NEUROTROPISM OF HUMAN CORONAVIRUS 229E

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ABSTRACT

The 299E prototype strain of human coronavirus (HCV-229E) has so far been mainly associated with infections of the respiratory tract. In the present study, we show evidence for infection of the central nervous system (CNS) by HCV-229E, both *in vitro* and *in vivo.*

Various human cell lines of CNS origin were tested for their susceptibility to infection by HCV-229E. Production of viral antigens was monitored by indirect immunofluorescence with monoclonal antibodies and infectious progeny virions by plaque assay on the L132 human embryonic lung cell line. The SK-N-SH neuroblastoma and H4 neuroglioma cell lines were highly susceptible to infection. The U-87 MG and U-373 MG astrocytoma cell lines were also infectable by HCV-229E. We could also demonstrate infection of the MO3.13 cell line, which was established by fusion of human oligodendrocytes with a thioguanine-resistant mutant of the TE671 (RD) human rhabdomyosarcoma cell line. An apparently more extensive infection of the MO3.13 cells, when compared to the parental cells, supports the notion that human oligodendrocytes are differentially susceptible to infection by this virus.

We also tested for HCV-229E gene expression in pathological brain specimens. For that purpose, we developed a reverse transcription-polymerase chain reaction (RT-PCR) assay to amplify a portion of the mRNA encoding the viral nucleocapsid protein. Using stringent laboratory conditions, viral RNA was detectable in brain tissue of 4 of 11 multiple sclerosis patients and none of 6 neurological and 5 normal controls.

These results strongly suggest neurotropism on the part of HCV-229E and emphasize the importance of further studies on the possible involvement of human coronaviruses in neurological diseases such as multiple sclerosis.

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INTRODUCTION

Human coronaviruses (HCV) are recognized as respiratory pathogens that are responsible for 15 to 35 % of common colds[1]. Two prototype strains, named 229E and OC43, represent the two known serotypes. Besides infections of the respiratory tract, other pathologies have sporadically been associated with human coronaviruses. A seroepidemiological study has linked them with some pneumoniae, perimyocardites, meningites and radiculites[2]. Also, their involvement in enteric infections was suggested from various reports of their presence in stool specimens of infants and children with severe diarrhea[3,4,5].

Another disease association that remains to be confirmed is the possible involvement of HCVs in neurological disorders, specifically multiple sclerosis (MS). The original report of coronavirus-like particles in the brain of an MS patient[6] was rapidly followed by the description of two coronaviruses isolated from two MS patients[7]. Despite the closer relationship of these isolates to murine coronaviruses[8], it was recently shown that their replication could be detected in brain tissue of some MS patients[9] and that they could cause a demyelinating disease in primates[10]. Moreover, antibodies to both serotypes of human coronaviruses were detected in the cerebrospinal fluids of MS patients, which could result from replication of HCVs within the central nervous system[11]. Finally, the neurotropism of some murine strains of coronavirus is well recognized and actually provides a very useful animal model of virus-induced demyelinating disorders, given the close analogy of the clinical manifestations of the disease in rodents to multiple sclerosis[12]. Together, these indications constitute enough stimulus for renewed interest in the study of the neurotropism of human coronaviruses and their possible involvement in multiple sclerosis.

Very few studies have attempted to verify the replication of human coronaviruses in neural and glial cells of the nervous system. Pearson and Mims[13] reported a productive infection of murine neurons in primary cultures by HCV-OC43, as well as the presence of viral antigen in astrocytes. However, infection of myelin-producing oligodendrocytes was not detected. In the same report, the authors also reported that human embryo brain cells, including astrocytes were susceptible to HCV-OC43 infection but did not produce infectious virus[13]. Collins and Sorensen[14] showed that the U87-MG human glioblastoma cell line could be persistently infected with HCV-OC43. No studies have been reported so far on the neurotropism of HCV-229E, a virus isolated from the human respiratory tract in 1966[15].

