CORONAVIRUSES AND MULTIPLE SCLEROSIS

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the human central nervous system (CNS) without known cause. A popular theory proposes that an exogenous stimulus initiates an immune response against self CNS proteins, possibly myelin structural protein(s). However, damage to the CNS as the result of an immune response to a chronic low grade CNS infection has never been ruled out. Therefore, demyelinating lesions in MS may represent the final direct or indirect immunopathological reaction to an infectious agent(s). Supporting this hypothesis are epidemiologic studies that strongly implicate an environmental factor in the development of MS (1). Particularly compelling is the MS epidemic in the Faroe Islands (2) and the recent study of offspring to immigrants to the United Kingdom from low incidence countries developing MS at rates similar to the indigenous English population (3).

Several common human RNA or DNA viruses have been implicated in the pathogenesis of MS but none have been definitively associated with the disease (4). Our laboratory and collaborators have been unsuccessful in detecting herpes simplex type I, cytomegalovirus, varicella zoster virus, Epstein Barr virus or borrelia burgdorferii (unpublished data), human T-cell lymphotropic virus (5,6), or measles, mumps, and rubella viruses (7) in MS brain by either in situ hybridization, immunohistochemistry or the polymerase chain reaction.

Coronaviruses have been implicated in MS since the report of virus isolation from the brains of two MS patients and the electron microscopic observation of a coronavirus like particle in an MS brain perivascular immunocyte (8,9). Coronaviruses are also known to cause demyelination (10,11) and are capable of stimulating T-cell mediated autoimmune reactions in rodents (12,13). Recently, we reported finding coronavirus RNA and antigen in active demyelinating plaques of MS autopsy brain thus further implicating coronaviruses in MS (14). This coronaviral RNA and antigen was detected primarily with cDNA probes and antibodies specific for MHV related coronaviruses.

Although these results were surprising they are plausible since we then demonstrated that coronaviruses JHM and SD could infect the CNS and cause demyelination following intracerebral inoculation in several species of primates (15). Additionally, coronavirus 229E has recently been detected in MS brain by PCR technology (16).

Our current goals are to specifically identify the coronavirus(es) found in human brain and investigate how these coronaviruses interact with human brain cells and immune system. We now report the initial results and development of 1) PCR amplification of coronaviruses directly from human brain, 2) an MS brain derived cDNA library and screening for CV clones and 3) an *in vitro* human brain cell - coronavirus infection system.

