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Detection of Tissue Culture-Adapted Theiler's Virus RNA in Spinal Cord White Matter Cells Throughout Infection

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The appearance of histological lesions and the localization of viral RNA in the central nervous system of mice infected with tissue culture-adapted Theiler's murine encephalomyelitis virus (WW strain) (TMEV-WW) was studied. Viral RNA was detected by autoradiography after in situ hybridization, using a ^{3}H-labeled DNA probe complementary to virion RNA, which was applied to deparaffinized sections of central nervous system tissues from infected mice. Subjacent histological sections of tissues were used to assess the location and extent of lesions. Lesions were first observed at 20 days post-inoculation and appeared to enlarge throughout infection. They consisted of infiltrates of mononuclear cells and lymphocytes in spinal cord white matter and leptomeninges; at 78 days post-inoculation severe necrotizing and demyelinative myelitis and gliosis were observed. In contrast to the pathogenesis of brain-derived TMEV-WW-infected mice, no lesions were found in the central nervous system gray matter of mice infected with tissue culture-adapted TMEV-WW at any time post-infection. Tissue culture-adapted viral RNA was found in the cells of spinal cord white matter throughout infection; only one neuron in close proximity to the injection site was found to contain viral RNA shortly after infection. At early times after infection, spinal cord white matter cells containing viral RNA were found before development of inflammatory lesions; at later days post-inoculation, positive cells were found within, at the periphery of, or at a distance from lesions. The number of infected cells and the amount of viral RNA per cell appeared to remain constant from 20 to 78 days post-inoculation despite the increasing intensity of the inflammatory response. The nearly exclusive spinal cord white matter tropism of tissue culture-adapted TMEV-WW appeared to directly correlate with the disease-inducing potential of this virus.

Theiler's murine encephalomyelitis viruses (TMEV) comprise a group of picornaviruses (13, 17) capable of producing central nervous system (CNS) diseases (17a). One group of brain-derived TO and TO-like TMEV, including the DA (5), TO (19, 20), BeAn8386, Yale (8, 9), and WW (21) strains can produce two sequential phases of CNS disease in weanling mice inoculated by the intracranial route. The first phase is an acute polioencephalomyelitis, which is manifested by hind limb paralysis and occasionally by encephalitis; signs begin within a few days post-inoculation (dpi) (5, 6, 17, 20). The survivors of the acute phase enter the second, or chronic, phase of CNS disease at about 30 dpi; this phase is a chronic, progressive necrotizing or demyelinative myelitis (or both) (5, 6). In those mice that do not exhibit residual atrophy from the acute phase, the chronic phase is characterized by an unusual waddling gait (6). The acute phase is characterized by production of high titers of free virus in the CNS; viral titers begin to decrease at about the time serum-neutralizing antibodies begin to appear (6). During the chronic phase, lower levels of free virus persist despite an inflammatory response in the CNS and persistently high serum titers of neutralizing antibody. Fluorescent-antibody studies have demonstrated viral antigens in brain and spinal cord gray matter during the acute phase (6), but have been inconclusive in demonstrating the source of the persistent virus during the chronic phase. Similarly, electron microscopic studies of chronically infected mice have not revealed CNS cells containing viral crystalline arrays. However, viral arrays were observed both in neurons and in oligodendrocytes of suckling mice during the acute phase of the disease after infection with the WW strain (15). These arrays probably correlate with the inclusions observed with this strain by light microscopy (21). By using a highly

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sensitive in situ hybridization assay (1), we recently showed that viral RNA is present in large amounts in neurons of the entire neuraxis during the acute phase and in small amounts in cells of the spinal cord white matter during both the acute and chronic demyelinating phase (2).

Once the TO and TO-like brain-derived TMEV are adapted to cell culture, a process requiring multiple blind subpassages (7), the encephalomyelitic potential is completely attenuated, yet the demyelinative potential of the virus is preserved (12). Upon intracranial inoculation of mice with tissue culture-adapted viruses, no clinical signs or histological lesions characteristic of acute disease have been observed. However, after a prolonged period, animals develop a waddling gait and demyelinating or necrotizing (or both) lesions of spinal cord white matter (12).