In the present study, we report on the replication of HCV-229E in neural and glial cell lines and the presence of its genome in parts of the brains of some MS patients.

MATERIALS AND METHODS

Virus and Cell Lines

The 229E strain of HCV was originally obtained from the American Type Culture Collection (ATCC; Rockville, MD), plaque-purified twice and grown on the human embryonic lung cell line L132, as described previously[16,17]. The following human neural and glial cell lines were obtained from the ATCC: H4 (neuroglioma, brain), SK-N-SH (neuroblastoma, metastasis to bone marrow), U-373 MG and U-87 MG (glioblastoma, astrocytoma). The MO3.13 human-human hybrid cell line was derived by lectin-enhanced polyethylene glycol-mediated somatic cell fusion between the thioguanine-resistant rhabdomyosarcoma mutant RD-TG.6, derived from the TE671 (RD) cell line (ATCC), and primary human oligodendrocytes obtained from cultures of human adult temporal lobectomies[18]. Similar to oligodendrocytes but unlike the parent tumor rhabdomyosarcoma line, MO3.13 cells have been demonstrated to express myelin basic protein and proteolipid protein by immunohistochemistry, Western immunoblotting and Northern blotting, and show surface immunoreactivity for galactocerebroside and myelin-associated glycoprotein.

These cell lines were grown as monolayers at 37°C, in a humidified atmosphere containing 5 % (v/v) CO_2, using Dulbecco's modified Eagle's medium containing high glucose, L-glutamine and sodium pyruvate, and supplemented with 10 % (v/v) heat-inactivated fetal calf serum, without antibiotics (Gibco BRL Life Technologies, Inc., Burlington, Ontario, Canada). Cells were passaged every four days at a concentration of 100,000 cells per ml.

Immunofluorescence Assay

For immunofluorescence, approximately 1.5 x 10^6 cells (obtained by trypsinization of cell monolayers grown on plastic 25-cm^2 flasks, followed by pelleting) were mixed with an equal volume of HCV-229E virus stock diluted to give an MOI of 1. Twenty-five microliters of this suspension was deposited into each well of a 12-well glass slide (Flow, ICN Biomedical Canada Ltd., Mississauga, Ontario, Canada) and infection allowed to continue for 20 or 40 h at 33°C (the optimal HCV-229E growth temperature[17]) and 5 % (v/v) CO_2. Slides were washed twice in Dulbecco's phosphate buffered saline (PBS) and fixed with cold acetone at -20°C for 20 min. Viral antigen was detected by adding a 1/10 dilution of ascites fluids containing HCV-229E-specific monoclonal antibodies, designated 3-10H.5 or 4-9H.5 (ELISA titers: 1/144,000 and 1/20,000, respectively), or control ascites fluids prepared with the parental myeloma cells. After incubation at room temperature in a humidified chamber for 2 h, the slides were washed twice with PBS and a 1/100 dilution of fluorescein isothiocyanate-conjugated $F(ab')_2$ goat antibodies to mouse immunoglobulins (Cappel, Organon Tecknika Inc., Scarborough, Ontario, Canada) were added for another 30 min. Fluorescence was observed with a Leitz fluorescence microscope after mounting the slides with glass coverslips, using glycerol:PBS (9:1).

Preparation of RNA and Reverse-Transcription Polymerase Chain Reaction

Brain tissues were collected from a total of 11 patients diagnosed with multiple sclerosis and 11 control patients, five with normal autopsy reports and six with indications of other neurological diseases (Montreal Brain Bank, Montreal, Quebec and University Hospital, London, Ontario). Total RNA was extracted from coded central nervous system (CNS) tissues by the method of Chomczynski and Sacchi[19]. Briefly, 50 to 300 mg of tissue were thawed and homogenized in 0.5 ml of 4 M guanidinium thiocyanate, extracted with phenol and chloroform and precipitated twice with ethanol. After washing with 70% (v/v) ethanol, the pellets were air-dried and resuspended in water. The extracted RNA was first tested for the presence of human myelin basic protein or actin mRNAs by reverse-transcription - polymerase chain reaction (RT-PCR) to insure that it was undegraded[20].