METHODS AND RESULTS

PCR Amplification of Coronaviruses from Human Brain

Human brain samples were derived from MS and control autopsy samples used in prior *in situ* hybridization and immunohistochemical screens for the presence of coronaviruses (15). All necessary precautions were taken to avoid contamination of RNA preparations with exogenous sources of RNA and DNA. Total RNA extracted from frozen human brain was reversed transcribed using the following reaction conditions. 1ug of RNA was mixed with 100pmoles of random hexamers (Boehringer Mannheim) in a total volume of 15ul, heated at 65°C for 5 min then cooled at room temperature. CVP1 5' TTGAAGGCTCTGGAAGGTCTGC and CVP2 5'CTGCAAGAATGGGGAAGTGTGG was the initial set of primers. The CVP1/P2 product is 374bp for MHV and 441bp for OC43. 5ul of P1/P2 reaction is removed, mixed into 100ul H₂O and then 5ul of this, i.e. approx. 0.05ul of original rxn, is used in second round PCR with P3/P4 primers. The nested set reaction conditions are as follows: 10ul of a enzyme/buffer/dNTP mix was added to the RNA/primer tube and the reaction was incubated at 43°C for 1 hr. The final reverse transcriptase reaction conditions were 40ug/ml RNA, 4uM random hexamers, 50mM Tris, pH 8.3, 100mM KCl, 10mM MgCl₂, 4mM DTT, 1 unit/ul RNasin (Promega), 1mM dNTPs, and 5 units of AMV reverse transcriptase (LifeSciences, Inc.). Reactions were heated at 97°C for 5 min then stored at -20°C. PCR reaction conditions were as follows. Tubes containing 85ul of buffer, primers, enzyme and approximately 100ul of mineral oil were heated to 80°C in the Perkin Elmer Thermocycler. 5ul of the cDNA from the reaction described above was mixed with 10ul of 2mM dNTPs in a separate tube and this was then added through the oil into the PCR reaction tube. The final reaction conditions were: 10mM Tris, pH 8.8, 50mM KCl, 1.5mM MgCl₂, 0.2mM dNTP, 0.1% Triton X-100, 0.5uM of each primer, and 5 units of Taq polymerase (Promega). The temperature profile for the reactions was as follows: 95°C/ 1 min, 58°C/1.5 min, 72°C/3 min for 3 cycles then 95°C/1 min, 62°C/1 min, 72°C/2 min for 36 cycles followed by incubation at 72°C for 7 min. The PCR primers, CVP3 and CVP4, used for these reactions were designed based on the published sequences of the nucleocapsid genes of MHV A59 and HCV OC43 (2,22). Viral cDNA amplified with CVP3, 5'IIAAATTGCTIITCTTGTCTGGC (I=inosine), and CVP4, 5' CCAAATTCTGATTAGGGCCTCTC, produce a 186bp PCR product. These primers amplify a region extending from nucleotide 858 to 1043 or 784 to 969 for MHV A59 or HCV OC43, respectively. The PCR reaction products were analyzed by gel electrophoresis and Southern-blot hybridization. The ³²P labeled probe used for hybridization, CVPP has the sequence 5'AAGCAIAITGCCAAIAAGTCAGICAGAAAATTTT; this hybridizes specifically to the 186bp CV PCR product.

Table 1. CV PCR amplification from MS brain.

<u>Sample</u>	<u>Diagnosis</u>	<u>Brain Region</u>	<u>PCR Product</u>
TO-1	MS	Cerebrum	Positive
TO-2	MS	Brain stem	Negative
ZO	MS	Cerebrum	Positive
ZOH	MS	Cerebrum	Negative
HO	MS	Cerebrum	Negative
KN-1	MS	Cerebrum	Negative
KN-2	MS	Spinal Cord	Negative
ME	MS	Cerebrum	Positive
SZ	MS	Cerebrum	Negative
SY	MS	Cerebrum	Negative
RO	MS	Cerebellum	Negative
TR	MS	Spinal Cord	Negative
SC	MS	Cerebrum	Negative
DI-cDNA library	MS	Cerebrum	Positive
HA	Cardiac	Cerebellum	Negative
OR	Cardiac	Cerebrum	Positive
MU	Cardiac	Cerebrum	Negative
LA	Sepsis	Cerebrum	Negative
MC	Cardiac	Cerebrum	Negative

Table 1 shows our initial PCR results for 12 MS patients with 4 having amplifiable coronavirus product. MS sample DI was amplified from a cDNA library made from mRNA from this patient's white matter. Sequencing of this amplified product demonstrated high homology with the published sequence of JHM. One of control brain samples had amplifiable product. To date these products have not been sequenced.

Coronavirus Infection of Human Brain Cells

Primary or continuous human and murine cell cultures were infected with coronavirus JHM, SD or sham inoculums. Origin and methods of growing coronaviruses JHM, JHM OMP1 and SD have been described (11,15). The human astrocyte cell line STTG-1 was obtained from the ATCC and maintained under standard cell culture conditions. Human primary brain cultures were derived from the resected epileptic cortex of two patients (F and V). Cells were grown in DMEM-10%FCS medium. DBT and 17Cl 1 cells growth and maintenance have been described (8,11). Cultures were observed for CPE and infectious virus production. Additionally, all cultures were analyzed for the presence of viral products by immunohistochemistry (14,15), western and northern blots and PCR amplification from isolated cell RNA as described (15). To inhibit infection, viral inoculums were pretreated with a polyclonal anti-coronavirus antibody (gift from J. Leibowitz).