In this report, we describe the localization of viral RNA after intracranial inoculation of tissue culture-adapted TMEV-WW by in situ hybridization. In contrast to the localization of viral RNA after infection of mice with brain-derived TMEV-WW (2), we demonstrate that tissue culture-adapted viral RNA is found almost exclusively in cells of spinal cord white matter. Possible explanations for the pathogenic differences between brain-derived and tissue culture-adapted TMEV are discussed.

MATERIALS AND METHODS

Viruses and animals. A single stock of brain-derived TMEV-WW, prepared as previously described (17), was adapted to grow in BHK-21(C-13) cells (ATCC CCL-10) by the blind subpassage method described by Lipton (7). Adaptation was achieved after the fourth subpassage, as defined by the production of cytopathic effect. A single final stock of tissue culture-adapted virus representing the fifth passage in cell culture was prepared by infecting cells at a multiplicity of infection of 0.1 to 1.0 and harvesting at 4 dpi by freezing and thawing monolayers of the cells three times. The infectious tissue culture fluid was clarified of cellular debris by centrifugation at 400 × *g* for 10 min. The final stock contained 10^6 PFU/ml as titered by the standard plaque assay described by Lipton (7) and by a tissue culture infectious dose assay. Fifty percent endpoints were calculated by the methods of Reed and Muench (16). Virus was stored at −70° until used.

A stock of the GDVII strain of TMEV (TMEV-GDVII) was grown in BHK-21(C-13) cells as previously described (18) and contained 10^7 PFU of clarified culture fluid per ml. When large quantities of virus were required to prepare viral RNA, BHK-21(C-13) cells were infected at a multiplicity of infection of 10 PFU per cell and harvested between 18 and 36 h postinfection, when maximum cytopathic effect developed.

ICR mice (19 to 21 days old), from Flow Laboratories, Dublin, Va., were inoculated intracranially in the left hemisphere with 20 μl of TMEV-WW containing $10^{4.3}$ PFU. Animals were observed at intervals after infection for signs of encephalitis (lethargy, ruffled fur, cachexia, circling behavior), paresis, flaccid paralysis, and the characteristic waddling gait observed in chronically TMEV-infected mice. At intervals after infection, three to six mice were anesthetized with ether, subjected to thoracotomy, and perfused transcardially with normal saline followed by a solution of 75% ethanol–25% glacial acetic acid. CNS tissues were immediately dissected and immersion fixed for 12 to 24 h. CNS tissues were blocked and embedded in paraffin for sectioning. The temperature of the paraffin was maintained at 60°C, and the tissue blocks were embedded within 1h. These steps were taken to ensure minimal heat degradation of viral RNA. Control mice were inoculated with 20 μl of Hanks balanced salt solution, sacrificed, and processed as described above.

Histology. Sections from the tissue blocks were prepared for routine histology and stained with hematoxylin and eosin stain (H & E).

Preparation of the [^{3}H]cDNA probe. TMEV-GDVII RNA was used as a template for the reverse transcription of the [^{3}H]cDNA probe. TMEV-GDVII was purified, and the RNA was extracted in 1% sodium dodecyl sulfate and 100 μg of proteinase K per ml as previously described (18). The [^{3}H]cDNA probe was synthesized complementary to viral RNA as described previously (2, 18). The probe had a specific activity of 2.5×10^5 dpm/ng.

In situ hybridization. Subjacent sections from selected tissue blocks sectioned for histological examination were used for the hybridization studies. The procedures for preparation of the tissue sections and the protocol for hybridization have been described in detail elsewhere (1, 2, 18). However, for these studies, the amount of hybridization mixture used was calculated from the surface area of the tissue to be hybridized to ultimately provide 4.42 μl of mixture, containing 0.13 ng of [^{3}H]cDNA per μl per cm^2 of cover slip. Hybridization was allowed to proceed to a C_0t of 0.57 mol·s/liter. After hybridization, the slides were washed, dehydrated, dipped in Kodak NTB-2 liquid emulsion, developed 1 or 3 weeks later, and stained with H & E as described previously (2, 18).

RESULTS

Clinical course. Weanling mice inoculated with $10^{4.3}$ PFU of tissue culture-adapted TMEV-WW appeared normal until 40 dpi, when about 1% of the mice began to exhibit a slight hind limb gait disturbance. When present, the gait disorder was best observed by forcing infected and control animals to climb an inclined test tube rack. The limb disturbance of infected mice was characterized by a failure to abduct the hind limbs during climbing. However, the disorder was often subtle. No progression of clinical status was noted in affected mice not sacrificed for studies over the nearly 3-month observation period. Mice were sacrificed for histological examination and hybridization studies without regard to clinical status.

