Novartis 238: Gastroenteritis Viruses.

Print ISBN 0-471-49663-4 eISBN 0-470-84653-4

Enteric infections with coronaviruses and toroviruses

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Abstract. Many enteric viruses are difficult or impossible to propagate in tissue culture. Coronaviruses and toroviruses are large, enveloped, plus-strand RNA viruses in the order Nidovirales that cause enteric disease in young pigs, cows, dogs, mice, cats and horses. Two different serogroups of mammalian coronaviruses cause frequent respiratory infections in humans, and coronaviruses and toroviruses have been implicated in human diarrhoeal disease by immunoelectron microscopy. However, there is as yet no consensus about the importance of these enveloped viruses in human diarrhoea, and little is known about their genetic variability. The large spike (S) glycoprotein is an important determinant of species specificity, tissue tropism and virulence of coronavirus infection. To infect enterocytes, both S glycoproteins and the viral envelope must resist degradation by proteases, low and high pH, and bile salts. One specific site on the S glycoprotein of bovine coronavirus must be cleaved by an intracellular protease or trypsin to activate viral infectivity and cell fusion. S glycoprotein binds to specific receptors on the apical membranes of enterocytes, and can undergo a temperature-dependent, receptor-mediated conformational change that leads to fusion of the viral envelope with host membranes to initiate infection. Analysing spike–receptor interactions may lead to new ways to propagate these enteric viruses as well as new strategies for development of novel antiviral drugs.

2001 Gastroenteritis viruses. Wiley, Chichester (Novartis Foundation Symposium 238) p 258–275

Viruses that cause gastroenteritis must be resistant to inactivation in the hostile environment of the enteric tract where they are exposed to proteolytic enzymes, bile salts, mucus, bacterial products and extremes of pH. Each type of gastroenteritis virus is optimized for replication in one or more types of specialized cells in the enteric tract. The virus must be able to bind to specific receptors on the membranes of the target cell, and the viral genome must encode an apparatus that allows the virion or its nucleocapsid to penetrate through the plasma membrane or endosomal membranes to initiate infection. The virus must be optimized for efficient replication in the specialized cells of the gastroenteric tract. In the infected cells, sufficient progeny virus must be made to infect other

cells or be shed from the infected animal before the infected cell is killed either by virus infection or immune responses, or by apoptosis due to differentiation of the enterocytes. Many enteric viruses must survive in the external environment in water or soil long enough to initiate infection by the faecal–oral route. Some viruses that cause enteric diseases are transmitted by the respiratory route, and the enteric tract is infected subsequent to virus replication in the respiratory tract.

Most viruses that infect the enteric tract are non-enveloped viruses with naked nucleocapsids designed to withstand the hostile environment in the enteric tract. In the *Coronaviridae* family of the order Nidovirales (Cavanagh et al 1993, Cavanagh 1997, Siddell & Snijder 1998), members of the coronavirus and torovirus genera cause enteric diseases of many species of domestic animals and possibly of humans. These are plus-strand RNA viruses that have envelopes as an essential component of the virions. The viral envelopes consist of lipoprotein bilayers and several virus-encoded envelope proteins or glycoproteins (Duckmanton et al 1998, Lai & Cavanagh 1997, Lai & Holmes 2000). In coronaviruses, the virion glycoprotein, S, which is found in the large trimeric spikes on the envelope is specialized for binding to specific receptors on enteric epithelial cells and for inducing receptor-mediated fusion of the viral envelope with host cell membranes that introduces the viral nucleocapsid into the cytoplasm of the target cell (Lai & Holmes 2000). The mechanisms by which these enveloped viruses survive in the enteric tract and bind to and enter their target cells are being explored.

Results and Discussion

Enteric diseases

Coronaviruses and toroviruses that cause enteric diseases and their natural host species are listed in Table 1 (Cavanagh et al 1993, Koopmans & Horzinek 1994). In general, most of these viruses cause disease only in one host species, and disease is more severe in infant animals than in adults. Inapparent infection is common in adults. Shedding of virus in the faeces may persist for weeks after inoculation. Animals can be infected by oral inoculation. Several of these viruses can replicate in the epithelial cells of the respiratory tract as well as the enteric tract, and they can be transmitted by the respiratory route.