For RT-PCR, HCV-229E primers were designed to amplify either of two regions of about 300 bases in the gene coding for the nucleocapsid protein of this virus, at positions 498 to 806 or 964 to 1265[21]. To confirm the identity of the amplified products, hybridization was performed with oligonucleotide probes derived from sequences located between each pair of primers (bases 693-716 and 1080-1103, respectively[21]). Approximately 1 µg of RNA was reverse transcribed at 37°C for 35 min with 20 U of Moloney murine leukemia virus reverse transcriptase (Pharmacia Canada Inc., Baie d'Urfé, Québec, Canada) and 50 pmol of both up- and downstream primers (to target both positive- and negative-stranded RNA) in 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1 % (v/v) Triton X-100 (1X *Taq* polymerase buffer; BIO/CAN, Mississauga, Ontario, Canada), 1.0 mM (each) dATP, dCTP, dGTP and dTTP (Pharmacia), 40 U of RNAguard (Pharmacia) and 4.0 mM $MgCl_2$. Twenty microliters of the reverse transcription was added to 80 µl of a PCR mix overlaid with mineral oil. This mixture contained 1X *Taq* polymerase buffer (BIO/CAN), 2.5 U of *Taq* polymerase (BIO/CAN), 50 pmol of both up- and downstream primers, 0.25 mM (each) dATP, dCTP, dGTP and dTTP (Pharmacia) and 2.4 mM $MgCl_2$. PCR was performed using a modification of the original method[22]. An amplification cycle of 1 min at 94°C, 2 min at 60°C and 2 min at 72°C was repeated 30 times and was followed by an extension period of 7 min at 72°C. Twenty µl of reaction products were loaded onto 1.5 % (wt/vol) agarose gels, allowed to migrate and transferred to nitrocellulose filters according to the method of Southern[23]. Blots were hybridized with

^{32}P-end-labeled oligonucleotide probes at 50°C for 16 h in a buffer containing 6x SSC, 1x Denhardt's solution, 0.05% (wt/vol) pyrophosphate and 100 µg/mL sonicated salmon sperm DNA. The blots were washed 3 x 15 min at room temperature and for 20 min at 60°C in 6x SSC, 0.05% (wt/vol) pyrophosphate and exposed to X-ray film (Kodak, Rochester, NY) at -70°C for 48 or 96 h.

The detectability level of the RT-PCR assay was evaluated by cloning the amplification product of RNA prepared from HCV-229E-infected L132 cells into the *Sma*I site of the pGEM 3Z vector (Promega, Fisher, Montréal, Québec, Canada). Five µg of *Hin*dIII-linearized plasmid were transcribed *in vitro* in the presence of 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM spermidine, 25 mM NaCl (transcription buffer; Stratagene, La Jolla, California), 0.4 mM (each) ATP, GTP and UTP, 20 U T7 DNA polymerase and 2 U RNase Block II (Stratagene) and 3 µM [α-^{32}P]CTP. The amount of transcript was evaluated by ^{32}P incorporation and the number of molecules detectable was estimated by performing RT-PCR on ten-fold serial dilutions of the transcript.

RESULTS AND DISCUSSION

Infectability of neural and glial cell lines

We found that all neural and glial cell lines tested could be infected with HCV-229E, as revealed by immunofluorescence with virus-specific monoclonal antibodies (MAbs). Representative results are shown in Fig. 1.

