Antigenic Assessment of Coronaviruses Isolated From Patients With Multiple Sclerosis

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• **Many studies have either supported or discounted the role of coronaviruses as etiologic agents in multiple sclerosis (MS). Two new approaches were applied to investigate this controversy. First, monoclonal antibodies specific for either murine coronaviruses (mouse hepatitis viruses) or human coronaviruses were used to characterize the antigenic features of MS-derived coronaviruses SK and SD. Both isolates were found to have a mouse hepatitis virus–type profile. Second, serum and cerebrospinal fluid antibodies to different coronaviruses, including SD, were measured in MS and control groups. No significant difference in antibody level to coronaviruses was found between MS and control samples. The results of these antigenic studies do not support a specific association between MS and coronaviruses.**

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The cause of multiple sclerosis (MS) remains unknown, although viruses have often been considered as possible etiologic agents of this disease.[1,2] After the electron microscopic identification of coronaviruslike particles in tissue from patients with MS coronaviruses have been among the viruses that have received particular attention.[3] For example, Burks et al[4] isolated two coronaviruses, designated SK and SD, from brain materials obtained at autopsy from two patients with MS and passaged either in murine cells or directly in mice.

The significance of these observations is still controversial. Serologic and virologic investigations have provided findings that tend either to support[4-6] or to discount[7-11] a pathogenic role for coronaviruses in MS. In addition, experimental neurologic infections of rodents by murine coronaviruses have revealed many pathogenic parallels to MS, including chronic demyelination,[12-16] a relapsing and remitting course in some instances,[17] genetic susceptibility to disease,[18,19] and immunologic abnormalities, including intrathecal antibody synthesis and oligoclonal cerebrospinal fluid (CSF) antibody production.[20,21] Interestingly, isolate SD causes demyelination after intracerebral inoculation in mice.[22] Finally, clinical reports have also shown that coronaviruses may cause acute neurologic diseases in humans,[23,24] and epidemiologic evidence has demonstrated that initial coronavirus infection and immunity frequently occur in late childhood,[25] the period thought to be critical in establishing environmental exposure to the putative MS agent.[26]

Most recently, Murray and colleagues[27] prepared a complementary DNA probe to MS-derived isolate SD. They found SD genome in nine (43%) of 21 MS autopsy cases, one (6%) of 16 normal control autopsy cases, and one (20%) of five other neurologic disease (OND) control autopsy cases. If confirmed, these important findings would strongly argue that isolate SD is of human origin and is closely associated with MS. In view of the potential significance of a virus that could be etiologically linked to MS, it would be of value to have alternative approaches to evaluate this association. In this regard, we studied SK and SD directly by serologic methods. First, panels of monoclonal antibodies were established with high specificity for either mouse hepatitis viruses (MHVs) or human coronaviruses (HCVs). Binding assays of the panels with isolates SK and SD indicate that SK and SD have the general antigenic features of murine coronaviruses rather than HCVs. Second, we extended previous serologic surveys by assaying patients with MS and controls for antibodies to SK and SD, as well as prototypic MHVs and HCVs. Patients with MS did not significantly differ from normal individuals or OND controls in their serum or CSF antibody responses to SK, SD, or other coronaviruses. Although these studies do not suggest an etiologic role for known coronaviruses in MS, they also do not rule out the possibility that MS may be caused by an uncharacterized or recombinant coronavirus.

MATERIALS AND METHODS

Viruses and Cells

For this study, HCV-229E was grown on monolayers of L132 cells.[28] The HCV-OC43 was propagated on HRT cells.[29] The identity of these viruses was confirmed in binding assays with the use of reference antisera (National Institutes of Health [Bethesda, MD] No. V-361-501-588 and V-360-701-562). The MHV-JHM and MHV-A59 were grown on DBT cells as previously described.[30] The isolation of two coronaviruses designated SK and SD from brain materials of patients with MS has been previously described.[4] These viruses were also propagated on DBT cells. Vesicular stomatitis virus (VSV) was used as a noncoronavirus control. Each virus was prepared under serum-free conditions, as previously described.[31] Supernatants from infected cultures were clarified by centrifugation at 400 g for ten minutes and stored at −70°C until use.