Virion structure and assembly

Coronaviruses have a helical nucleocapsid that consists of a 27–32 kb plus-strand RNA genome encapsidated by the nucleocapsid phosphoprotein N. The structure of torovirus nucleocapsids is unique. Thin sections of torovirus virions show that the electron-dense nucleocapsid is shaped like a doughnut or torus. No RNA-dependent RNA polymerase protein is found in virions of coronaviruses or

TABLE 1 Coronaviruses and toroviruses that cause enteric disease

Coronaviridae genera	*Host species*
Coronavirus	
Bovine coronavirus (BCoV)	Cattle
Mouse hepatitis virus (MHV)	Mice
Haemagglutinating encephalomyelitis virus (HEV)	Swine
Transmissible gastroenteritis virus (TGEV)	Swine
Feline enteric coronavirus (FCoV)	Cats
Canine coronavirus (CCoV)	Dogs
Human enteric coronavirus (HECoV)	Humans (?)
Torovirus	
Berne virus (ETV)	Horses
Breda virus (BoTV)	Cattle
Porcine torovirus (PoTV)	Swine
Human torovirus (HTV)	Humans

toroviruses because immediately after uncoating in the infected cell, the plus-strand RNA genomes are translated to make the viral polymerase polyprotein.

Coronaviruses do not bud from the plasma membrane. Instead, coronavirus envelopes are acquired by budding of the nucleocapsid at membranes of a pre-Golgi complex called the budding compartment where viral glycoproteins S, M, E and, in some coronaviruses, HE, are incorporated into the viral envelope (Bos et al 1997). The intracellular localization of the viral membrane glycoprotein, M, appears to play a key role in determining the site of coronavirus budding. The small envelope glycoprotein, E, is required for virus budding (Bos et al 1996). The lipid composition of the coronavirus envelope reflects that of the membranes of the budding compartment, which is different from the lipid composition of the plasma membrane of the same cells (van Genderen et al 1995). The unusual lipid composition of coronavirus envelopes may play a role in making these viral envelopes more resistant to degradation by bile salts than envelopes of unrelated viruses that bud from the plasma membrane.

TGEV virions treated with non-ionic detergent release a spherical core that contains the viral nucleocapsid and M protein, but lacks the viral spike glycoprotein, S. Although virions of coronaviruses appear spherical while budding and soon afterwards, they mature and change to a flattened disk shape after being released into the lumen of the intracytoplasmic vesicles (Salanueva et al 1999, Holmes et al 1984). The vesicles filled with virions apparently fuse

TABLE 2 Coronavirus receptors

Virus	*Receptor*
MHV	Murine CEACAM1a and related murine glycoproteins
BCoV	9-*O*-acetylated sialic acid moieties
HCoV-OC43	9-*O*-acetylated sialic acid moieties
TGEV	Porcine aminopeptidase N (pAPN)
FCoV	Feline aminopeptidase N (fAPN)
HCoV-229E	Human aminopeptidase N (hAPN)
CCoV	Canine APN (cAPN) (?)

with the plasma membrane to release virions by an exocytosis-like process. In polarized epithelial cell lines grown on filters, coronaviruses can be released either at apical or basolateral membranes, or both, depending on the virus and the cell line (Rossen et al 1997).

Spike glycoprotein interactions with virus receptors

The spike (S) glycoproteins of coronaviruses bind to specific cell membrane glycoproteins that serve as virus receptors and induce fusion of the viral envelope with host cell membranes. At least three types of membrane molecules are used as receptors for various coronaviruses as shown in Table 2. The S glycoproteins of MHV strains utilize as receptors murine glycoproteins in the carcinoembryonic antigen (CEA) family of glycoproteins in the immunoglobulin superfamily (Holmes & Dveksler 1994). The prototype receptor CEACAM1a (formerly called MHVR or Bgp1a) is expressed on apical membranes of endothelial cells and many epithelial cells including respiratory, enteric, and thymic epithelial cells, and on hepatocytes, macrophages, B cells and activated T lymphocytes (Godfraind et al 1995). Transfection of non-murine cells with cDNA encoding murine CEACAM1a renders the cells susceptible to MHV infection (Dveksler et al 1991).

Carbohydrate moieties, principally 9-*O*-acetylneuraminic acid, are recognized by both the haemagglutinin-esterase glycoprotein (HE) expressed on the envelopes of some coronaviruses including BCoV, HEV, some strains of MHV and toroviruses, and by the S glycoproteins of some coronaviruses (Schultze & Herrler 1993, Duckmanton et al 1999). Removal of the carbohydrate moiety from susceptible cells by treatment with neuraminidase or esterase markedly

reduces the infectivity of the virions, but does not eliminate infection altogether (Vlasak et al 1988). It is not yet certain whether the binding of HE or S glycoprotein to 9-*O*-acetylneuraminic acid can directly mediate membrane fusion, or whether an unidentified co-receptor may be required for virus entry.

