serological groups. This, however, was misleading, as some members e.g. HCV-229E and PEDV, had only low antigenic cross-reactivity with other members of group 1. Until recently TCV had been in group 2 but recent sequence and antigenic analyses have indicated that it should be assigned to group 3 (Stephenson *et al.*, 1999).

Only one strain of BEV has been isolated. Strains of BRV have been divided into two serotypes and BEV and BRV share some epitopes. Immune electron microscopy has revealed relationships between these viruses and human torovirus (Beards *et al.*, 1984; Duckmanton *et al.*, 1997). Much remains to be done to establish the extent of variation among human toroviruses.

### Growth *In Vitro*

Previously OC43 and serologically related strains of HCV were considerably more difficult to propagate in cell culture than 229E-related strains and so organ cultures were used for OC43, hence the letters 'OC'. Now, however, cell lines are available for the propagation of both of the laboratory-adapted HCV types (consult HCV sequence papers referred to above), although primary isolation remains difficult (Myint, 1994, 1995). Cultures of fetal and adult astrocytes were infected by both types of HCV but infectious virus was only released from the fetal cells (Bonavia *et al.*, 1997). Human torovirus has not been grown *in vitro*.

### CELL RECEPTORS FOR HCVs

#### Attachment of Coronaviruses to Cells

The attachment of a virus to host cells is a crucial early step in infection, and specificity of attachment can be a major factor in determining host cell range and hence pathogenesis. This has been elegantly demonstrated by the transfer of coronavirus receptors from susceptible cells to other cells, rendering the latter susceptible to infection. It is the S protein that serves to attach the virus to cells. The extent to which the HE protein, when present, is also responsible for cell attachment is not clear. This topic has been reviewed by Holmes and Compton (1995) and Lai and Cavanagh (1997).

Human aminopeptidase N (APN) has been identified as a receptor for HCV-229E (Yeager *et al.*, 1992). This protein is a metalloprotease located on the surface of epithelial cells, including those of the intestine, lung and kidney. Porcine APN is the receptor for TGEV (Delmas *et al.*, 1992). TGEV replicates mostly in enterocytes of the small intestine

and produces its greatest tissue damage at this site but it also replicates to a lesser degree within the respiratory tract. This correlates with the incidence of APN, which is greater in the small intestine than in lung tissue.

The cellular receptor for MHV is a member of the carcinoembryonic antigen (CEA) family of glycoproteins in the immunoglobulin superfamily (reviewed by Lai and Cavanagh, 1997). This CEA protein is a 424 amino acid glycoprotein with four immunoglobulin-like domains. Human and hamster cells, refractory to infection by MHV, became susceptible to MHV when this receptor was expressed following transfection with the murine CEA gene (Dveksler *et al.*, 1991). Chen *et al.* transfected COS-7 cells, which lack a functional receptor for MHV, with genes of human CEA and human biliary glycoprotein; the cells were then susceptible to MHV (Chen *et al.*, 1997). Experiments with chimeras of human and murine CEAs revealed that the immunoglobulin loop of human CEA conferred virus-binding specificity. Different isoforms of murine CEAs exist. These have extensive differences in the amino-terminal immunoglobulin-like domain to which MHV binds, and bind MHV to different extents. Analysis of chimeras indicated that amino acids 38–43 were key elements for binding MHV and virus-induced membrane fusion (Rao *et al.*, 1997).

Human cells which were not susceptible to CCV or FCV became susceptible when transfected with a human/canine chimera of APN (Benbaccer *et al.*, 1997). The critical, carboxy-terminal domain was formed by amino acids 643–841 of the canine APN. When amino acids 255–348 of porcine APN were replaced by amino acids 260–353 of human APN, the resulting chimeric protein was able to function as a receptor for HCV-229E (Kolb *et al.*, 1996). Thus different parts of APN molecules may function as receptors for different coronaviruses within viruses of group 2 (Table 10.1). Expression of feline APN in rodent cells rendered the cells susceptible not only to FCV but also to HCV-229E, CCV and TGEV (Tresnan *et al.*, 1996). Previous work had shown, however, that human APN would bind HCV-229E but not TGEV, while porcine APN would bind TGEV but not HCV.