Histology and in situ hybridization. (i) Histopathology. At 7 and 11 dpi, a few animals had small perivascular cuffs of mononuclear cells near the

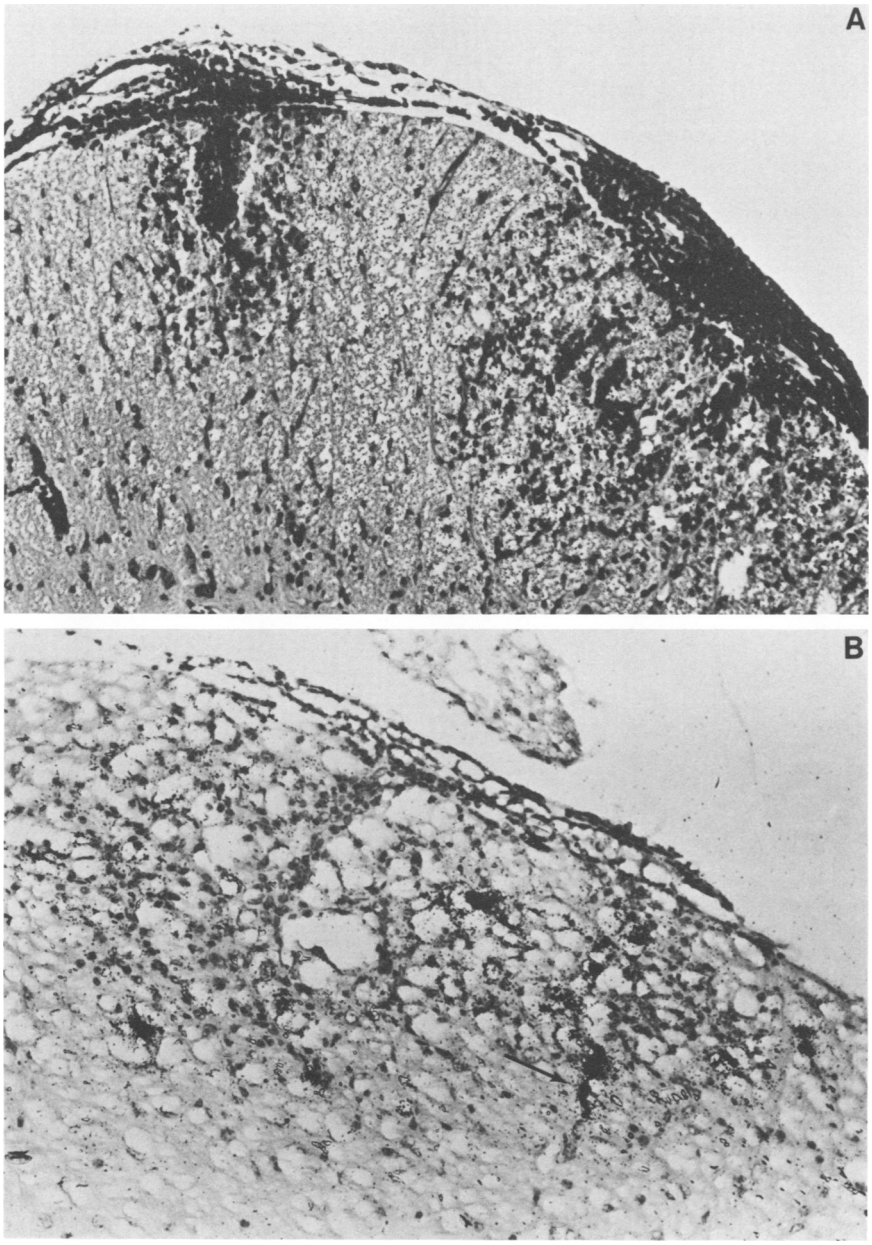

FIG. 1. Histopathology and localization of viral RNA in the spinal cord of a mouse sacrificed at 20 dpi with tissue culture-adapted TMEV-WW. (A) The leptomeninges were severely infiltrated with lymphocytes and mononocytes (top). Inflammation of the white matter parenchyma also occurred and appeared to follow nerve tracts toward the grey matter (bottom). Some perivascular infiltrates were observed at the boundary of the grey and white matter (bottom left). The anterior aspect of the spinal cord is at the right of the figure (H & E, ×63). (B) Detection of viral RNA in a spinal cord section subjacent to A by in situ hybridization, using a TMEV-specific [^{3}H]cDNA probe. Several infected cells were detected within and at the periphery of lesions. The autoradiographic grains occasionally followed nerve tracts (arrow). Many infected cells had extensive cytoplasmic processes (H & E, ×250). Autoradiographic exposure was for 3 weeks.