The third type of receptor used by coronaviruses is aminopeptidase N (APN), a large Class II membrane metallo-glycoprotein expressed on the apical membranes of respiratory and enteric epithelial cells, macrophages, and at synaptic junctions (Yeager et al 1992, Delmas et al 1992, Wessels et al 1990). The coronaviruses in antigenic group I, including TGEV, HCoV-229E, FCoV and probably CCoV, apparently all utilize APN proteins as receptors (Tresnan et al 1996, Benbacer et al 1997). TGEV uses pAPN, but not hAPN, and HCoV-229E uses hAPN, but not pAPN as a receptor. Thus the specificity of receptor interactions can determine the species specificity of coronavirus infection. All four viruses in serogroup 1 can utilize feline APN as a receptor in cell culture, suggesting that this may have been the original receptor for group I coronaviruses (Tresnan et al 1996). It is likely that conserved elements of the S glycoproteins of these viruses bind to conserved elements of APN. Mapping of the sites on APN that determine the species specificity of virus receptor activity was done using chimeric porcine and human APN glycoproteins (Benbacer et al 1997). Substitution of an eight amino acid region near the N-terminus of hAPN for the corresponding region of the pAPN glycoprotein conferred susceptibility to HCoV-229E. In contrast, a region in the C-terminal domain determines receptor activity for porcine, feline and canine coronaviruses (Hegyi & Kolb 1998). Possibly these two regions are adjacent in the as yet unknown three-dimensional structure of APN. Site directed mutagenesis that introduced a single *N*-linked glycosylation site at amino acid 291 of human APN, similar to that found in the corresponding region of porcine APN, was sufficient to block infection by HCoV-229E (D. Wentworth & K. Holmes, unpublished results). Thus, genetic drift among APN glycoproteins of different species that serve as coronavirus receptors can affect host susceptibility to coronavirus infection.

The S glycoproteins of coronaviruses and toroviruses are highly glycosylated. A coiled-coil in the stem of the spike glycoprotein mediates the formation of trimers of S on the viral envelope. For some coronaviruses, including MHV and BCoV, the coiled-coil domain of S is required for membrane fusing activity, as shown for fusion glycoproteins of unrelated enveloped viruses (Hernandez et al 1996, Baker et al 1999). In MHV and BCoV virions, near the middle of each molecule of S protein in the spikes, a single trypsin cleavage site is exposed. Cleavage at this site by trypsin extracellularly or by a furin-like protease inside the Golgi yields the S1 and S2 glycoproteins (Lai & Holmes 2000, Lai & Cavanagh 1997). The N-terminal domain of S1 is required for receptor binding (Suzuki & Taguchi 1996), and the S2 domain is required for membrane fusion. This cleavage event activates

coronavirus-induced membrane fusion and viral infectivity for some cell lines (Storz et al 1981), but is not essential for viral infectivity since mutants of these viruses and different coronaviruses that lack the protease cleavage site are infectious (Bos et al 1997, Hingley et al 1998). In the small intestine, trypsin is available to cleave the viral S glycoprotein if the cleavage site is available. By analogy with other viral fusion glycoproteins such as HA of influenza, HN of Sendai virus, and gp120/41 of HIV1, it is likely that the cleavage event that forms S1 and S2, also induces a conformational change of the coronavirus spike glycoprotein that poises it to undergo further conformational changes leading to membrane fusion in response to alkaline pH or receptor binding.