Patient Samples

A total of 59 paired serum and CSF samples were obtained from the National Multiple Sclerosis Society Human Neurospecimen Bank (Neurology Service, Veterans Administration Wadsworth Hospital, Los Angeles). Twenty-one samples from patients meeting the Schumacher et al[32] criteria for clinically definite MS and 21 samples from normal controls were used. In addition, the following OND samples were tested: Parkinson's disease (n = 4), Huntington's chorea (n = 1), epilepsy (n = 5), and dementia (n = 7). The mean age and percentage of male patients, respectively, for each group were as follows: MS, 45.6 years (95%); normal controls, 21.6 years (95%); and OND, 56.1 years (88%). (In some individuals age was not known: MS, n = 4; normal controls, n = 11; and OND, n = 1). All samples were stored at −70°C. The samples were heat-inactivated (56°C for 30 minutes) before use.

Antibodies

All monoclonal antibodies used were produced in this laboratory. The production and characterization of monoclonal antibodies against MHV-JHM[31] and MHV-A59[33] have been described pveviously. Detailed analyses of monoclonal antibodies reactive with HCV-229E and HCV-OC43 will be presented elsewhere (J.O.F., unpublished data, 1988). Although the

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monoclonal antibodies to HCV-229E are not yet fully characterized, radioimmunoprecipitations indicate that all three major structural coronavirus proteins (E1, E2, and N)[34] are recognized by our panel of monoclonal antibodies to MHV-JHM, MHV-A59, and HCV-OC43. Mouse antisera against MHV and HCV were prepared by hyperimmunization with viruses propagated in serum-free media.

Binding Assays

The relative binding of monoclonal antibodies to different coronaviruses was assessed by solid-phase radioimmunoassay,[31] as previously described. In each assay, initial studies were performed with polyvalent serum to ensure that different viruses were compared at equivalent antigenic densities.

To evaluate clinical samples, serum and CSF were tested in triplicate in at least two separate enzyme-linked immunosorbent assays (ELISAs).[33,35] Each value was corrected for background binding by subtracting the average optical density (OD) at 490 nm determined without antigen from the OD obtained when viral antigen was included in the assay. As epidemiologic surveys have indicated that HCV infections are common in the general population,[25,36] we were unable to obtain adult human serum that would serve as known negative reference standards. Instead, reactivities to VSV, which is not a human pathogen, were used to determine a positive-negative cutoff OD of 0.20. In ELISA testing with VSV antigen, we found that this value was approximately 3 SDs above the mean OD obtained with normal serum diluted 1:100 or normal CSF diluted 1:2. For each CSF sample positive for a virus by ELISA, the titer relative to the corresponding serum sample was determined by the multiple of normal activity method.[37] Serum and CSF albumin and IgG, as well as total intrathecal IgG synthesis, were measured as previously described.[38]

RESULTS

Antigenic Characteristics of Isolates SK and SD

Initial radioimmunoassays, in which all monoclonal antibodies were tested against the reference coronaviruses, showed little cross-reactivity between the anti-MHV and anti-HCV panels (Fig 1). Thus, only two of 25 anti-MHV monoclonal antibodies bound to either HCV-OC43 or HCV-229E, and none of 22 anti-HCV monoclonal antibodies, had significant binding to MHV-JHM or MHV-A59.

In view of the high species specificity of our reagents, we next sought to determine the antigenic characteristics of coronaviruses SK and SD, which had been isolated from patients with MS. Ten of 25 anti-MHV monoclonal antibodies reacted with SK, and 12 of 25 reacted with SD, indicating a high degree of cross-reactivity (Fig 1). By contrast, there was little reactivity between SK and SD and the panel of anti-HCV monoclonal antibodies (1/22 and 0/22, respectively). These results suggest that SK and SD are antigenically much more closely related to murine coronaviruses than to the two prototype HCVs. Further support for this assertion was provided in binding experiments with polyvalent MHV antisera, which reacted much more strongly with SK and SD than did National Institutes of Health reference HCV antisera (data not shown). Since SK and SD showed similar patterns of reactivity with the anti-MHV panel, it is likely that these isolates are closely related to each other.

