

Human Coronavirus Gene Expression in the Brains of Multiple Sclerosis Patients

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Total RNA extracted from both white and gray matter of brain tissue from multiple sclerosis (MS) patients and controls was analyzed using a reverse transcription-polymerase chain reaction for the presence of the nucleic acid of human coronavirus (HCV) 229E and OC43, the two strains characterized to date and associated with respiratory infections. HCV-229E viral RNA was detectable in the central nervous system tissue of 4 of 11 MS patients and in none of 6 neurological and 5 normal controls. No HCV-OC43 nucleic acid was detected in any of the specimens. These results suggest a neurotropism on the part of the 229E strain of human coronavirus and underline the importance of further studies on its tissue distribution. The fact that it was detected only in tissue from MS patients illustrates the need for continued studies on the possible role of coronaviruses in the etiology of MS. © 1992 Academic Press, Inc.

Even though the cause of multiple sclerosis (MS) is unknown, an environmental agent, particularly a virus, has been postulated on the basis of epidemiologic studies (1). Human coronaviruses (HCV) number among potential pathogens due to the observation of coronavirus-like particles in the perivascular cuffing of an MS plaque (2), intrathecal synthesis of antibodies to HCV-229E and HCV-OC43 in a proportion of MS patients (3), and the isolation of coronavirus from two MS brains (4). Also, murine coronaviruses serve as models of demyelinating diseases in rodents (5), thus HCVs may produce a similar pathology in humans. However, Sorensen *et al.* were unable to detect HCV-OC43 in four MS brains by classical molecular hybridization (6). HCVs may be implicated in neurological diseases, particularly MS, either by persistent infection of oligodendrocytes or astrocytes (7, 8) or by some means of autoimmune stimulation such as molecular mimicry (9). Therefore an attempt should be made to detect very low concentrations of HCV RNA, characteristic of persistent infections, in tissues from patients with MS and other neurological disorders. Indeed, Murray *et al.* have recently detected murine-related coronavirus RNA and antigen in MS brains (10). In the present pilot study, we employed a reverse transcription-polymerase chain reaction (RT-PCR) technique to detect the RNA of HCV-229E and HCV-OC43, the two strains of HCVs characterized and partially sequenced to date in frozen gray and white matter of MS patients and in the white matter of controls.

MS tissues were collected from identifiable plaques and adjacent or nonadjacent normal-appearing white matter and from gray matter (Montreal Brain Bank, Montreal, Quebec, and University Hospital, London, Ontario). Seven specimens of plaque tissue, 3 of gray matter, and 11 of white matter were collected from a total of 11 MS brains and 1 specimen of white matter each from individuals whose neuropathological report at autopsy indicated normality (five individuals), Alzheimer's disease (four patients), subacute meningoencephalitis of apparent bacterial origin (one patient), or ischemic vascular disease (one patient). RNA extracted from coded central nervous system (CNS) tissues was first tested for the presence of human myelin basic protein (MBP) mRNA and human γ -actin mRNA by RT-PCR to ensure that the RNA was sufficiently intact (11). Specimens in which mRNA could not be detected were excluded from the study. Total RNA was extracted from 50- to 300-mg frozen CNS tissue by the method of Chomczynski and Sacchi (12) modified such that processing was performed in Eppendorf tubes and 10% of the extract was used for RT-PCR.

Total RNA extracted from L132 cells infected with HCV-229E as described previously (13) and a preparation of genomic HCV-OC43 RNA provided by Dr. S. Dea (Institut Armand-Frappier) were employed as positive controls in the study. Three microliters of RNA extract containing roughly 1 μ g of RNA was added to 17 μ l of master reverse transcription mix to achieve final concentrations of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% (vol/vol) Triton X-100 (1 \times Taq polymerase buffer; BIO/CAN, Mississauga, Ontario, Canada); 1.0 mM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia Canada Inc., Baie d'Urfé, Québec, Canada); and 4.0 mM MgCl₂. The mixture was supplemented with 20 U

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of Mo-MuLV reverse transcriptase (Pharmacia), 40 U of RNAGuard (Pharmacia), and 50 pmol of both up- and downstream primers and was incubated at 37° for 35 min. PCR was performed using a modification of the original method (14). The entire volume of the reverse transcription mixture was added to 80 μ l of a PCR master mix overlaid with mineral oil. This mixture contained 1 \times *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 MgCl₂. An amplification cycle of 1 min at 94°, 2 min at 60°, and 2 min at 72° was repeated 30 times and was followed by an extension period of 7 min at 72°. Twenty microliters of reaction product was loaded onto 1.5% (wt/vol) agarose gels, allowed to migrate, and transferred to nitrocellulose filters according to the method of Southern (15). Blots were hybridized with a ³²P-end-labeled oligonucleotide probe (2 \times 10⁶ cpm/ml) at 50° for 16 hr in a buffer containing 6 \times SSC, 1 \times Denhardt's solution, 0.05% (wt/vol) pyrophosphate, and 100 μ g/ml sonicated salmon sperm DNA. The blots were washed 3 \times 15 min at room temperature and for 20 min at 60° in 6 \times SSC, 0.05% (wt/vol) pyrophosphate, and exposed to X-ray film (Kodak, Rochester, NY) at -70° for 48 or 96 hr.