Fusion glycoproteins of HIV, Ebola virus and influenza A undergo programmed conformational changes in response to receptor binding or low pH like that found in endosomes (Weissenhorn et al 1999). The receptor-binding domain swings aside, uncovering a hydrophobic fusion peptide at the new N-terminus of the coiled-coil domain that was generated by protease cleavage. The coiled-coil domain extends and the fusion peptide inserts into the cell membrane. A second conformational change follows which brings the transmembrane anchor in the viral envelope into close proximity to the fusion peptide anchored in the cell membrane, and then fusion of the lipid bilayers occurs leading to virus infection. Our laboratory showed that for the enterotropic murine coronavirus MHV, binding to purified soluble CEACAM1a receptor glycoprotein at 37 °C neutralized viral infectivity (Zelus et al 1998). Neutralization by soluble receptor was accompanied by an increase in hydrophobicity of virions shown by strong association with liposomes in sucrose density gradients (B. D. Zelus & K. Holmes, unpublished results). This did not occur when virions and soluble receptor were incubated at 4 °C. The temperature-dependent, receptor-dependent change in viral hydrophobicity was associated with a conformational change in the S2 protein that made it susceptible to cleavage by trypsin at 4 °C. Virions incubated with or without soluble receptor at 37 °C were then incubated with trypsin at 4 °C and the protease-resistant S peptides were detected by immunoblotting with monoclonal antibodies to S1 or S2. We found that incubation of virions with soluble receptor at 37 °C, but not at 4 °C, caused a conformational change in the S2 glycoproteins that made them susceptible to degradation to protease at 4 °C, while the S1 proteins were not digested by trypsin. Conformational change in the MHV S2 glycoprotein was also observed in virions treated at pH 8.0 and 37 °C in the absence of soluble receptor. This alkaline pH-dependent, temperature-dependent conformational change in the coronavirus spike protein may be facilitated by the high pH in the small intestine. MHV infection of mouse intestine results in formation of multinucleate giant cells on the tips of villi. Thus, the murine coronavirus S glycoprotein is ideally designed to be activated by proteases and/or the mildly alkaline pH in the small intestine to induce

membrane fusion leading to virus entry and cell-to-cell spread of virus infection by cell fusion. MHV strains differ significantly in the amino acid sequences of the S glycoprotein, in epitopes expressed on S1 and S2, in susceptibility to protease cleavage at the S1/S2 boundary, in the stability of the interactions between S1 and S2, in the ability of S2 to induce cell fusion, and in the stability of viral infectivity under different host conditions. These variations in S are important determinants of the tissue tropism and virulence of the MHV strains in mice (Phillips et al 1999).

Role of coronaviruses and toroviruses in human enteric disease

Coronaviruses of many animal species have been shown to cause diarrhoea in their natural hosts following oral inoculation. Many, but not all, of these animal coronaviruses can be isolated from faeces of infected animals using cell lines from the natural host species or HRT18 cells, a human rectal tumour cell line. Presumably these cells must express the appropriate receptor for the virus. Addition of trypsin to the culture medium sometimes facilitates virus isolation and cytopathic effects of some primary coronavirus isolates such as BCoV (Storz et al 1981). The enzyme probably potentiates the temperature-dependent, receptor-induced or alkaline pH-induced conformational changes in the S protein that lead to membrane fusion and virus entry. Enteric infection is confirmed by isolation of the infectious virus from faeces, detection in faeces of viral RNA by RT-PCR, virus-encoded proteins by immunolabelling, or virions by immunoelectron microscopy using antibodies directed against viral envelope glycoproteins, or convalescent sera from patients or animals (Tsunemitsu et al 1999).

Human coronaviruses have been implicated in the aetiology of viral diarrhoea by several lines of evidence. Coronavirus-like particles were observed by immunoelectronmicroscopy in the faeces of humans, particularly infants, with diarrhoea. However, in negatively stained preparations, the pleiomorphic coronaviruses with their large petal-shaped spikes strongly resemble other enveloped viruses such as toroviruses, and also fragments of intestinal brush border membranes studded with cellular glycoproteins that are released into the intestinal lumen. Coronavirus antigens in human diarrhoeal stools in sporadic small outbreaks of enteric disease have most often been associated with HCoV-OC43 (Battaglia et al 1987). However, viral antigens can also be detected in some healthy contacts of infected individuals, making it difficult to unequivocally implicate the virus in the aetiology of the enteric disease. Isolation of human enteric coronaviruses has been more difficult than isolation of many animal coronaviruses. Some isolates could only be propagated for one round of replication, suggesting that some essential elements of the cell culture were not appropriate for serial propagation of the virus (Clarke et al 1979). A report of

isolation from an infant with necrotizing enterocolitis of a human enterotropic coronavirus in primary organ cultures of human fetal intestine has not yet been confirmed in other labs (Resta et al 1985). Several isolates of putative human enteric coronaviruses in cell cultures were subsequently found to be very similar to BCoV, suggesting that either the patient had acquired a bovine virus infection, the samples were contaminated with bovine virus, or a BCoV-like virus circulates in the human population (Zhang et al 1994). Serological studies have not unequivocally proven the existence of a human enterotropic coronavirus. Because people are repeatedly infected with respiratory coronaviruses, adults usually have antibody to coronaviruses related to HCoV-229E and HCoV-OC43. To prove that a viral isolate is the aetiological agent of the enteric disease from which the virus inoculum was obtained, it is necessary to demonstrate a rise in titre of specific anti-viral antibody. Quantitative data are required for a reliable clinical assay. Based on enteric coronavirus infections of other species, infants are the likeliest population to show serious coronavirus-induced enteric disease. However, the immune response to coronaviruses in infants is not robust, so it is often difficult to use serology to prove coronavirus infection in infants. RT-PCR has been used to detect coronavirus RNA in faecal samples of infected animals, and such assays are also likely to be useful for the study of human enterotropic coronaviruses, particularly since the nucleotide sequences of human coronaviruses have been determined. New and sensitive assays to detect HCoV infection will soon be available to aid in studies on the incidence, epidemiology and pathogenesis of enterotropic HCoV infection in infants.