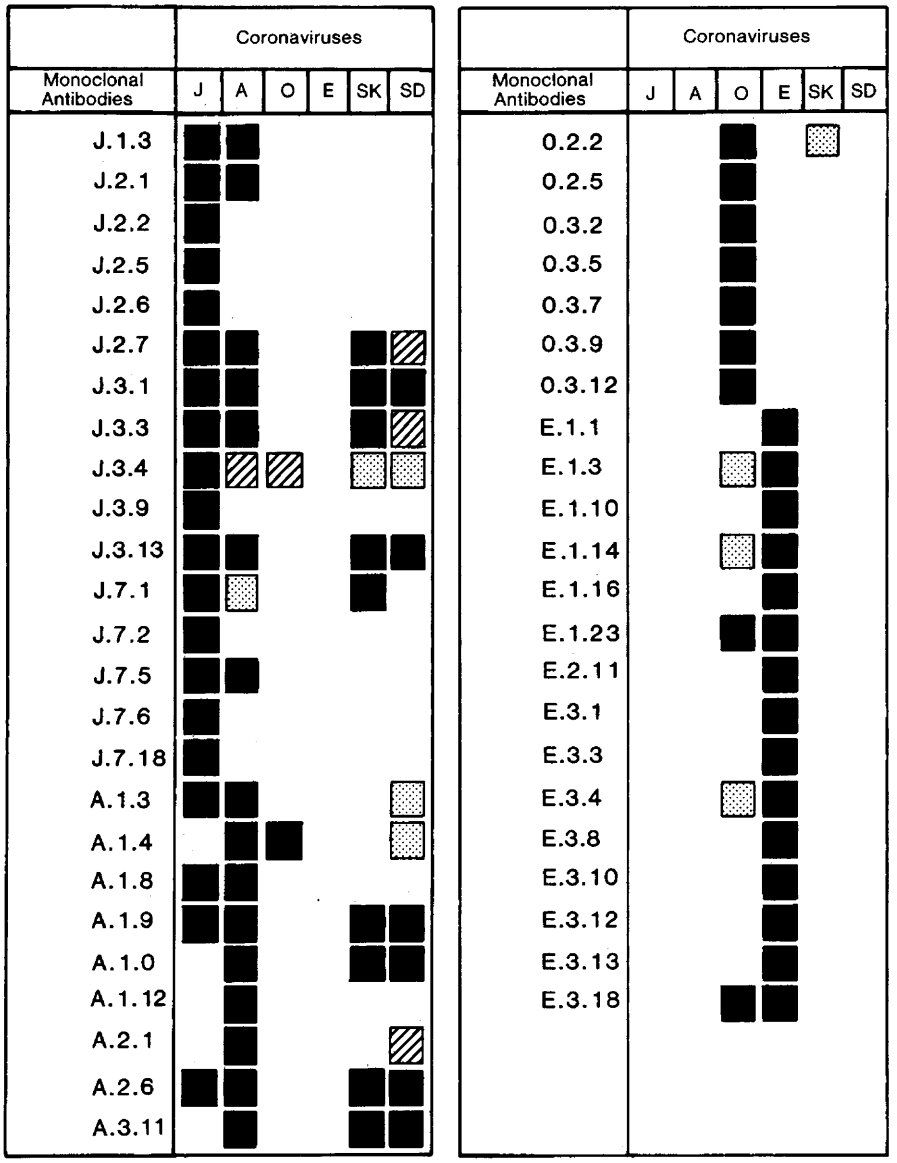

Fig 1.—Binding of monoclonal antibodies to prototypic coronaviruses and multiple sclerosis-derived isolates SK and SD by radioimmunoassay. J indicates mouse hepatic virus (MHV)-JHM; A, MHV-A59; O, human coronavirus (HCV)-OC43; and E, HCV-229E. First letter of each monoclonal antibody designation indicates immunizing virus used in production of that monoclonal. Squares that are closed, crosshatched, shaded, and open indicate strong, moderate, weak, and negative binding, respectively.[31]

Antibodies to Coronaviruses in Patients With MS and Controls

Serum and CSF antibodies to different coronaviruses were measured by ELISA. Because of the limited quantity of available CSF, we were not able to assess CSF antibodies to SK. In view of the data above, suggesting that SK and SD are similar antigenically, we did not think this omission had any decisive bearing on our investigations. The results of these assays are presented in Figs 2 and 3 and are summarized in Table 1. The most frequent and marked serum reactivities in all three patient groups were to HCV-OC43, as has been noted