injection site and in the hippocampal fissure, but the characteristic lesions of polioencephalomyelitis (microglial nodules and neuronophagia) were never seen; no lesions were observed in brain or spinal cord white matter. Inflammatory lesions in the CNS were limited to the spinal cord white matter and leptomeninges and were first detected at 20 dpi in two of the six animals

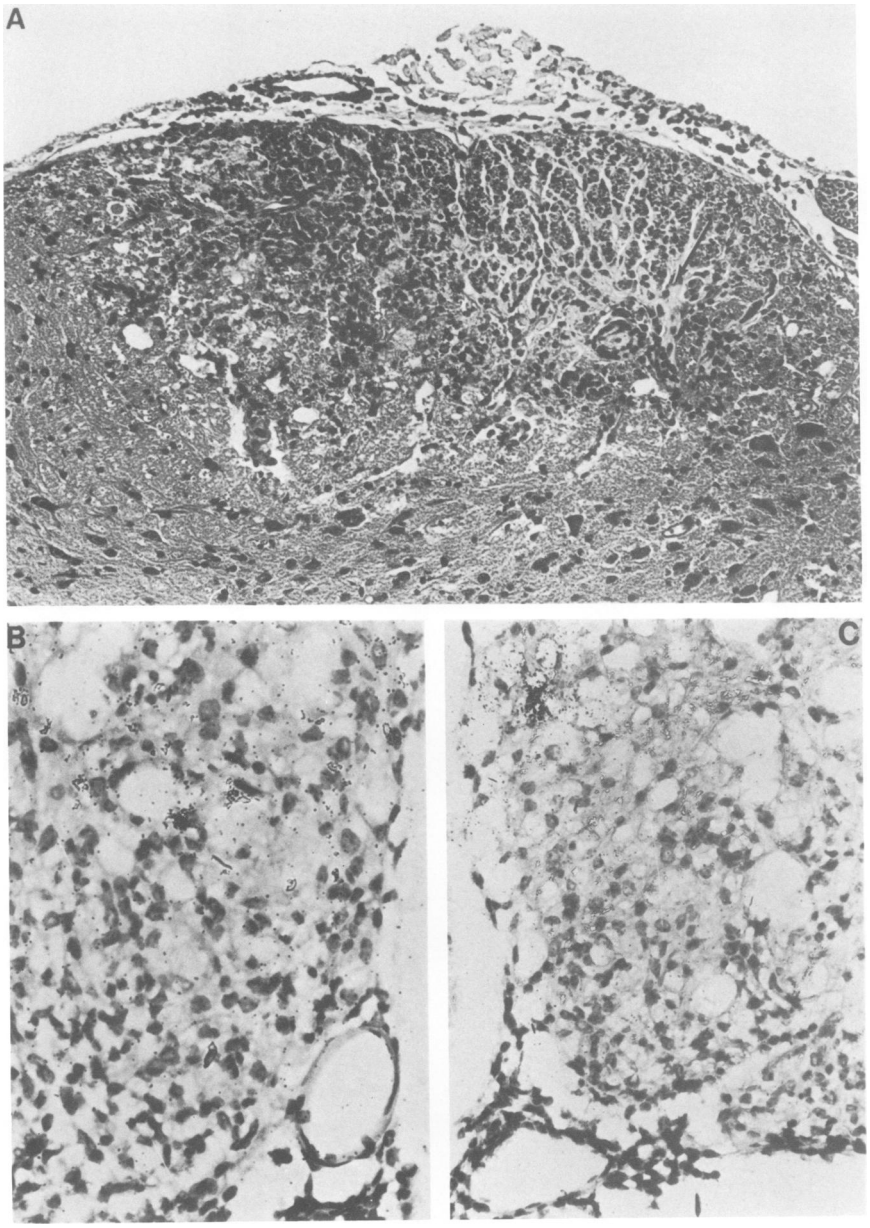

FIG. 2. Histopathology and localization of viral RNA in the spinal cord of a mouse at 78 dpi with tissue culture-adapted TMEV-WW. (A) The lateral white matter was severely inflamed. Numerous monocytes and lymphocytes were present in the white matter and leptomeninges. The lesion exhibited gliosis (arrow). The grey matter (bottom) is uninvolved. The posterior aspect of the spinal cord is to the left of A (not shown) (H & E, ×63). Viral RNA was detected in spinal cord cells of sections subjacent to A by in situ hybridization, using a TMEV-specific [^{3}H]cDNA probe (B and C). Infected cells were found at the periphery of inflammatory foci (B and C); some of these cells had extensive processes that contained autoradiographic grains (C). Autoradiographic exposure was for 3 weeks. The cells shown in B and C are adjacent to the anterior median sulcus (H & E, ×250).

sacrificed (33%) (Fig. 1A); lesions were easily detected in all of the mice sacrificed up to 78 dpi (Fig. 2A), the last examination point. Lesions were found at multiple levels of the spinal cord, although most of the largest lesions were found in the cervical-upper thoracic and lumbar-sacral segments. It is of interest that the animal shown in Fig. 2A had a barely detectable gait distur-