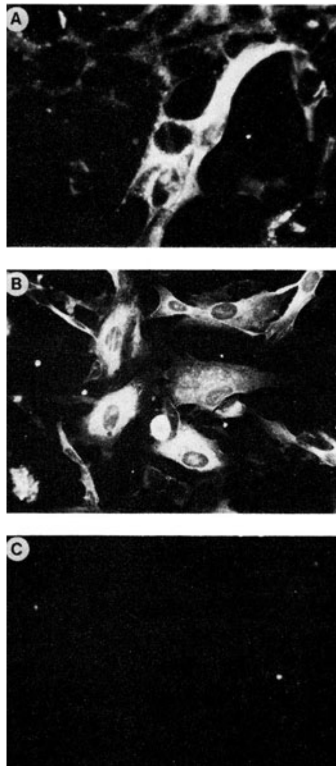

Figure 1. Immunodetection of HCV-229E antigens in neural and glial cell lines. Panel A: MO3.13 cells at 40 h post-infection, revealed with the 3-10H.5 MAb. Panel B: SK-N-SH cells at 20 h post-infection, revealed with the 4-9H.5 MAb. Panel C: SK-N-SH cells at 20 h post-infection, revealed with control antibody (magnification = 100X).

Varying degrees of antigen expression were observed in the different human neural and glial cells lines, as summarized in Table 1.

It is noteworthy that cells related to neurons, astrocytes and oligodendrocytes were susceptible to infection by the human coronavirus 229E, since the same situation is observed in the mouse with the neurotropic strains of murine hepatitis virus[24]. Indeed, this is the first report on the infection of such cells by HCV-229E. Interestingly, it appears that immortalized human oligodendrocytes were infected with this virus. The more extensive infection observed in these cells when compared to the parental rhabdomyosarcoma cell line, at least at 40 h post-infection (Table 1), suggests that the oligodendrocyte phenotype

Table 1. Percentage of infected cells after infection with an MOI of 1 for 20 or 40 h.

Cells		Percent infected cells at each time post-infection (%)	
Line designation	Type	20 h	40 h
SK-N-SH	Neural	90	90
H4	"	18	20
U-373 MG	Astrocyte	3	60
U-87 MG	"	2	60
MO3.13	Oligodendrocyte	6-10	20
TE671 (RD)	MO3.13 parental	6	3
L132	Lung	70-80	80

was involved in susceptibility. Even though final proof on the susceptibility of human oligodendrocytes to coronavirus infection will require the use of primary oligodendrocyte cultures and immunohistochemistry on human CNS tissue sections, the infection of these myelin protein-producing cells is indeed relevant to the possibility of the induction of demyelinating disease in humans by coronaviruses.

Detection of viral RNA in brain samples

Having demonstrated infection of human and glial cells *in vitro* by HCV-229E, we wanted to test for the presence of this virus in CNS tissue of multiple sclerosis patients. A previous study had used classical hybridization to search for the RNA of HCV-OC43 in four MS brain autopsy samples, with negative results[25]. Thus, we wanted to develop a more sensitive assay, capable of detecting very low amounts of viral nucleic acid, characteristic of persistent infections. For that purpose, we developed a polymerase chain reaction (PCR) assay, modified to include a reverse transcription step from extracted RNA (RT-PCR). As shown in Fig. 2, the detectability level of our assay was less than 60,000 molecules. Moreover, this number is most likely an overestimation since the *in vitro* transcription may have produced a proportion of incomplete transcripts which could not be amplified by RT-PCR. Similar detectability levels of RT-PCR were reported in other systems[26]. Since the target sequence is located on the most abundant viral mRNA as well as on the genome, and since we designed the assay to amplify both positive- and negative-sense RNAs, we estimate that a single infected cell should be detectable in our procedure.

We applied our HCV-229 RT-PCR assay to RNA extracted from frozen brain autopsy samples from both MS and control patients. Positive signals were obtained from four of eleven MS patients and none of eleven controls, which included five histopathologically normal patients, and six patients whose autopsy report indicated Alzheimer's disease (four patients), subacute meningoencephalitis (one patient) and ischemic vascular disease (one patient). Representative results are shown in Fig.3.