Toroviruses were first discovered in rectal swabs of a horse, yielding the prototype Berne strain which can be propagated in cell culture (Weiss et al 1983). Subsequently, similar viruses or virus-like particles have been observed in bovine, porcine and human diarrhoeal stools by electron microscopy although these viruses cannot be grown in cell culture (Duckmanton et al 1997, Jamieson et al 1998). Torovirus virions or VLPs aggregated by convalescent antibody or antibodies directed against glycoproteins encoded by S or HE open reading frames on the viral genome can be detected by immunoelectron microscopy (Jamieson et al 1998, Krishnan & Naik 1997). Nucleotide sequences of genes of several toroviruses have been determined, virus-encoded proteins have been expressed and used to raise antibodies, and these are now being used to develop sensitive diagnostic tests for torovirus infection in the enteric tract (Koopmans et al 1993, Duckmanton et al 1998, 1999). Bovine and porcine torovirus antigens and RNAs have been detected in diarrhoeal stools, and at much lower frequency in control stools, by immunoblotting and RT-PCR (Koopmans et al 1993). Antibodies directed against bovine torovirus cross-react with antigens in virions of human toroviruses (Koopmans et al 1997, Duckmanton et al 1998). In a case-control study, toroviruses were identified in 35% of paediatric gastroenteritis cases

TABLE 3 Possible mechanisms for survival of enveloped coronaviruses and toroviruses in the intestinal lumen

Resistance to	*Possible mechanism*
Bile salts	Lipid bilayer of viral envelope is derived from intracellular membranes
Proteases	Extensive glycosylation blocks numerous potential protease sites. This protects spikes from degradation, but allows one specific protease site to be cleaved, activating membrane fusion for some viruses
pH changes	Programmed pH-dependent conformational changes in structure and function of S glycoprotein is associated with membrane fusion
Mucus	Haemagglutinin-esterase activity of HE glycoprotein removes potential receptor moieties from glycoproteins in mucus and aids virus elution

and 14.5% of controls. Patients shedding torovirus in their stools were more frequently imunocompromised than patients shedding astroviruses or rotaviruses (Jamieson et al 1998). These data suggest that toroviruses may be an important cause of diarrhoea in children, particularly in immunocompromised patients.

While it is clear that enveloped coronaviruses and toroviruses commonly cause enteric infection in a variety of species, the molecular mechanisms that allow these enveloped viruses to survive and replicate in the enteric system have not yet been elucidated. Several possible specializations of the coronavirus and torovirus envelopes and S and HE envelope glycoproteins that may play key roles in survival of virions in the intestine are suggested in Table 3. When reverse genetics systems become available for coronaviruses and toroviruses, these hypotheses can be directly tested by mutational studies on the viral envelope glycoproteins.

Summary

New techniques for isolation in cell cultures of human enterotropic coronaviruses and toroviruses from diarrhoeal stools will elucidate the importance of these agents in human enteric diseases. Sensitive and specific diagnostic tests are becoming available to analyse the roles of these two types of viruses in human enteric diseases and to compare the viruses associated with different outbreaks of disease. Experiments that focus on the interactions between viral envelope glycoproteins and their cellular receptors are likely to elucidate the molecular mechanisms by which these enveloped viruses can cause enteric diseases.

Acknowledgements

The author is grateful for discussions with Bruce Zelus, David Wentworth, Dianna Blau, Jeanne Schickli, Aurelio Bonavia, Larissa Thackray and Brian Turner. This work was supported by NIH grant #AI 25231.

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DISCUSSION

Greenberg: Is the mechanism of diarrhoea in animals with coronavirus presumed to be lysis of cells and loss of absorptive epithelium? Is there any other putative mechanism for diarrhoea for the coronaviruses?

Holmes: Not yet. We actually stumbled on the receptor story while we were looking for a viral enterotoxin years ago. What you mention is the generally accepted model for viral diarrhoea, but I don't think we know all there is to know about the pathophysiology of viral diarrhoea.

