Pathogenesis of Coronavirus SD in Mice

I. Prominent Demyelination in the Absence of Infectious Virus Production

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• Following intracerebral inoculation of 3- to 4-week-old C57 B16/J mice with coronavirus SD, 23% exhibited neurologic signs within the first week. However, only 6% died. Within the first week after inoculation (AI), we noted a panencephalitis. Prominent demyelination detected in the spinal cord on day 6 continued through day 29 Al. Demyelinated lesions in the spinal cord were either subpial with few inflammatory cells except for macrophages or perivascular with prominent accumulation of lymphocytes, plasma cells, and macrophages. Beginning on day 6 Al, IgG was detected in the lesions. Although an infectious virus was detectable in the CNS only through day 12 Al. viral antigen expression continued through day 24. We concluded that coronavirus SD persists in a nonrecoverable form throughout the initial phase of demyelination, day 6 to day 24 Al.

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Human demyelinating diseases such as progressive multifocal leukoencephalopathy, subacute sclerosing panencephalitis, and postinfectious or postvaccinal encephalomyelitis are known to be initiated by viral

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agents.¹ A viral cause has also been suspected for one of the most common human demyelinating diseases, multiple sclerosis (MS).^{1,2} We previously reported the isolation of two coronaviruses while working with CNS tissue of two patients with MS.' Both isolates were closely related serologically to the human coronavirus OC43, as well as to the nonneurotrophic murine coronavirus A59.4 Following intracerebral inoculation, most murine produce coronaviruses dramatic, acute hepatitis.' However, the neurotrophic murine coronavirus strain JHM produces a chronic demyelinating, remyelinating disease following intracerebral (IC) inoculation of mice and rats.⁶⁻¹² It has also been reported that the three togaviruses-Semliki Forest virus,¹³ Ross River virus, and Venezuelan equine encephalomyelitis virus¹³—can cause demyelination in mice. Since coronavirus SD is antigenically distant from strain JHM, it was interesting to determine its pathogenesis in mice. We describe demyelination produced by coronavirus SD isolate following IC inoculation of weanling mice.

MATERIALS AND METHODS

We obtained 3- to 4-week old, male, specific pathogen-free C57 B16/J mice. All animals were maintained under specific pathogen-free conditions throughout the study. Uninoculated control mice, housed with the study animals, served as monitors of these conditions.

Neutralizing antibody was detected by the 50% plaque neutralization assay on delayed brain tumor (DBT) cells.⁴ Serum was pooled at the time of collection, inactivated at 56 °C for 30 minutes, stored at -70°C, and thawed at the time of assay. Serum from control mice, preimmune mice, and fetal calves was run for negative controls. All serum was run in duplicate. A total of 110 infected mice at 24 time points (average, 4.6 mice) and 32 control mice at 17 time points (average, 1.9 mice) were tested. Preimmune serum titers to coronavirus SD were undetectable.

We inoculated 388 mice IC with 10,000 to 100,000 plaque-forming units (0.03 mL) of plaque-purified SD virus. An additional 137 mice were inoculated IC with uninfected DBT cell extract. All inoculations were performed using ether anesthesia.

For viral isolation studies, two or three mice were killed at each time point. Blood and tissue were collected while the mice were under ether anesthesia. Whole blood and tissues were immediately ground with a mortar and pestle on ice and suspended to 10% in Hanks' balanced salt solution supplemented with penicillin and streptomycin. Suspensions were clarified by centrifugation at 12,800 g for three minutes and stored at -70 °C. Supernatant virus was detected by plaque assay on DBT cells.⁴ Brain and spinal cord tissue was minced and co-cultivated on monolayers of DBT cells or cultured directly for later cell fusion with 3T3 (17Cl-1) cells.¹³