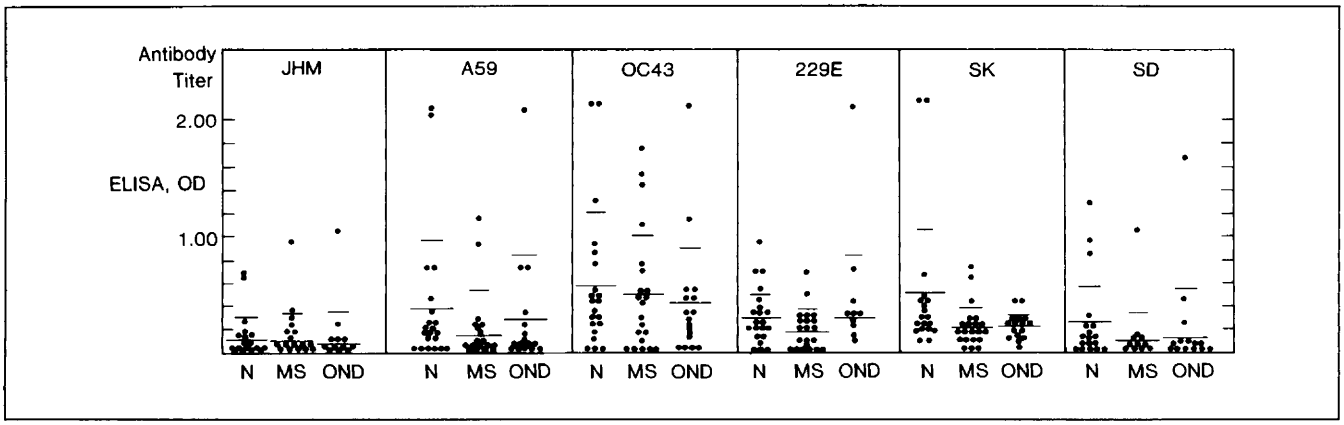

Fig 2.—Serum antibodies (1:100 dilution) to different coronaviruses. Mean enzyme-linked immunosorbent assay (ELISA) optical density (OD) and 1 SD are indicated by horizontal lines. Absorbances of greater than 0.20 OD units were considered positive. N indicates normal controls; MS, patients with multiple sclerosis; and OND, patients with other neurologic disease.

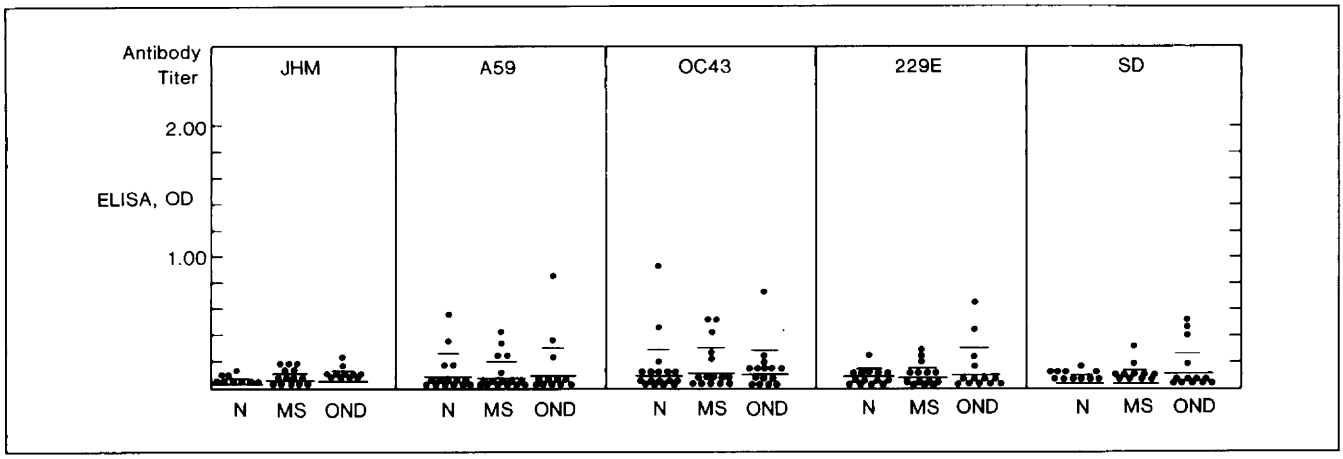

Fig 3.—Cerebrospinal fluid (1:2) antibodies to different coronaviruses. Titers are indicated as in Fig 2. See Fig 2 for explanation of abbreviations.

in most previous reports.[8,25,36] In general, serum antibodies reactive with either MHV-JHM or MHV-A59 were detected in a minority of patients. It is probable that these apparent anti-MHV antibodies may represent cross-reactivities to highly conserved antigenic determinants of HCV strains. In general, CSF antibodies to coronaviruses were infrequently detected and were usually of low titer (Fig 3).

