DETECTION OF CORONAVIRUS RNA IN CNS TISSUE OF
MULTIPLE SCLEROSIS AND CONTROL PATIENTS

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The cause of multiple sclerosis (MS) remains unknown. One of the leading hypothesis states that MS may result from the direct or indirect effects of a CNS viral infection. The hallmark of MS is the demyelinating lesion which may represent the final immunopathological reaction to many viral or non-viral precipitants. We are investigating coronaviruses in MS. Previously, two CV's were isolated from the brains of two patients with MS after passage through murine systems (1). CV are widely distributed in nature and are common human and animal pathogens. In addition, CNS demyelination results from CV infection of rodents (2,3) and in one report primates (4). The putative MS isolates (CV-SD and CV-SK) are antigenically related to the human CV OC43 and the murine CV A59 (5). To date no species specific marker has been identified and serologic data have not definitively resolved the species origin of CV-SD or CV-SK (5-7). Direct virus isolation from tissue is difficult, therefor to evaluate whether CV are present in human CNS tissue, the method of in situ hybridization (ISH) was performed using cDNA probes to detect CV-RNA. We report here the presence of CV-RNA sequences in human CNS tissue. In addition, CV-RNA is much more frequent in MS than non-MS tissue. These findings raise the question of a potential role for CV in MS.

We previously reported the following ISH results utilizing a cDNA probe prepared from purified CV-SD RNA by the random primer method (8-11). Tissue from 21 MS patients, 16 non-neurological disease (NND) and 5 other neurological disease (OND) control patients were examined. The OND cases included one of each of the following: amyotrophic lateral sclerosis (ALS), post-infectious encephalomyelitis, bacterial meningitis, subacute sclerosing panencephalitis (SSPE) and radiation-induced cerebral vasculitis.

All tissues were rapidly frozen and stored at -80°C. Average duration from patient death to tissue acquisition and freezing (autolysis time) for MS and control patients was 5.0 hours and 6.1 hours, respectively. The total number of MS and control tissue sections tested was 442 and 404 respectively.

Results for each experimental group are shown in Table 1. CV RNA was detected in the CNS from 11 of 21 MS patients (52%) and 2 of 21 non MS patients (9.5%). Significance was determined by chi square (χ^2) = 9.02, 1 degree of freedom with $p < 0.005$. Specifically, CV RNA was detected in CNS tissue from 1 of 16 (6%) NND patients and in 1 of 5 (20%) OND patients. The OND patient positive for CV RNA genome had radiation induced cerebral vasculitis. Both multiple sclerosis patients' CNS tissue from which CV-SD and CV-SK were originally isolated were found positive for CV RNA.

Table 1. PATIENTS POSITIVE FOR CV RNA

<u>Patients</u>	<u>N</u>	<u># positive(%)</u>
MS	21	11* (52)
Non-MS	21	2 (9.5)
-NND	16	1 (6)
-OND	5	1 (20)

* $p < 0.005$

Tables 2 and 3 summarize the total number of brain sections sampled from MS and control patients. The positive NND and OND patients were both found to have CV RNA in frontal cortex. CV RNA was not easily detected in the CNS of MS patients as only 49 (11.1%) of 442 sections tested contained viral genome. Genome was identified in both plaque and non-plaque areas of cortex, brainstem and spinal cord. CV RNA was found in only 3 (0.8%) frontal white matter sections of 376 sections examined from the 21 control patients.

We then repeated the above experiment utilizing cloned MHV-A59 cDNA (12) (kindly provided by S. Weiss and J. Leibowitz) as a probe. MHV-A59 has extensive homology with CV-SD (6). The 1800 base pair fragment was excised from pst1 site of clone g344, agarose purified, and end_g labelled (13) with ³²P-ATP to high specific activity (1×10^6 cpm/ng). The probe was shown to be specific for CV-SD and MHV-A59 (Fig. 1). There was no cross reactivity to human or murine nucleic acids.