Less work has been done on the receptor for HCV-OC43. Collins (1995) has demonstrated that OC43 binds to major histocompatibility complex class I molecules.

## Involvement of Glycans

Both the S and HE proteins of BCV bind to cell surface components, a vital component of which is the glycan component *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac<sub>2</sub>). This residue acts as a receptor not only on erythrocytes but also on susceptible tissue culture cells (Schultze and Herrler, 1992). The S protein binds more efficiently than HE to Neu5,9Ac<sub>2</sub> and it has been proposed that the BCV S protein is responsible for the primary attachment to cells (Schultze and Herrler, 1992). The HEF glycoprotein of influenza C virus also binds to Neu5,9Ac<sub>2</sub>, while influenza A and B viruses do not (Vlasak *et al.*, 1988).

It has been proposed that the attachment of coronaviruses might be a two-step process. Primary attachment might be mediated by a first receptor, e.g. Neu5,9Ac<sub>2</sub>, for some coronaviruses, a second receptor, e.g. APN or CEA proteins, bringing the virus and cell membranes closer together. The latter step might be necessary for the fusion of the two membranes for the release of the virus genome. Some receptors might fulfil both functions for some coronaviruses.

IBV (and TGEV) attaches to  $\alpha$  2,3-linked neuraminic acid on erythrocytes and, provided that the virions are first treated with neuraminidase (from *Vibrio cholerae* or *Newcastle disease virus*), can cause haemagglutination (Schultze *et al.*, 1992). This removes  $\alpha$  2,3-linked neuraminic acid, which is present on the S protein of the virus and interferes with the attachment of the S protein to neuraminic acid on the erythrocyte surface. In view of the finding that TGEV attaches to porcine enterocytes, as opposed to erythrocytes, via APN, it may be that either  $\alpha$  2,3-linked neuraminic acid does not play a role in the attachment to these susceptible cells or that it may function as a primary receptor, insufficient for efficient infection, part of APN forming a secondary receptor essential for efficient infection.

The role played by the HE protein where present, e.g. in HCV-OC43, is not clear. Although it can mediate binding to erythrocytes, the main function of HE might be to remove neuraminic acid from the virus and cell surface. The esterase activity of the HE, and HEF protein of influenza C, specifically cleaves Neu5,9Ac<sub>2</sub> and does not hydrolyse the receptors for influenza A and B. Following haemagglutination at 4°C, subsequent incubation at 37°C

results in the destruction of the neuraminic acid and subsequent elution of the virus and reversal of the haemagglutination.

## CLINICAL FEATURES

Diseases associated with coronaviruses in humans have been reviewed by Myint (1994).

### Upper Respiratory Tract Disease

Most information on the clinical features of HCV infections in humans has been obtained from volunteers experimentally infected with HCVs, and epidemiological studies using paired sera collected from individuals during and after naturally acquired HCV infections. HCVs are believed to be second only to rhinoviruses with respect to the percentage of colds that they cause.

HCVs generally cause mild upper respiratory tract infections in humans, and up to 30% of common colds are caused by HCVs. The incubation period of HCV infections is relatively short, with a range of 2–5 days to the development of symptoms. Illness—nasal discharge, mild sore throat, sneezing, general malaise, perhaps with headache (Tyrrell *et al.*, 1993)—then lasts for an average of 6–7 days, sometimes lasting for 18 days. Fever and coughing may be exhibited in 10% and 20% of cases, respectively. No difference is observed between 229E and OC43 strains in the pathology of infection. Generally symptoms are indistinguishable from those of colds caused by rhinoviruses. Virus excretion reaches a detectable level at the time symptoms begin and lasts for 1–4 days. Subclinical or very mild infections are common and can occur throughout the year.

An electron microscopic examination of the nasal epithelium of a person infected by HCV revealed virus particles within and outside the ciliated cells but neither within or outside the goblet cells or other cells of the nasal mucosa (Afzelius, 1994). The ciliated cells seemed not to be destroyed, although in many cases the cilia were withdrawn. This loss of cilia was considered likely to cause rhinorrhoea.

### Lower Respiratory Tract Disease

There have been some reports indicating a more serious lower tract involvement in young children and old people (Isaacs *et al.*, 1983). It is not clear that HCVs infect the lower respiratory tract but the occurrence of HCV upper respiratory tract infections coupled with other factors may cause more serious disease. Up to 30% of acute wheezing episodes in asthmatic children may be due to coronavirus infection.