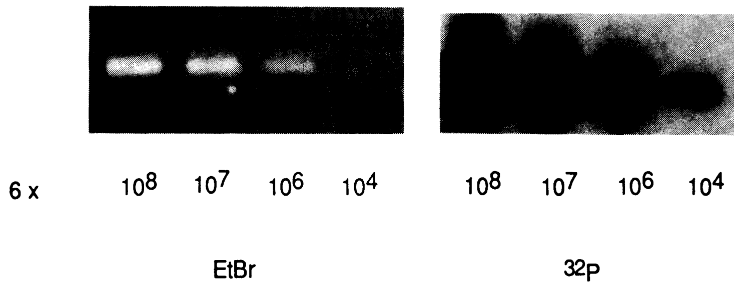

Figure 2. Determination of the detectability of the HCV-229E RT-PCR. The estimated amounts of transcripts indicated on the figure were amplified by RT-PCR and analyzed on agarose gels, which were stained with ethidium bromide (EtBr), followed by Southern hybridization with an internal radiolabeled oligonucleotide probe (^{32}P).

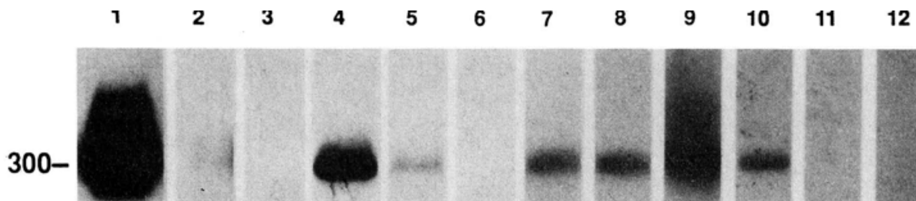

Figure 3. Detection of HCV-229E in brain samples by RT-PCR. The templates in each lane are RNA extracted from: lane 1, L132 cells infected with HCV-229E; lane 2, mock-infected L132 cells; lane 3, no RNA; lane 4, mouse brain mixed with 170 pg RNA extracted from HCV-229E-infected L132 cells; lane 5, mouse brain mixed with 1.7 pg RNA extracted from HCV-229E-infected L132 cells; lane 6, mouse brain; lanes 7-12, specimens from MS patients (positive samples were from: lanes 7 and 9, white matter; lane 8, gray matter; lane 10, plaque tissue). Blots were exposed to X-ray film for 48 (lanes 1-6) or 96 h (lanes 7-12). The migration position of the 300 bp HCV-229E amplified product is indicated on the left.

The results of our study indicate for the first time that the 229E strain of human coronavirus, which has so far only been associated formally with respiratory infections, has the capacity to infect cells of the central nervous system, as monitored by *in vitro* infection of transformed cell lines. Moreover, its genome is expressed in some human brains. Interestingly, it was shown in the animal model of coronavirus-induced demyelinating disease that neurotropic coronaviruses can gain access to the central nervous system from the respiratory tract, through the olfactory and trigeminal nerves[27]. Thus, it is conceivable that the same type of spread from the upper respiratory tract to the CNS could occur in humans and be relevant to the development of a neurologic disease in genetically predisposed individuals[28,29].

An association of HCV-229E with multiple sclerosis cannot be inferred from our pilot RT-PCR study, given the small number of clinical samples analyzed. Moreover, the establishment of an etiologic link with the disease will require large scale studies involving an epidemiological component. Nevertheless, we believe that research efforts are needed to establish the pathological relevance of the HCV-229E neurotropism suggested by our study. Interestingly, we recently found evidence for the sharing of amino acid sequences between HCV-229E and myelin basic protein[16]. Since this protein appears to be a target for the immune system of MS patients[30], the possibility that molecular mimicry of myelin antigens by a virus, be it a coronavirus or another virus sharing similar mimetic properties, could be involved in the establishment of this degenerative autoimmune neurologic disease also warrants attention.

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