In only two instances were antibodies to coronaviruses overrepresented in the MS group. In both cases, the magnitude of the finding was small and did not achieve statistical significance by the Fisher exact test for small samples. In the first case, serum antibodies to MHV-JHM were marginally more common in patients with MS (19%) than in normal subjects (10%) or patients with OND (12%) (Table 1). In the second case, 29% of patients with MS were found to have CSF antibodies to HCV-OC43, while only 14% of normal subjects and 12% of patients with OND had CSF antibodies reactive with this virus. While these data suggest increased HCV-OC43 reactivity of the CSF in patients with MS, the P values of these comparisons (.45 for MS vs normal; .26 for MS vs OND) are, as noted, statistically insignificant.

Analysis of individual patients revealed that only five of 21 with MS and two of 17 with OND showed a marked increase in CSF antibodies to any coronavirus (Table 2). In most instances, the highest titer of anticoronavirus antibody was directed against an HCV (data not shown). Also, most of these patients had a modest elevation in CSF albumin level, indicating a moderate degree of blood-brain-CSF-barrier breakdown; at present we have no explanation for these abnormalities in the patients with OND. As antibodies to coronaviruses were measured in relative ELISA titers and CSF IgG was measured in weight per volume units, we were unable to relate these two quantities directly. Nevertheless, it seems clear that high levels of intrathecal anticoronavirus antibodies did not consistently characterize our patients with MS as a group.

COMMENT

Recently, Murray and colleagues[27] used sensitive in situ hybridization techniques to demonstrate the presence of coronavirus SD in central nervous system tissue from patients with MS. In view of the controversy concerning the association of coronaviruses in general, and isolates SK and SD in particular, with MS, we sought to evaluate two hypotheses that might be predicted to be true if coronaviruses are etiologically involved in MS. These hypotheses concerned (1) the antigenic characteristics of isolates SK and SD and (2) the antibody responses of patients with MS to SK, SD, and other coronaviruses.

In the first case, coronaviruses typ-

Table 1.—Antibody Titers of Normal, MS, and OND Groups to Different Coronaviruses*

	MHV-JHM	MHV-A59	HCV-OC43	HCV-229E	SK	SD
			CSF			
Normal						
Mean ± SD OD	0.01 ± 0.02	0.08 ± 0.14	0.11 ± 0.20	0.10 ± 17	NT	0.02 ± 0.04
% positive	0	10	14	5	NT	0
MS						
Mean ± SD OD	0.04 ± 0.06	0.07 ± 0.13	0.15 ± 0.17	0.08 ± 0.9	NT	0.02 ± 0.08
% positive	0	14	29	14	NT	5
OND						
Mean ± SD OD	0.03 ± 0.17	0.09 ± 0.21	0.12 ± 0.17	0.10 ± 0.17	NT	0.08 ± 0.18
% positive	6	18	12	18	NT	12
			Serum			
Normal						
Mean ± SD OD	0.12 ± 0.18	0.39 ± 0.59	0.59 ± 0.61	0.31 ± 0.23	0.47 ± 0.58	0.24 ± 0.35
% positive	10	48	76	71	71	29
MS						
Mean ± SD OD	0.10 ± 0.22	0.16 ± 0.52	0.49 ± 0.52	0.19 ± 0.18	0.22 ± 0.17	0.08 ± 0.23
% positive	19	29	62	53	43	5
OND						
Mean ± SD OD	0.11 ± 0.25	0.27 ± 0.54	0.41 ± 0.53	0.31 ± 0.51	0.23 ± 0.10	0.16 ± 0.40
% positive	12	29	65	47	65	18

*All titers are expressed as optical density (OD) determinations by enzyme-linked immunosorbent assay. Positive individuals are expressed as a percentage of each group. MS indicates multiple sclerosis; OND, other neurologic disease; MHV, mouse hepatic virus; HCV, human coronavirus; and NT, not tested.