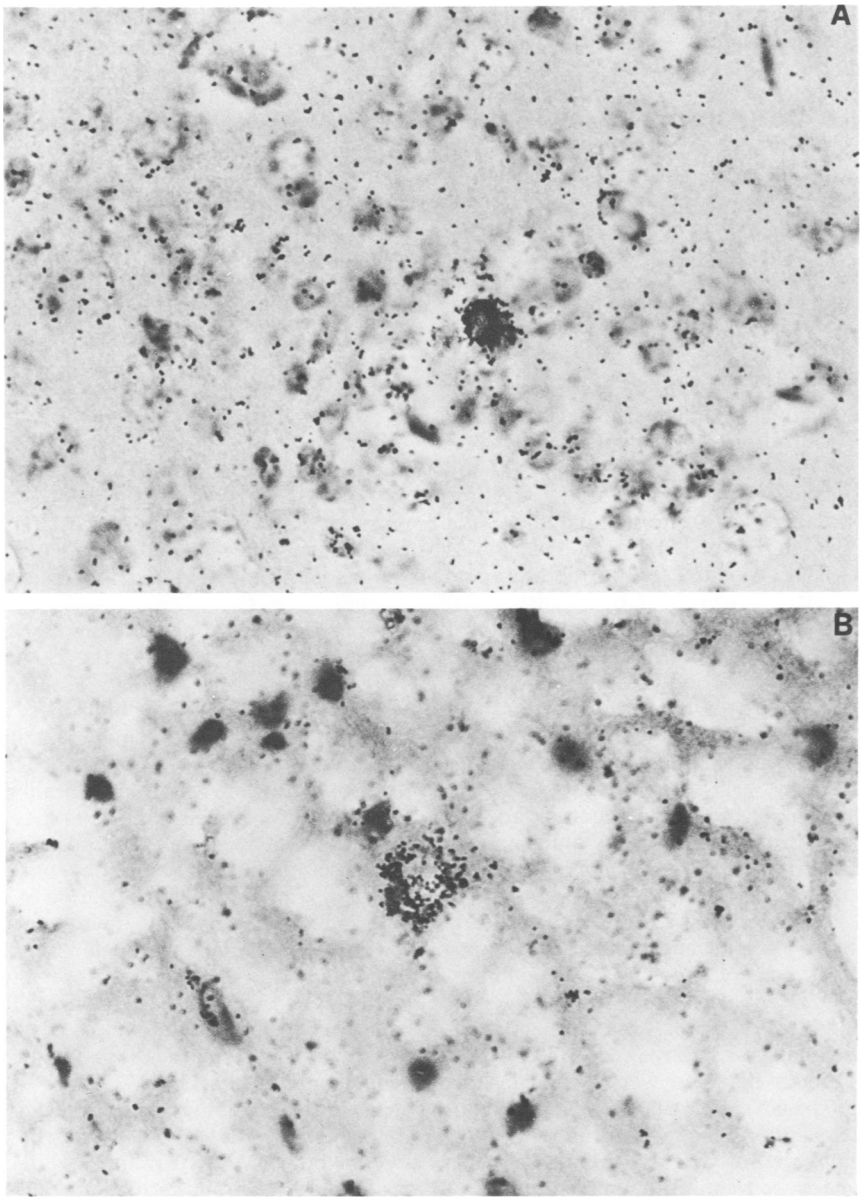

FIG. 3. Detection of tissue culture-adapted TMEV-WW in the CNS of a mouse sacrificed at 7 dpi. Tissue sections were hybridized in situ, with a TMEV-specific [^{3}H]cDNA probe. (A) Infected neuron of cerebral cortical layer III superior to the injection site. This was the only neuron containing viral RNA found at any time postinfection (H & E, ×400). (B) Infected cell in the white matter of the lateral column of the spinal cord. No inflammatory cells were observed in any section examined (H & E, ×400). Autoradiographic exposure was for 3 weeks.

bance, despite the large inflammatory and necrotic lesion in the spinal cord. In general, no definite correlation between clinical status and pathological lesions was apparent. Even though the spinal cord lesions appeared to increase in size and severity throughout the infection, the clinical status of infected mice did not detectably deteriorate. Gliosis was observed in one animal sacrificed at 78 dpi (Fig. 2A). All of the brain sections examined between 11 and 78 dpi appeared normal. These confirm the earlier studies of Dal Canto and Lipton (3) and Lipton and Dal Canto (12) that the encephalitogenic potential of this strain of TMEV is completely attenuated after tissue culture adaptation.

(ii) In situ hybridization. Since the histopathol-

ogy of tissue culture-adapted TMEV-WW infection was limited primarily to spinal cord white matter, in situ hybridization was performed to determine whether viral replication was similarly restricted. A [^{3}H]cDNA probe complementary to viral RNA was used to locate viral RNA in tissues, and hence the site of viral replication, at various times post-inoculation. Characterization of the probe and its specificity for TMEV-WW-infected cells has been described (see below; 2, 18). For the purposes of these studies, sections subjacent to those shown in Fig. 1, 2, and 3 were processed for in situ hybridization. Approximately 60 to 120 μm of tissues from each of two mice sacrificed at each time point were used for hybridization. The sections used for the hybridization experiments were representative of the average histopathology found among the three to six mice sacrificed at the time after infection studied. Autoradiographic exposure was allowed to proceed for 1 or 3 weeks. At 7 dpi, viral RNA was detected in the brain and spinal cord after 3 weeks of autoradiographic exposure; no positive cells were found after 1 week of exposure. One of the infected cells was a cortical neuron superior to the hippocampus (Fig. 3A); this cell was near the injection site. The remaining positive cells were found in uninflamed areas of spinal cord white matter (Fig. 3B).