A study in a neonatal intensive care unit revealed that all premature infants infected with coronaviruses had symptoms of bradycardia, apnea, hypoxaemia, fever or abdominal distention. Chest X-ray revealed diffuse infiltrates in two cases (Sizun *et al.*, 1995).

### Molecular Bases of Tropisms

Little is known about the molecular basis of the tropism of human coronaviruses and toroviruses. Observations made with MHV are instructive in this regard. Variation in the sequence of S would appear to have marked effects on tissue tropism and pathogenicity. Mutations in S of MHV were associated with the rate of spread of the virus in neurons and whether the infection resulted in demyelination or encephalitis. Persistent infection of mouse glial cells by MHV resulted in the production of mutants with uncleaved spike protein. These did not cause cell–cell fusion and consequently spread slowly (Gombold *et al.*, 1993). These and other changes in tropism and pathogenicity probably did result from changes in the S gene, although the possibility that mutations had occurred elsewhere in the genome cannot be excluded (Cavanagh, 1995). Gallagher (1997) has shown that a tissue culture-adapted mutant of MHV JHM, the JHMX strain, did not elute from its receptor at 37°C, whereas the parental JHM strain did do so. This difference in interaction with the receptor was associated with a large deletion from the S protein of JHMX.

### Epidemiology

Generally HCV epidemics occur during the winter and early spring but the peak period may vary by

several months. However, other periods of high infection rates have been observed and it appears that major peaks may occur at any time of the year. All age groups are infected with HCVs and infection rates have been shown to be relatively uniform for all age groups (Monto and Lim, 1974). This is different from the situation with some other respiratory viruses, such as respiratory syncytial virus, where there is a distinct decrease in infection rates with increase in age.

The incidence of OC43 and 229E types of HCV has been studied in depth among approximately 1000 people in Tecumseh, USA, over a period of 4 years. Serology revealed that 17% of individuals had a cold caused by OC43 in any one year (Monto and Lim, 1974). Infections by 229E in the same community averaged 8% each year, while double this rate was detected elsewhere in another study. In some years the incidence of disease was much greater than the average. In a study in the Rhone Alpes region of France, virus was isolated from approximately one-third of nasal swabs, 18% of these yielding HCV (Lina *et al.*, 1996).

There is a periodicity of infections caused by 229E and OC43 group viruses which follows a complex pattern, although they usually cause peaks of infection with intervals of 2 or 3 years. In general, high infection rates in any particular year are caused by either 229E or OC43 group viruses, with only the occasional sporadic HCV infection belonging to the other group. HCV infections have been shown to occur throughout the world with the same cyclic pattern.

Certain individuals are more prone than others to HCV infections. Reinfection of individuals with the same HCV serotype often occurs within 4 months of the first infection, suggesting that homologous HCV antibodies are protective for about 4 months. In addition, there are indications that antibodies to one HCV group may not be protective against infection with viruses from the other HCV group.

The serological response to both naturally acquired and experimentally induced infections is extremely variable and depends on a number of factors, including the infecting HCV strain and the serological test employed. Many individuals have high antibody levels after infection, and reinfection with the same or related strains is common.

### **Coronaviruses Associated with the Human Enteric Tract**

There has been an increasing number of reports describing coronavirus-like viruses isolated from faecal specimens from humans (Macnaughton and Davies, 1981; Myint, 1994; Duckmanton *et al.*, 1997). Some of these viruses were isolated from infants with necrotizing enterocolitis, patients with non-bacterial gastroenteritis and from homosexual men with diarrhoea who were symptomatic and seropositive for human immunodeficiency virus. Some isolates were shown to be serologically related to OC43. The discovery that a protein found in enterocytes functions as a receptor for HCV-229E strengthens the possibility that coronaviruses might replicate in the human alimentary tract.

### **Toroviruses Associated with the Human Enteric Tract**

Duckmanton and colleagues (1997) have isolated and purified toroviruses from faeces of paediatric patients with gastroenteritis, extending previous work (Beards *et al.*, 1984; Koopmans and Horzinek, 1994). That the viruses detected were indeed toroviruses was demonstrated by nucleotide sequencing. More studies of these little-known viruses may not be expected.