Greenberg: Coronaviruses have a genome that could easily encode a toxin.

Koopmans: The difference is, though, that in infected animals you readily find antigen in many epithelial cells.

Greenberg: Rotavirus is found in epithelial cells in large amounts.

Saif: TGEV is found throughout the entire small intestine, and it wipes out the entire intestinal epithelium (Saif & Wesley 1999).

Farthing: About 15–16 years ago, Professor Mathan in Vellore identified coronaviruses in the faeces of patients with tropical sprue. However, when he looked at control patients without diarrhoea, he found the same prevalence of coronaviruses. I guess from your talk that you are cautious about saying whether this is a pathogen in humans or not. It clearly is a pathogen in animals: there is tremendous homology between these viruses, particularly between pig and human intestine. They are virtually identical. Can you speculate on why there are these clear differences?

Holmes: I'm glad that you asked that question. There is no question that the human coronaviruses HCoV-229E and HCoV-OC43 cause respiratory infections. They cause up to 30% of colds in people. The question is whether they can also cause enteric infection. The porcine coronavirus, TGEV, is a very efficient cause of diarrhoea, particularly in young animals. A naturally occurring variant of TGEV, called PRCoV, has deletions of more than 200 amino acids in the receptor binding domain of the viral spike glycoprotein (Ballasteros et al 1995). PRCoV causes only respiratory infection. It may serve as a natural vaccine to protect animals from fatal enteric infection. HCoV-229E, the human coronavirus that we are working with, is one of only a handful of human coronavirus strains that are available for study. The sequence of the S gene of HCoV-229E resembles

this porcine respiratory coronavirus. Possibly the region of S that is present in TGEV but is absent in PRCoV may have something to do with a difference in the stability of these viruses in the enteric tract. The feline coronavirus doesn't have that deletion in S, and grows very nicely in the cat enteric tract.

Saif: There is more to this story. PRCoV is largely avirulent, and TGEV is highly virulent (Saif & Wesley 1999). There are two major mutations: one is in the region you showed in the spike, which seems to relate to tissue tropism, but there is also a change in mRNA 3. Since Dr Luis Enjuanes of Spain has an infectious clone for TGEV we can now clearly address these two scenarios with the coronaviruses. One is the tissue tropism and the other is the virulence. I think these studies will be extremely interesting in the future. I was wondering, Kay, whether you could comment on the mechanism of persistence of these coronaviruses?

Holmes: Persistence of mouse hepatitis virus (MHV), particularly the enterotropic strains, usually occurs in mice that are immunosuppressed, such as nude mice or infants (Compton et al 1993). The viruses that are isolated from mice or murine cells with persistent MHV infection have mutations in the gene that encodes the spike glycoprotein (S) such that the cleavage between S1 and S2 is much less likely to occur, and therefore the virus particles are more stable. I think that a combination of viral factors and host factors are required for coronavirus persistence.

Bishop: What was the source of your human coronavirus?

Holmes: It is a human respiratory coronavirus. There have been a few reports of human enteric coronaviruses being propagated in cell culture, but several of them have turned out to be very closely related to or identical to bovine coronavirus strains (Moscovici et al 1980, Zhang et al 1994).

Kapikian: I remember at the meeting here on novel diarrhoea viruses in 1986 (Ciba Foundation 1987), there was great excitement because Dr Sylvia Resta and Dr James Luby had described the growth and passage of a human coronavirus from stools of infants with necrotizing enterocolitis in human intestinal culture. The agent was serially passaged and further characterized (Resta et al 1985). We were unable to obtain this virus. I believe that you are the only person I know who received the virus from Dr Luby's group. What has happened to this virus?

Holmes: We were never able to grow that virus in any cell line. Dr Resta had grown it in human fetal intestinal organ cultures. We tried to adapt it to many different cell lines without success. We could not show the cross-reactivity of the amount of material that we got with HCoV-OC43, HCoV-229E or animal coronaviruses. Dr Luby has recently made the putative human enterotropic coronavirus available to people through the ATCC (Luby et al 1999). Perhaps unfortunately, this was done after adapting this virus to a mouse macrophage cell line and a mosquito cell line. I have not studied that variant strain yet. It will be important to obtain nucleotide sequence information from this isolate.

Kapikian: Coronaviruses and their association with human gastroenteritis have been a very difficult area of research. In 1982, a report from France described the detection by electron microscopy (EM) of coronavirus in stools of newborns with necrotizing enterocolitis (Chany et al 1982). As noted in the proceedings of the Novel diarrhoea viruses symposium (Ciba Foundation 1987), it was later thought that this virus was a bovine coronavirus. I am not aware that a human coronavirus has been associated conclusively with enteric disease.