By 20 dpi, the degree of inflammation and the amount of viral RNA per cell were much greater (Fig. 1A and 1B). The latter point could be appreciated by the fact that the number of individual grains per positive cell could be discerned after 1 week of autoradiographic exposure. After 3 weeks of exposure, the total number of infected cells was easier to observe, but the large number of grains obscured appreciation of individual grains above each cell. Most of the positive cells detected were within lesions (Fig. 1B) and had small, ovoid, basophilic nuclei, but it was impossible to determine whether these cells were of CNS or hematogeneous origin. Some were found in uninflamed areas of spinal cord near nerve tracts or at the periphery of lesions (Fig. 1). These positive cells had small basophilic nuclei characteristic of glial cells and extensive dendritic cytoplasmic processes, but positive identification of these cells as oligodendroglia was not possible on simple morphological grounds. At the same time, no cells containing viral RNA were found in any section of the brain or in spinal cord gray matter. Examination of CNS tissues from mice inoculated with brain-derived TMEV-WW and sacrificed at 29 dpi revealed positive cells both in spinal cord white matter and in neurons (data not shown).

At 78 dpi, spinal cord lesions were extremely large (Fig. 2A); however, the relative number of positive cells and the number of grains per cell had remained at about the same level as those observed at 20 dpi, but the location of these cells had changed (Fig. 2B and 2C). For example, in one set of matched specimens, the mean number of grains per cell after 1 week of exposure and at 20 dpi was 55 ± 4.2; at 78 dpi it was 49.5 ± 7.8. Most of the positive cells detected at 78 dpi were found at the periphery of the lesions or in uninvolved white matter parenchyma (compare Fig. 1B and Fig. 2B). The positive cells at the periphery of lesions had the same morphological characteristics as those of the cells seen at 20 dpi.