For in situ viral antigen studies, brains and spinal cords were immediately snap frozen in a dry ice-alcohol bath and stored at -70 °C. Four-micrometer cryostat sections were fixed in acetone for ten minutes at -20 °C and air dried. The sections were soaked for ten minutes in phosphate-buffered saline (PBS) solution with a pH of 7.0. Preimmune or viral specific antisera⁴ were applied (50 µL). Siliconized coverglasses were added and the primary antisera allowed to react for 30 minutes at room temperature in a humidified chamber. Coverglasses were then gently soaked off. Following two ten-minute rinses in PBS, 50 µL of either fluorescein-conjugated sheep anti-guinea pig IgG, Staphylococcus aureus protein A labeled with iodine 125 (50,000 counts per minute per section), or rabbit anti-guinea pig IgG 1251

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Fig 1.—Infectious virus detected in 10% homogenates of brain (squares), spinal cord (triangles), and spleen (circles) by plaque assay on delayed brain tumor cells after intracerebral inoculation of SD virus in wean-ling C57 B16/J mice. Al indicates after inoculation; PFU, plaque-forming units.

(50,000 counts per minute per section) were added. Coverglasses were placed on the sections, and labeling continued for 30 minutes at room temperature. Protein A ¹²³I had a specific activity of 60 mCi/mg.

Rabbit anti-guinea pig IgG was iodinated using an iodogenic method17 and free iodine was removed using desalting columns as described by Christopherson et al.¹⁸ Average specific activity of iodinated rabbit anti-guinea pig IgG was from 1 million to 10 million counts per minute per microgram of protein. For autoradiographic techniques, unreacted iodine was removed by two ten-minute washes in PBS. followed by an overnight rinse at 4 °C in 4 L of PBS. Sections were then dehydrated with two ten-minute washes of 70% alcohol and two ten-minute washes of 95% alcohol, air dried, and stored at 4 °C in sealed slide boxes containing anhydrous calcium sulfate. For autoradiography, the sections were soaked for ten minutes in PBS and dipped at 42 °C in photographic emulsion (NTB-2) diluted 1:1 in distilled water containing 2% glycerol. Our autoradiographic antigen detection methods were described in detail elsewhere¹⁹ and were similar to those of Moar et al.20 Following a seven-day exposure at 4 °C, slides were developed, rinsed, and fixed. Tissue sections were stained using a modified hematoxylin-eosin procedure as described by Baserga and Malamud.²¹

For histopathologic studies, anesthetized mice underwent perfusion through the left ventricle of the heart with buffered formaldehyde solution until blanching of the liver occurred. Representative tissues from all major organ systems were removed and stored in excess perfusate at room temperature. They were then embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin-eosin for routine screening. Serial sections of the brain and spinal cord were also stained with Luxol fast blue PAS-hematoxylin, phosphotungstic acid-hematoxylin, and the Bodian method.

Mice processed for electron microscopy



Fig 2.—Autoradiographic detection of viral antigen in focal areas of increased cellularity in temporal cortex of infected mouse four days after inoculation (AI). Top left, Light microscopy (original magnification ×410). Top right, Darkfield microscopy (original magnification ×410). Bottom, Background level of exposed silver grains observed by light microscopy (left) and darkfield microscopy (right). This section was reacted with preimmune guinea pig antiserum and rabbit anti-guinea pig IgG labeled with iodine 125, then exposed for seven days prior to development. Similar background levels were obtained on uninfected tissue reacted with guinea pig antiviral antisera and rabbit anti-guinea pig IgG ¹²⁶ or sections of brain or spinal cord reacted with protein A ¹²⁶ (original magnification ×410).

were first heparinized with 10 units. Each animal was perfused with 5 mL of cacodylate buffer (pH, 7.3), followed by 30 mL of half-strength Karnovsky's fixative at room temperature. Selected tissues were removed, diced to 1-mm cubes, and stored in excess perfusate overnight at 4 °C. Tissue blocks were postfixed in 1% osmium tetroxide and 1.5% potassium ferricyanide²² for one hour. Dehydrated tissues were subsequently embedded in epoxy resin. Thin sections were stained with uranyl acetate-lead citrate.