### **Coronaviruses in the Central Nervous System**

Multiple sclerosis (MS) is a chronic disease of the central nervous system involving multifocal regions of inflammation and myelin destruction. A number of environmental factors have been proposed for triggering MS, foremost among them being infectious agents (Kurtze, 1993). Many viruses have been linked with MS, including coronaviruses. Coronavirus-like particles were observed in the brain of an MS patient and titres of antibodies to HCVs were greater in cerebrospinal fluid from MS patients compared to control subjects. Two putative coronaviruses (CV-SD and CV-SK) were isolated after passage in mice of brain material from two MS patients. Doubt has been cast on whether the SD and SK isolates are of human, rather than murine,



origin. However, analysis of brain tissue from MS and non-MS patients by *in situ* hybridization using DNA probes complementary to CV-SD and MHV RNA revealed the presence of nucleic acid to which the probes bound (Murray *et al.*, 1992a). The incidence of this RNA was fivefold greater in the MS patients. Stewart *et al.* (1992) analysed RNA from brain tissue of MS patients using polymerase chain reactions (PCRs) specific for HCVs OC43 and 229E. RNA of the latter type was detected four of 11 MS patients and in none of six neurological and five normal controls. No OC43 RNA was detected.

Although these observations do not prove that coronaviruses cause MS in humans, it should be remembered that strains of MHV cause both acute and chronic infections, including demyelination, of the central nervous system of mice and rats (Dales and Anderson, 1995; Houtman and Fleming, 1996). Moreover, intracerebral inoculation of Owl monkeys and African green monkeys with either MHV or the CV-SD isolate resulted in acute to subacute panencephalitis and/or demyelination. Replication of these viruses was demonstrated by detection of viral RNA and proteins (Murray *et al.*, 1992b). Experiments with MHV and rodents have shown that the virus was able to enter the central nervous system from peripheral trigeminal or olfactory nerves after *intranasal* inoculation (see references in Murray *et al.*, 1992b).

Infection by OC43 in primary cultures of fetal astrocytes and of adult microglia and astrocytes was demonstrated by fluorescence (Bonavia *et al.*, 1997). This technique failed to detect 229E but RNA replication of this type of HCV was detected by PCR and probing of the resultant DNA. Interestingly, only the fetal astrocytes released infectious virus. Clearly HCVs have the capacity to replicate in some cells of the CNS of primates.

One feature of MS is an increased frequency of myelin-reactive T cells, a characteristic also of rodents infected with neurotropic strains of MHV. Transfer of these rodent T cells to naive animals triggered experimental allergic encephalomyelitis (EAE), an autoimmune disease that is used as a model for MS, triggered by injection of myelin basic protein (MBP) (Watanabe *et al.*, 1983). Epitopes common to MBP and MHV—molecular mimicry—have been invoked as the basis of this autoimmunity. HCV-229E has a non-structural protein which has a short amino acid sequence identical to one in MBP (Jouvenne *et al.*, 1992).

Almost one-third of T cell lines established from MS patients, but < 2% from normal control subjects, showed an HLA-DR-restricted cross-reactive pattern of antigen activation after *in vitro* selection with either MBA or HCV-229E antigens (Talbot *et al.*, 1996). This further supports the possibility that molecular mimicry is involved in MS, HCV being one of several pathogens that have exhibited this phenomenon with MBA (reviewed by Talbot *et al.*, 1996).

## DIAGNOSIS

Diagnosis of HCV infections is not routinely done in most diagnostic virus laboratories. This is mainly due to their fastidious growth requirements in cell culture and to their inadequate immunological characterization, which makes serological diagnosis difficult. In addition, the infections caused by HCVs have so far proved to be largely of minor clinical significance and therefore have not merited extensive study. However, the application of nucleic acid technology, described below, promises to make detection of human coronaviruses, and toroviruses, much easier.

Diagnostic procedures which have been used comprise virus isolation in cell and/or organ cultures, and serological diagnosis on paired sera from infected individuals (Myint, 1994, 1995).

## Virus Isolation

Virus isolation can be done from nasal and throat swabs, and nasopharyngeal aspirates taken from infected individuals.