Negative controls included deionized water and RNA extracted from uninfected L132 cells and mouse brain. Recommended precautions to prevent carryover contamination were implemented and were successful (16, 17). Briefly, they consisted mainly of performing RNA extractions and PCR reactions in a separate laboratory, using positive-displacement pipettes and gloves, processing positive controls last, autoclaving solutions when possible, and confirming positive results in a second test.

All target sequences were roughly 300 bp in length. Primers were derived from bases 274–307 (exon 3) and 550–573 (exon 7) of the human MBP gene (18) and bases 1358–1381 (exon 3) and 1712–1735 (exon 4) of the human γ -actin gene (19). HCV-229E primers were 5'-AGGCGCAAGAATTCAGAACCAGAG-3' and 5'-AGCAGGACTCTGATTACGAGAAGG-3' derived from base sequences 498–521 and 783–806, respectively, on mRNA 7 of HCV-229E (20). A second set of primers were 5'-AACTTTGGAAGTGCAGGTGTTGTG-3' and 5'-TGGTTCAGCAGTTGCAGGTGAAGT-3' derived from base sequences 964–982 and 1242–1265, respectively, on mRNA 7 of HCV-229E (20). Primers for HCV-OC43 were 5'-CCCAAGCAAAGTCTACCTCTCAG-3' and 5'-GTAGACTCCGTCAATATCGGTGCC-3', corresponding to bases 215–238 and 497–520, respectively, on the mRNA coding for the nucleoprotein of HCV-OC43 (21). Oligonucleotide probes for hybridiza-

tion with the amplification product were derived from sequences located between each pair of primers. They were 5'-ATGAAGCAGTTGCTGCGGCTCTT-3' (bases 693–716) for the first and 5'-GAGTCAGGCAA-CACTGTGGTCTTG-3' (bases 1080–1103) for the second pair of primers for HCV-229E (20). An oligonucleotide probe for hybridizing the amplification product of HCV-OC43 was 5'-GATGGCAACCAGCGTCAACTGCTG-3' (bases 419–442) (21).

The detectability level of our assay was evaluated as follows: the amplification product from human coronavirus RNA was treated with the Klenow fragment of DNA polymerase I and cloned into the *Sma*I site of the pGEM 3Z vector (Promega; Fisher, Montréal, Québec, Canada) by blunt-ended ligation (22). Five micrograms of *Hind*III-linearized plasmid was transcribed *in vitro* in the presence of 20 mM Tris-HCl (pH 8.0); 4 mM MgCl₂; 1 mM spermidine; 25 mM NaCl (transcription buffer; Stratagene, La Jolla, CA); 0.4 mM (each) ATP, GTP, and UTP; 20 U T7 DNA polymerase, and 2 U RNase Block II (Stratagene) and 3 μ M [α -³²P]CTP. The amount of transcript was evaluated by ³²P incorporation and the number of molecules detectable was estimated by performing RT-PCR on 10-fold serial dilutions of the transcript.

HCV-229E RNAs from infected cells were readily detected using the RT-PCR (Fig. 1, lane 1). The assay could detect less than 10⁴ target molecules (results not shown). Dilutions of RNA mixed with 1 μ g of RNA extracted from normal mouse brain tissue were as easily detected (Fig. 1 lanes 4 and 5). Two or more RT-PCR reactions for the detection of HCV-229E were performed on each brain specimen using one or both primer pairs. Positive and negative results arising from clinical specimens can be seen in Fig. 1. Only specimens giving positive results in at least two different RT-PCR tests were considered to be confirmed positives (Table 1). No positive hybridization signals were obtained from any of the specimens tested for the presence of HCV-OC43 RNA, despite an apparently similar detectability level.

Four of 21 specimens from MS brains tested for HCV-229E nucleic acid consistently gave positive results while none of the 11 control brain specimens did so. However, only 1 sample per control patient was available, whereas 1 to 5 samples per MS patient could be tested in this pilot study. Three of the four positive samples were detected with both primer pairs. The confirmed positive samples came from 4 of 11 different MS brains. The positive specimens were obtained from a white matter plaque, normal-appearing white matter, gray matter, and cervical cord tissue which included both gray and white matter.