Holmes: Dr Storz's lab recently isolated a virus from a child with diarrhoea in Louisiana (Zhang et al 1994). This also looks like a bovine coronavirus. It may be that some of these coronaviruses from animals can cause disease in human contacts, but there appears to be no serial transmission of these viruses in humans.

Saif: There is a report from France in which the fifth cell culture passage (in HRT-18 cells) of coronavirus-like particles from a child with necrotizing enterocolitis caused diarrhoea in a calf, typical of a bovine coronavirus infection (Patel et al 1982). Coronavirus was re-isolated from the faeces and coronavirus-positive immunofluorescence was observed in the colon and rectum, suggesting that either the human enteric coronavirus infects cattle or that it was a bovine coronavirus strain that infected the child. In addition, there is a report of a lab worker infected with bovine coronavirus who developed diarrhoea and shed coronavirus particles in faeces (Storz & Rott 1981). In some recent work I have done with Mo Saif, we gave his student a well characterized DB2 strain of bovine coronavirus that we had isolated, passaged in gnotobiotic calves and adapted to cell culture. His student put this virulent calf-passaged bovine coronavirus into some turkey poults and baby chickens, and it caused diarrhoea in the turkeys but not the chicks (Ismail et al 2000).

There may be a less restrictive host specificity for bovine coronaviruses that means they can sometimes infect other hosts, including humans and turkeys. Perhaps this relates to their possession of a haemagglutinin with a high sequence homology to the haemagglutinin of influenza C viruses, which is also present on the human respiratory coronavirus, OC43 (Parker et al 1989, Zhang et al 1992).

Holmes: There is a coronavirus of elk that is very much like bovine coronavirus (BCoV; Daginakatte et al 1999). Perhaps they share pastures. The BCoV is one of the coronaviruses that binds to the 9-*O*-acetylated sialic acid. Perhaps this common receptor moiety gives the virus a broader host range.

Saif: We isolated coronaviruses antigenically indistinguishable from bovine coronaviruses from the faeces of wild ruminants with a dysentery-like diarrhoea syndrome (Tsunemitsu & Saif 1995). These species included white-tailed deer, sambar deer and a waterbuck. Mule and white-tailed deer also had antibodies to bovine coronavirus. The coronavirus isolates infected and caused diarrhoea in inoculated calves suggesting that wild ruminants harbour coronaviruses antigenically similar to bovine strains and transmissible to cattle.

Glass: We have seen lots of examples of rotaviruses and caliciviruses transmitted between animals and humans. Do any group of animal handlers such as veterinarians have antibodies against coronavirus that might suggest exposure? I ask this because in 1991, we had an EM conference in Atlanta. Al Kapikian was there with Owen Caul and Laura Aurelian who each saw coronaviruses frequently. We said that we wouldn't call something seen by EM a coronavirus ever again unless we had an independent confirmatory assay such as the ELISA that Marion Koopmans has worked on. This was nine years ago, and we still don't have consistent, confirmed cases that we are certain about. At some point we either need the confirmatory assays or we have to say we don't think that there are enteric coronaviruses in humans. Antibodies in veterinarians or livestock handlers would take us a step further.

Saif: I'm not sure that anyone has ever looked at that.

Holmes: Wallace Rowe found antibodies in humans to MHV, a murine coronavirus (Hartley et al 1964), which were probably antibodies to the antigenically related human coronavirus HCoV-OC43.

Saif: There is the problem of cross-reactivity in both groups. There is the HCoV-OC43 human coronavirus that cross-reacts with bovine coronavirus and there is the HCoV-229E human coronavirus that cross-reacts with the porcine TGEV coronavirus.

Holmes: As people are beginning to sequence more and more coronavirus genomes, they have found naturally occurring recombinants between canine and feline coronaviruses, where there is a cross-over in the spike protein that allows a dog virus to adapt to cats. I think that jumping species is something that we should be concerned about with coronaviruses. There are five animal species in which fatal neonatal diarrhoea can be caused by coronaviruses. Some of these Elvis-like sightings of coronavirus have been seen in human infant diarrhoeal samples. It is possible that they could jump, but it is unlikely.

Koopmans: Martin Petric has gathered data about human toroviruses. He has obtained sequences from some of the torovirus-like particles, and finds almost identical sequences in some regions to the equine toroviruses. The problem with these data is that they are not reproducible in other labs. He speculates about zoonotic transmission (Jamieson et al 1998, Duckmanton et al 1997).