229E and related strains can be isolated in roller culture monolayers of human embryonic lung fibroblasts, such as W138 and MRC5 cells. In virus-positive cultures a gradual cytopathic effect consisting of small, granular, round cells appears throughout the monolayers, although especially around the periphery of the monolayers. However, cell sheets are rarely destroyed completely on initial isolation. Isolates are generally confirmed as being 229E-related by standard serum neutralization tests. Other cell types that have been used are described by Myint (1995).

OC43-related strains usually cannot be grown in

cell cultures, at least on initial isolation, and for these strains isolation has to be performed in organ cultures of human embryonic tissues. Small pieces of tracheal or nasal epithelium with the ciliated surface uppermost are inoculated with the respiratory specimens and the culture examined for ciliary activity daily for up to 2 weeks. The medium from these preparations is then harvested and examined after negative staining by direct electron microscopy or by immune electron microscopy using convalescent-phase serum from the infected patient. Immobilization or reduction in ciliary activity of infected organ cultures in comparison to that in controls has also been used to identify HCV infection, but this is not a reliable measure of infection.

### Serological Diagnosis

229E- and OC43-type infections can be identified using paired serum samples taken from acute and convalescent stages of infection; tests used include virus neutralization, complement fixation and haemagglutination inhibition (for OC43-related strains). A more sensitive method for detecting significant changes in HCV antibody in paired human sera is ELISA.

### Detection of HCV Proteins

HCV particles can be detected in epithelial cells shed from the nasopharynx of individuals with coronavirus infections. Indirect immunofluorescence assays have shown that coronavirus fluorescence can be visualized in nasopharyngeal cells from volunteers inoculated with HCVs. An antigen-capture ELISA has been used to detect HCV antigens in nasal and throat swabs, and nasopharyngeal aspirates, taken from children suffering from acute respiratory infections. Nasal swabs were the best specimens for obtaining suitable quantities of antigen for the assay.

### Detection of HCV RNA

Cloning of the N gene of HCV-229E made possible the production of a radiolabelled nucleic acid probe which was used in a slot-blot technique to detect

HCV in nasal washings from volunteers infected with 229E. This technique was of comparable sensitivity to standard culture. However, when a non-radioactive probe was used, preferable in a diagnostic laboratory, there was loss of sensitivity (Myint, 1995).

A much more sensitive method of detection is the PCR or, strictly speaking, the reverse transcriptase PCR, as the RNA of HCV must first be copied into DNA by the enzyme reverse transcriptase (RT). Stewart *et al.* (1995) have described a RT-PCR, followed by Southern blotting, for the detection of HCV-229E and OC43 in cerebral autopsy material. Cristallo *et al.* (1996) have also described a RT-PCR and probing method for detecting HCV-OC43. Myint *et al.* (1994) have used a nested RT-PCR to detect HCVs on nasal swabs. This approach, which does not require probing to detect the PCR product, was much more sensitive than cell culture or probe methods. Of all the methods used to detect HCV, the nested PCR would seem to be the one most likely to be used in future. PCR technology is commonly used in major diagnostic laboratories for many purposes. The specific reagents required for HCV detection—oligonucleotides corresponding to an HCV gene—are readily and inexpensively made as the nucleotide sequences of both 229E and OC43 are in the public domain. This approach can also be used to detect coronaviruses in faeces. It is possible, however, that some human coronaviruses present in faeces might have nucleotide sequences significantly different from those of 229E and/or OC43. Degenerate oligonucleotides, possibly based on sequences in the more conserved polymerase gene, might be useful in this regard (Stephenson *et al.*, 1999).

The sequencing of part of the genome of toroviruses present in human faeces is a great advance for the study of these little known viruses, making possible the application of RT-PCR analogous to that described for HCV (Duckmanton *et al.*, 1997).

## TREATMENT

As yet there are no treatments which prevent the development of HCV infections. When a human cerebral cortical neuron cell line, in which HCV-OC43 replicated very poorly, was treated with in-

terferon  $\gamma$ , the susceptibility to OC43 was increased 100-fold. This was due to membrane expression of HLA class I which acts as a receptor for OC43 (Collins, 1995). Current studies of the various enzymatic activities within the large polymerase protein of HCV may lead to the development of drugs that would inhibit HCV but have no adverse effect on human cells.

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