MS and control brains were cut into four micron frozen sections, placed onto pretreated slides, fixed with 3:1 v/v ethanol/acetic acid solution, dehydrated through graded alcohols and pretreated to improve probe diffusion (10). The hybridization mixture contained 0.6M NaCl, 50% formamide, 10% dextran sulphate, 1x Denhardt's, and 100ug/ml of denatured human nucleic acids to decrease any nonspecific tissue background (9). ISH was carried out at 42°C for 24 hours with 1×10^6 cpm's of probe per tissue section. Coverslips were removed and all sections washed

Table 2. MS TISSUES POSITIVE FOR CV RNA

<u>CNS REGIONS</u>	<u>positive/total (%)</u>
CORTEX PLAQUE	17/230 (7.4)
CORTEX NON-PLAQUE	16/142 (11.3)
BRAIN STEM PLAQUE	2/16 (12.5)
BRAIN STEM NON-PLAQUE	6/20 (30.0)
SPINAL CORD PLAQUE	8/32 (25.0)
SPINAL CORD NON-PLAQUE	NOT DONE

TOTAL	49/440 (11.1)

Table 3. NON MS TISSUE POSITIVE FOR CV RNA

<u>CNS REGION</u>	<u>positive/total (%)</u>
CORTEX	3/376 (0.8)
BRAINSTEM	0/58 (0.0)

TOTAL	3/404 (0.7)

for 8 hours in 50% formamide; .01M Tris and 0.6M NaCl. Sections were then dehydrated in graded alcohols containing 0.3M ammonium acetate, air dried, coated with NTB-2 photographic emulsion (Eastman Kodak Co., Rochester, N.Y.), air dried, and placed in a desiccated container at 4°C. Slides were developed 3 to 5 days later and counter stained with hematoxylin and eosin. Positivity was determined by finding a significant number of silver grains developed over cells compared with background. Pretreatment of all tissues with ribonuclease significantly decreased or abolished the amount of positive hybridization.

Results with the MHV-A59 probe were similar to those obtained with the CV RNA probe. Fig.1 shows areas of positive hybridization in the white matter cells from MS patients brains. These patients were the donors from which CV RNA and CV-SK were isolated.

These results indicate that CV RNA is present in human CNS tissue and in a significantly higher proportion of MS patients than controls. There was no predilection for plaque or non-plaque areas. Even in sections that contained viral genome, there was a paucity of positive cells. This may explain previously negative results by less sensitive technology (14) and the difficulty of consistent successful CV isolations from human CNS tissue.

Although human CV's are generally associated with upper respiratory infections, more recent reports indicate that these viruses may cause gastroenteritis (15) and childhood meningitis (16). In humans, CV are ubiquitous with 100% of some populations seropositive for CV-OC43 by the age of 6 years (17). Human CV's are difficult to grow, presumably due to restrictive species and/or tissue tropisms. However, human CV OC43 productively infects suckling mice and in vitro infects and persists both in mouse astrocytes and human embryonic astrocytes (18,19). Murine coronaviruses are known to cause demyelination in rodents (2,3)

Fig. 1. Results of ISH with 32-P labelled A59 cDNA probe. All photos are 400x magnification before reduction. All sections were developed after 3 days of exposure. A) Uninfected DBT cells. B) A59 infected DBT cells treated with RNase resulting in no detectable signal. C) A59 infected DBT cells showing positive cytoplasmic signal. D) CV-SD infected DBT cells showing a multinucleated giant cell with diffuse positive cytoplasmic signal. E and F) MS patients SD and SK showing positive signal within the cerebral white matter.

and in one reported study primates (4). CV-SD also causes demyelination in mice (3). Murine coronaviruses are also capable of inducing class II antigen expression on cultured rat astrocytes rendering them capable of participating in the immune response (20). Demyelination can also be adoptively transferred to naive rodents from CV infected rodents suggesting a role for cross reactive epitopes between CV antigens and myelin proteins (21). The above suggest that the full spectrum of CV induced human disease may not be fully appreciated and that CV may play a role in MS.

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