Table 2.—Patients With Marked Increase in Anticoronaviral CSF Antibodies*

Patient	Serum/CSF Anticoronavirus Antibody Ratio†	CSF Total IgG, mg/dL‡	CSF Albumin, mg/dL§	Intrathecal IgG Synthesis, mg/d‖
MS-9	140:1	7.6	47.4	14.3
MS-12	175:1	10.8	29.0	31.0
MS-14	200:1	9.6	50.0	9.7
MS-23	200:1	10.9	37.5	26.4
MS-24	130:1	21.6	80.0	38.5
OND-1 (Parkinson's disease)	200:1	16.4	90.0	6.9
OND-13 (seizure disorder)	200:1	17.5	105.0	15.7

*CSF indicates cerebrospinal fluid; MS, multiple sclerosis; and OND, other neurologic disease.

†Relative anticoronavirus titers, determined by enzyme-linked immunosorbent assay, as described in the text. In some instances, patients had reduced ratios to several coronaviruses; in these cases, the lowest ratio only is listed. A marked reduction in this ratio, eg, less than 200:1, is consistent with either blood-brain-CSF-barrier breakdown or intrathecal antibody synthesis.[37]

‡Normal values, 1 to 8 mg/dL.

§Normal values, 5 to 34 mg/dL.

‖As determined by the Tourtellotte formula.[37] Normally this value should be less than 3.0 mg/d.

ically have a relatively narrow range of hosts, ie, a given coronavirus will usually cause disease in a small number of closely related species.[39] Thus, it seems probable that a coronavirus causing MS would be of human origin and have the antigenic features of an HCV. Most HCVs have been isolated from respiratory tract infections and usually have been shown to be antigenically related to either HCV-OC43 or HCV-229E.[40,41] Recently, however, HCVs have been increasingly implicated in gastrointestinal tract infections,[42] and some of these enteric HCVs may be antigenically distinct from respiratory HCVs (J.O.F., unpublished data, 1988). Thus, our monoclonal antibody panel would be expected to recognize most, but not all, HCVs. A previous report on the antigenic properties of SK and SD used polyvalent serum in neutralization and radioimmunoprecipitation tests; this study was inconclusive and suggested serologic relatedness of SK and SD with MHV-A59, MHV-JHM, and HCV-OC43, but not HCV-229E.[43] To determine the antigenic characteristics of isolates SK and SD, we produced two panels of monoclonal antibodies. One panel was highly specific for MHVs and was found to react strongly with SK and SD. The other set of monoclonal antibodies was specific for HCVs and was shown to have essentially no reaction with SK and SD. Thus, our analysis of the antigenic characteristics of SK and SD is consistent with the findings of Weiss[10] at the nucleic acid level, which suggested a murine, rather than human, origin for these viruses.

In the second case, we reasoned that if SK, SD, or a closely related virus were etiologic agents of MS, patients with MS should show a particularly high degree of seropositivity to these antigens. In fact, serum and CSF antibodies to any coronavirus, including SK and SD, were not significantly elevated in the group of patients with MS when compared with normal subjects and patients with OND. Although we found a modest increase in the frequency of positive CSF responses to HCV-OC43 in our patients with MS, consistent with the previous report of Salmi et al,[5] in our survey this finding was not statistically significant. The difference between our observations and those of previous reports may be due to the number of patients tested, the sensitivities of the assays employed, or the criteria used to designate significant or positive antibody titers. In any event, no strong, specific serologic reaction of patients with MS to any of the coronaviruses, including SK and SD, has been established.

Logically, no set of experiments can absolutely rule out the possibility that coronaviruses (or any other agent) are etiologic in MS; as Johnson[1] pointed out, the "absence of evidence cannot be misconstrued as evidence of absence." Thus, the cause or causes of MS are unknown, and we cannot say with certainty what does not cause MS.[44] It is conceivable that MS may be caused by a coronavirus that is either of murine origin or from an uncharacterized HCV. In this regard, it has been shown that different coronaviruses recombine in vitro[45] and in vivo.[46] These recombinations occur at a high frequency,[47] and in some instances multiple crossover points are formed.[48] By analogy, it is possible that a genuine HCV persists in a highly attenuated form during chronic MS and was rescued during murine passage by recombination with an MHV residing in mouse cells. If this occurred, SK and SD would be expected to have variable amounts of both HCV and MHV RNA. Such an MHV-HCV recombination event in vivo or during isolation would be consistent with both our data and the hybridization study of Murray et al.[27]

In conclusion, the availability of the monoclonal antibodies presented herein provides a valuable means of characterizing SK and SD, as well as other viruses that may be isolated from human central nervous system tissue in the future. At present, however, the preponderance of evidence does not support an etiologic association of known coronavirus isolates with MS.

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