Table 2 shows the results of infection with JHM of human and murine cell lines. Primary brain cultures and the human astrocytoma cell line STTG could be infected. The infection was of a very low grade and complete CPE would not develop for 5 to 6 days post adsorption. In contrast, murine DBT cells and 17Cl-1 cells could be productively infected by JHM. Similar results were seen with CV SD and the primate isolate JHM OMP1. In general only low levels of virus were detectable in human cells suggesting that these cells were prone to being persistently infected. However the exact mechanisms will require further investigation.

Table 2. Infection of Cell Lines with Coronavirus.

System	CPE	Virus	PCR	Northern	IF	Western
17Cl-1	-	-	-	-	-	-
17Cl + JHM	+	+	+	+	+	+
V Primary Brain	-	-	-	-	-	-
V + JHM	+	ND	+	-	+	-
F Primary Brain	-	-	-	-	-	-
F + JHM	+	ND	+	-	+	-
STTG	-	-	-	-	-	-
STTG + JHM	+	+	+	-	+	-
DBT	-	-	-	-	-	-
DBT + JHM	+	+	+	+	+	+

a. Pretreatment with antibody prevents CPE in all cells

b. Similar results with CV SD and JHM-OMp1

DISCUSSION

Despite the intensive investigations of neurotropic coronaviruses in rodents, human neurologic disease resulting from coronavirus infection has not been proven. However, accumulating data, in addition to the results presented here, suggest that coronaviruses may infect human CNS tissue and that these viruses may differ from the prototypical upper respiratory human coronaviruses. In one case, a coronavirus (Tettnang virus) was isolated from the cerebrospinal fluid of a young girl with viral meningitis following an upper respiratory infection (17). This human coronavirus isolate was suspected by the authors to be more closely related to murine coronavirus than to human coronavirus. Recently, Parkinsons disease patients were reported to have high cerebrospinal fluid antibody titers specific to the murine coronaviruses MHV A59 and MHV JHM when compared to patients with other neurologic disease or non-disease controls (18). Burks et al reported the isolation of two coronavirus (SD & SK) from MS autopsy brain tissue after passage through suckling mice or murine cell culture (8). Although these isolates have serologic cross reactivity to murine coronavirus and HCV OC43 (19) others have demonstrated that these putative MS coronavirus isolates are antigenically and genetically more closely related to murine coronavirus than to the human coronavirus (19,20); our results show a 97% sequence identity in the 3' end of the genome between coronavirus SD and MHV JHM (unpublished data). Additionally, we have demonstrated murine-like coronaviruses directly in demyelinating plaques of autopsy MS brain. We have also reported the CNS infection of primates (14). Taken together these data suggest that coronaviruses can infect the human CNS and may play a role in multiple sclerosis pathogenesis.

The results presented in this paper demonstrate the value of PCR as a sensitive method to screen human brain and cell lines for the prescence of minute amounts of coronavirus RNA. Additionally this represents a third method of identifying "murine" related CV in multiple sclerosis brain. Coronavirus specific PCR products were amplified from one MS cDNA library and sequenced. The sequence of this product had a 94% identity to JHM. This cDNA library will now allow us to "rescue" other

regions of this coronavirus. Hopefully we will glean data on the possibility of a human-murine recombinant virus versus a mutant murine coronavirus. Our in vitro results show that JHM is able to infect at low levels human STTG-1 cells and primary human brain culture cells. The infection takes several days to develop and can be inhibited by pretreatment of virus with neutralizing antibody. Infection of STTG-1 cells will be important in studying the human cellular immune response to these viruses. This will be possible since these cells are HLA DR2 and are known to present foreign antigens to T-cells.

The data presented here do not establish coronaviruses as an etiological agent for MS, but they do indicate that coronaviruses are present in the human CNS and are preferentially detected in MS tissue. However the stage is set for identifying the antigenic structure of these coronaviruses and to begin investigations of the cellular immunity to these antigens in HLA DR2 MS patients.

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