Comparison of signal intensities between positive

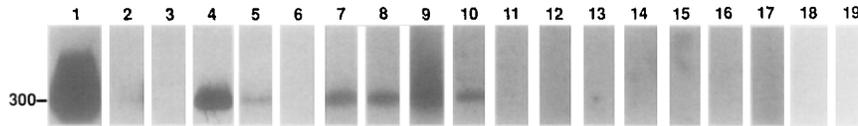


Fig. 1. Typical results of hybridization of amplification products with oligonucleotide probe specific for HCV-229E. The templates in each lane are RNA extracted from: lane 1, L132 cells infected with HCV-229E; lane 2, mock-infected L132 cells; lane 3, no RNA; lane 4, mouse brain mixed with 170 pg RNA 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 7–14, specimens from MS patients (lanes 7 and 9, white matter; lane 8, gray matter; lane 10, plaque tissue); lanes 15–19, specimens from control brains (lane 15, normal; lanes 16 and 18, Alzheimer's disease; lane 17, ischemic vascular disease; lane 19, subacute meningoencephalitis). Blots were exposed to X-ray film for 48 (lanes 1–6, 18, 19) or 96 hr (lanes 7–17). The migration position of the 300-bp amplified product is indicated on the left.

clinical specimens (Fig. 1, lanes 7–10) and control samples (Fig. 1, lanes 4 and 5) suggests the presence in MS brains of less than 10 pg of viral RNA per microgram of total RNA, since the strength of the signals from clinical samples fell between the intensities of the signals obtained from 170 and 1.7 pg of total RNA extracted from HCV-229E-infected cells, in which the proportion of viral RNA is unknown.

Recently, Murray *et al.* (10) reported the detection of coronaviruses in 12 of 22 MS brains by *in situ* hybridization using a probe derived from this group's MS isolate, which is more closely related to murine coronaviruses than to either HCV (23). It is important to note that, in the present study, we would not detect such nucleic acid in the event that it was present since the RT-PCR technique employed here is specific for HCV-229E and HCV-OC43 only. Indeed, our HCV-OC43 primers did not amplify MHV-A59 RNA (results not shown), whereas the HCV-OC43 probe used by Murray *et al.* (10) did hybridize weakly to it. Interestingly, our results on the detection of HCV-OC43 by RT-PCR are consistent with previous negative results with classical hybridization (6). Surprisingly, an HCV-229E mRNA 7 probe used by Murray *et al.* (10) did not give any positive signals by *in situ* hybridization. This discrepancy with our positive results may be due to their preselection of tissues for the presence of murine-related coronavirus RNA, or to differences in the techniques used by our two groups.

TABLE 1

RESULTS OF RT-PCR FOR THE DETECTION OF HCV-229E IN CNS TISSUES

Specimens	Total number of samples	Total number of patients	Number of confirmed positives
MS	21	11	4
Normal	5	5	0
Alzheimer's disease	4	4	0
Ischemic vascular disease	1	1	0
Encephalitis	1	1	0

Given the existing murine coronaviral model for demyelinating disease it would be tempting to speculate about a possible role for HCV-229E in the etiology of MS, particularly in view of the ability of some coronaviruses to produce demyelination in primates (24). However, this is premature, considering that the mere presence of the virus does not necessarily indicate the existence of a causal relationship.

Not all samples from an MS patient could be confirmed positive for the presence of coronavirus RNA and both gray and white matter specimens were among the positives. This is consistent with findings in the animal models of murine coronavirus chronic neurologic disease, where *in situ* hybridization revealed regional localization of viral genomes in the CNS (25, 26). Moreover, the estimated amount of viral RNA present in the clinical specimens (less than 10 pg/ μ g) correlates well with estimates made in the murine model where viral persistence in the CNS was observed after infection of the upper respiratory tract (27). Since our RT-PCR method detected less than 10^4 molecules and the target sequence is located on the most abundant viral mRNA as well as on the genome, we estimate that a single infected cell should be detectable using this assay.

We cannot rule out the possibility that the positive results we have obtained in tissue homogenates may arise from peripheral blood lymphocytes (PBLs) present in the brain vasculature and not in the neural cells themselves. While HCV infection of PBLs has not yet been addressed in the literature, preliminary evidence in our laboratory indicates that the PBLs of some individuals harbor HCV-229E RNA.

We do not propose a coronaviral etiology for MS based on these results; however, a neurotropism on the part of HCVs is likely, warranting further studies to identify the type of cells harboring the viral genome. The mere presence of HCV-229E nucleic acid in CNS tissues, PBLs, or both is in itself of significance because this virus has formerly only been associated with infections of the respiratory tract. The recent identification of the HCV-229E receptor, which is present in

brain synaptic membranes, macrophages, and granulocytes (28), as well as the infectability of cultured neural cells (unpublished data), further supports the results obtained in our study. The fact that the RNA of HCV-229E was detected in CNS specimens from multiple sclerosis patients is an issue which must be explored in a large-scale study including controls from a wide variety of inflammatory neurological diseases.

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