Holmes: He is looking by immunoelectron microscopy at fecal specimens of children with diarrhoea that are hospitalized. He finds torovirus-like particles in 20–30%. The RT-PCR and sequencing of other regions of the viral genome may tell the tale.

Koopmans: I have spent some time at CDC trying to figure out what was going on with fringed particles in stools (Koopmans et al 1993). We did a blinded study where Martin collected stool samples containing viruses that he felt resembled

toroviruses on the basis of the pictures that we have been sending him. We ran gradients in parallel with the animal torovirus, and tested those gradient fractions by ELISA using hyperimmune sera from cattle. From this it was clear that there is something in human faecal samples that in gradients, co-migrates with the animal toroviruses and is immunoreactive with the animal sera. We also found them in association with clinical symptoms in a study in Brazil (Koopmans et al 1997). This is as far as those studies got. We have also tried to amplify some of the genomes from this work. This never worked, but Martin has described that from similar particles they have genomic information (Duckmanton et al 1997). The difficulty is that a lot of the fringed particles were not confirmed as something that looks like a torovirus. I think that some of the fringed particles are viral but a lot of them aren't.

Holmes: We were interested in looking by EM at the interaction of beautiful coronaviruses, which have characteristic large, petal-shaped spikes, with brush border membrane vesicles that we have isolated from mouse small intestine. We first examined virus preparation and the membrane vesicles separately. We were surprised that we couldn't tell the coronaviruses and membrane vesicles apart because the brush border membranes have some large oligomeic protein complexes, such as amino peptidase N, that stick out from the membrane and look just like viral spikes.

Koopmans: I have looked at many toroviruses by EM but still wouldn't dare to diagnose them by this technique.

Glass: David Brown, do you still have the EM surveillance in the UK that would allow you to identify sightings?

Brown: We have a network of EMs, and two to three times a year someone sends in a micrograph showing fringed particles that could be coronavirus or torovirus. I had the good fortune to spend a year working with Professor V. I. Mathan in Vellore. It gets very hot in Vellore, and I found that I did develop an enthusiasm for EM because it was the only room in the hospital that had air conditioning. I spent a long time trying to characterize these particles. I think that these fringed particles were mostly bits of brush border and cell membrane. We do see them, but I have not seen convincing evidence that these are human torovirus or coronavirus. There is no serological evidence of infection in humans, either with the toroviruses or with the bovine coronavirus.

Holmes: Martin Petric has serological evidence from immunoelectron microscopy.

Brown: Immune EM on fringed particles is a difficult technique to interpret, because the particles do tend to stick together in any case. Perhaps Al Kapikian would be able to comment on this. When I was working with Tom Flewett in Birmingham, we did try to do immune EM on fringed particles, and I wouldn't advocate it as a way to spend your life.

Kapikian: You are right that they can spontaneously aggregate. However, we did detect a putative new human respiratory coronavirus using immune EM (Kapikian et al 1973). We were able to show that the particles characteristically aggregated following incubation with a convalescent serum. EM studies with fringed particles have been particularly difficult and require careful evaluation. I commend Martin Petric in his studies with the toroviruses. He has been extremely cautious, meticulous and careful in his studies with these fringed viruses. I was at a meeting in Montreal and he presented his data cautiously with so many caveats that the chairperson said, 'You have wonderful data, why all these concerns?' Martin replied, 'You don't know my colleagues in the gastroenteritis field!'

Saif: We routinely use immune EM to discriminate in bovine faecal specimens between Breda virus (a torovirus) and coronavirus. This is the only way we can do it, because of their morphogenetic similarity but antigenic distinctiveness.

Farthing: Were all your receptor studies done with intestinal epithelium?

Holmes: Those that I showed were done with cloned soluble receptor proteins purified from cultured cells. The anchored proteins are expressed on the apical brush border membranes of the epithelium.

Farthing: Is there a receptor on the bronchial epithelium?

Holmes: Yes, the same receptor glycoprotein is on apical membranes of respiratory epithelium.

Estes: I was interested in your concept that these viruses may be resistant to bile salts because of the intracellular membranes that they have picked up. Is there any evidence that these are associated with lipid rafts? Are they resistant to cold Triton X100?

Holmes: No, they are not resistant to Triton X100 as the virus envelope can be solubilized by it. Enteric coronaviruses are apparently resistant to bile salts, which would be found in the intestine. The pH 8 in the small intestine may help to activate the spike proteins of these viruses for entry into epithelial cells.

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