RESULTS

Clinical signs were first observed between days 4 and 9 AI, during which 91 (23.4%) of 388 infected animals displayed either extreme hyperexcitability or hind limb paresis. Twenty-

five (6.4%) of the infected mice exhibiting symptoms died during this first stage. Subsequently, all mice became free of clinical signs, except for continued growth retardation in a few recovered animals, until day 30 AI. From day 30 to day 60 AI, 26 (26.8%) of 97 infected mice had clinical evidence of hind limb anesthesia and/or paresis. Hind limb anesthesia was shown by a lack of response to light touch or pinprick sensation, whereas paresis was noted when animals were weak and dragged their hind limbs. Of these animals, 12 eventually became moribund and died. Only six had evidence of previous disease between days 4 and 9 AI. Neutralizing antibody to coronavirus SD was detect-

Histopathology Following Intracerebral Inoculation of Coronavirus SD*									
· · · · · · · · · · · · · · · · · · ·	Days After Inoculation								
	1-5	6-10	11-15	16-20	21-25	26-30	31-60	61-90	91-120
No. of mice studied (No. of controls)	22 (10)	20 (10)	16 (8)	6 (3)	3 (1)	6 (3)	10 (5)	2 (1)	6 (2)
Histopatologic evidence, No. (%) Meningitis				a (a)	A (A)	- (a)		a (a)	- (-)
Cerebral	20 (90.1)	15 (75)	2 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Encephalitis	18 (82)	20 (100)	14 (87.5)	1 (17)	2 (33)	0 (0)	0 (0)	0 (0)	0 (0)
Myelitis	16 (72)	17 (85)	7 (44)	4 (67)	1 (33)	2 (33)	0 (0)	0 (0)	0 (0)
Demyelination†	0 (0)	6 (30)	9 (56)	4 (67)	1 (33)	2 (33)	0 (0)	0 (0)	0 (0)
Hydrocephalus	0 (0)	0 (0)	5 (31)	3 (50)	2 (67)	3 (50)	4 (40)	1 (50)	0 (0)

*Numbers represent the number of animals for which the given lesion was observed.

The frequency of demyelination is that observed in longitudinal sections of formaldehyde solution-fixed, paraffin-embedded spinal cord. Extensive examination of 1-µm cross sections of epoxy resin-embedded spinal cord showed demyelination in two of four mice on day 6 after inoculation, four of four on day 8, four of four on day 12, four of four on day 28, and two of five on day 90.

able beginning on day 4 AI. The 50% plaque neutralizing titer increased to a mean of 1:2,560 by day 9 AI, remaining at this level for the duration of the study. Neutralizing antibody was never detected in control animals.

Infectious virus was recoverable in high titer from the brain and spinal cord, and in considerably reduced titer from the spleen and liver for the first four days AI (Fig 1). Virus could not be isolated at any time from the blood, heart, lung, thymus, kidney, or duodenum. Infectious virus was not detected in the liver by day 5, the spleen by day 6, and the brain and spinal cord by day 7. Virus was detectable by co-cultivation of minced brain tissue on either DBT or 3T3 (17Cl-1) cells as late as day 12 AI, after which all methods of isolation, tissue homogenate, co-cultivation, and cell fusion proved unsuccessful. An extensive search for viral particles by electron microscopy revealed little except an occasional viral particle in necrotic cell debris of the temporal cortex on days 4 through 6 AI.

Evidence of viral antigenic expression was seen in sections of brains and spinal cords by indirect immunofluorescence for the first ten days AI. Until day 24 AI, virus could be detected using anti-guinea pig IgG 125I and autoradiography. Viral antigen was widely distributed in the meninges and predominated in the white and gray matter of the brain and spinal cord. However, distribution tended to be heaviest and persisted the longest in temporal cortex gray matter of the brain (Fig 2) and in spinal cord white matter. Using protein A ¹²⁵I, IgG was found to infiltrate brain and spinal cord lesions beginning on day 6 AI. Serial sections of spinal cord lesions showed both IgG and viral antigen expression in identical lesions. Prior to day 6, viral antigen but no IgG was found in areas of inflammation.

To determine if viral antigen expression represented persistence of infectious virus or expression of a latent or defective virus, the frozen tissue blocks adjacent to antigen-positive areas were thawed, minced, and co-cultivated with 17Cl-1 cells. With this method, infectious virus could be detected through day 12 AI, but it was not recovered from any tissue blocks between days 12 and 24 AI.

Histopathology of the Brain

Histopathologic evidence of CNS involvement was encountered in 95% of infected mice examined during the first 29 days AI (Table). A light cellular infiltrate predominantly composed of neutrophils and occasional multinucleate syncytial cells was seen in the cerebral meninges and near the inoculation wound in the left cerebral hemisphere on day 1 AI. Both the hippocampus and temporal cortex were prominently involved by day 2 AI. Neuronal necrosis was striking, especially in the temporal cortex, and both areas were heavily infiltrated by neutrophils. Acute panencephalitis developed in animals examined on day 4 AI and persisted through day 8. By day 9, the inflammatory cell response in the brain was limited to the temporal cortex and its overlying meninges. The nature of the response had changed in that mononuclear cells now predominated and neuroglial nodules were evident. Portions of the temporal lobes, which on day 7 AI were acutely necrotic, underwent liquefaction on days 9 through 13 AI. Mineral deposits were occasionally observed in the temporal cortex beginning on day 12 AI. The destructive changes in the temporal cortex led to the development of compensatory hydrocephalus. During this early

period, myelin destruction was noted, primarily in the temporal cortex.

Histopathology of the Spinal Cord

Although inflammatory lesions were found in the spinal cord and the surrounding meninges from days 1 through 17 AI, they lacked the intensity of the cerebral lesions and remained focal. Demyelination was a striking feature that was first observed on day 6 AI and persisted until day 29 AI. Initial lesions were small and subpial. By day 17 AI, large areas of demyelination extended over several vertebral segments (Fig 3, top). Subpial edematous lesions were not usually associated with inflammatory cells other than macrophages. By electron microscopy, myelin stripping by these macrophages was noted (Fig 4, bottom right). Other lesions, particularly prominent at day 12 AI, tended to be larger and deeper and were adjacent to prominently cuffed vessels (Fig 3, bottom). The perivascular cuffs consisted of lymphocytes, macrophages, and plasma cells (Fig 4, top and bottom left). In areas of both types of demyelination, axon preservation was shown by both light and electron microscopy (Fig 3, top right, and Fig 4, bottom left).

COMMENT

Following IC inoculation of coronavirus SD into weanling mice, prominent foci of demyelination were detected in the spinal cord. The development of demyelinating lesions following infection was especially interesting since coronavirus SD was isolated while we were working with MS autopsy brain material,³ and demyelination occurred in the absence of infectious virus production.³

Although the cause of MS is unknown, considerable evidence suggests viral involvement in its patho-



Fig 3.—Demyelination in spinal cord following intracerebral inoculation of coronavirus SD in weanling mice. Top left, Longitudinal section of diffuse spinal cord demyelination 13 days after inoculation (AI) (hematoxylin-eosin, original magnification ×40). Top right, Longitudinal section of focal spinal cord demyelination with numerous gitter cells and well-preserved axons 20 days AI (Bodian method, ×40). Bottom left, Cross section showing perivascular demyelination and mononuclear cell cuff in spinal cord white matter 12 days AI. Embedded in epoxy resin (toluidine blue, original magnification ×164). Bottom right, Mononuclear cell cuff and adjacent demyelination in the spinal cord wite matter 12 days AI.

genesis.^{1,2} Coronaviruses have been specifically implicated as a possible cause since (1) Tanaka et al²³ found coronaviruslike particles by electron microscopy in the brain tissue of one patient with MS; (2) coronaviruses JHM and SD are capable of causing demyelinating disease in mice⁶⁻¹²; (3) coronaviruses were isolated while working with fresh autopsy material from two patients³; (4) serologic investigations showed elevated CSF coronavirus antibody levels in patients with $MS^{24,25}$; and (5) coronaviruses were isolated from the CSF of patients suffering from an acute encephalitic syndrome.²⁶ While a role for coronavirus SD in the pathogenesis of MS has yet to be established, a comparison of SD-induced demyelination in mice with MS and other virus-induced demyelination in animal models is appropriate.

The characteristic lesions of MS are focal areas of demyelination in which axons appear relatively well preserved and oligodendroglia are destroyed.²⁷ Acute lesions often appear edematous and may be associated with perivascular accumulations of lymphocytes, plasma cells, and macrophages. Lipid-filled macrophages are numerous, especially at the margins of acute lesions. Although the distribution of lesions varies widely within the CNS, they are typically located in subpial, subependymal, and/or perivascular areas. $^{\rm 27}$

One theory of the cause and pathogenesis of MS postulates that it results from an immunopathologic process in genetically predisposed persons in response to a viral infection. Evidence of an immunopathologic process consists of observation of perivascular lymphoid cells in acute lesions,²⁷ decreased numbers of circulating suppressor T lymphocytes during exacerbations,²⁸ accumulation of immunoglobulin associated with demyelinated lesions,²⁹ and the presence of elevated oligoclonal IgG in the CSF of patients with MS.²⁵

Multiple sclerosis is a disease unique to humans. In fact, naturally occurring demyelinating diseases of animals are rare. Animal models of demyelination depend on the viral strain involved, the age and strain of animal infected, and the inoculation route.^{6-12,31} Two distinct mechanisms of demyelination have been demonstrated. One involves a direct viral cytolysis of oligodendroglia, the second an immunopathologic process.

Murine hepatitis viral infection (strain JHM) is the main example of demyelination resulting from viral cytolysis of oligodendroglia. Characteristically, lesions appear as randomly distributed foci of demyelination lacking inflammatory cells other than lipid-filled macrophages. Immunoglobulin G is absent from these lesions; however, viral antigen is abundant. By electron microscopy, viral particles are recognized in oligodendroglia.⁹ Immunosuppression does not prevent demyelination.¹¹

Intracerebral inoculation of mice with the DA strain of Theiler's virus,³² selected temperature-sensitive mutants of vesicular stomatitis virus,³³ or Chandipura vesiculovirus³⁴ results in a prominent mononuclear inflammatory reaction adjacent to foci of demyelination in the spinal cord. The cell bodies of oligodendroglia are unaffected. Immunosuppression reduces the inflammatory reaction and demyelination.³² Viral particles are usually not observed by electron microscopy.

The primary demyelination following inoculation of coronavirus SD is morphologically similar to that of acute lesions in MS. The demyelinating lesions are subpial or perivascular. Mononuclear and prominent IgG infiltration occur concomitantly with demyelination. The pathogenesis observed indicates that both oligodendroglia destruction and subsequent immunopathologic reactions may occur. Virologic studies during the peri-



Fig 4.—Electron micrograph showing perivascular cell infiltration and phagocytic myelin stripping. Top left, Mononuclear cells migrating into perivascular area of demyelination 12 days after inoculation (AI), including plasma cell (lower right corner) and macrophage containing lipid vesicles and myelin debris (×4,205). Top right, Plasma cells and demyelinated axon in adjacent area (×4,205). Bottom left, Area of demyelination 12 days AI with numerous denuded axons (×3,472). Bottom right, Phagocytic cell actively stripping myelin from axon on day 8 AI (×3,413).

od of demyelination indicate that the virus persists in a form not readily recoverable. However, demyelination is associated with a low level of viral antigenic expression. Despite extensive electron microscopy, viral particles have not been observed in brain or spinal cord cells following coronavirus SD infection.

Regarding the possible role of coronavirus SD in MS, the observation

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that this virus persists in a nonrecoverable state during the period of murine demyelination suggests that virus isolation may not be the best approach for demonstrating viral information in human tissue. Standard antigen detection methods such as immunofluorescence also may lack sensitivity for viral antigen expression. Therefore, the definitive method for establishing a coronavirus etiolo-

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gy for MS will require the direct demonstration of viral antigen or nucleic acid in MS tissue. In addition, tissue from autopsies in controls should be negative for viral genome.

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