DISCUSSION

In his first report on the isolation of TMEV, Theiler (19) demonstrated that brain-derived stocks of the virus were capable of establishing a persistent CNS infection. Much later, other investigators confirmed and extended these observations with other TO and TO-like TMEVs and demonstrated that these viruses can cause a chronic demyelinating disease (2, 5, 6, 17a). Tissue culture adaptation of the TO and TO-like TMEV has been shown to attenuate their encephalitogenic potential, but does not affect their demyelinogenic potential or ability to persist in vivo (8, 9, 12, 17a). It has proven difficult to demonstrate in which cells brain-derived or tissue culture-adapted TMEV persists during the chronic phase by immunofluorescence and ultrastructural studies. We have recently described the histopathology and localization of viral RNA after infection of mice with brain-derived TMEV-WW (2) and have demonstrated the dual tropism of brain-derived virus for white and gray matter cells. The GDVII strain of TMEV has been found to replicate exclusively in neurons by in situ hybridization (18) and immunofluorescence studies (14); TMEV-GDVII is extremely neurovirulent and does not establish a persistent CNS infection (8, 9). The studies of tissue culture-adapted TMEV-WW reported here have (i) confirmed that brain-derived stocks capable of producing the typical TMEV biphasic disease are rendered devoid of their encephalitogenic potential after tissue culture adaptation and (ii) have demonstrated that tissue culture-adapted TMEV-WW replicated nearly exclusively in cells of spinal cord white matter throughout infection. The strict neurotropism of TMEV-GDVII, the dual gray matter and white matter tropism of brain-derived TMEV-WW, and the nearly exclusive white matter tropism of tissue culture-adapted TMEV-WW appear to directly correlate with the disease-inducing potential of these viruses.

For the in situ hybridization studies, TMEV-GDVII was used as the source of RNA which

served as the template for reverse transcription of the probe. This strain was chosen because it replicated to very high titers in vitro compared with the WW strain (W. G. Stroop, unpublished data) and therefore served as an abundant source of template. The use of GDVII RNA was not considered a drawback, since all of the TMEV strains share cross-reactive antigens (7), indicating conservation of genetic information among the strains tested. Additionally, commercially prepared TMEV-GDVII antiserum (Microbiological Associates, Rockville, Md.) strongly reacted to TMEV WW-infected BHK-21(C-13) and CNS cells in an immunofluorescent-antibody assay (W. G. Stroop, unpublished data). Moreover, as further evidence of the specificity of the [^{3}H]cDNA probe used for these studies, we have previously shown that the probe hybridized to both TMEV-WW- and GDVII-infected tissue culture cells and TMEV-WW acutely infected CNS tissues, but did not hybridize to uninfected cells or tissues. Also, a [^{3}H]cDNA probe synthesized from a heterologous virus RNA template did not hybridize to TMEV-infected cells (2, 18).

Viral RNA was found nearly exclusively in spinal cord white matter cells; the single positive neuron was in an area superior to the injection site. Before the appearance of the inflammatory response (7 dpi), viral RNA was detected in spinal cord white matter, suggesting that the positive cells were glia. However, because the fixative used to perfuse the tissues dissolves much of the myelin (18), it was impossible to ascertain whether the cells were oligodendroglia, astroglia, or microglia. At late times postinfection, some of the positive cells were found at a distance from inflammatory foci. Some of these positive cells (as well as some cells within lesions themselves) appeared to be morphologically similar to those observed at 7 dpi. As the infection proceeded from 20 to 78 dpi, the cells containing RNA appeared to be located more frequently at the periphery of the lesions, suggesting that immune cells were actively responding to infected cells, but that the infection was proceeding radially faster than immune clearance. Thus, at early times postinfection, the isolated infected cells were probably glia, but at later times postinfection, the positive cells could have been glia, or activated macrophages, or both. Evidence that supports an early glial infection by TMEV-WW comes from the ultrastructural observations of Penney and Wolinsky (15), who demonstrated virus-infected oligodendrocytes during the acute phase of infection of suckling mice. A comparison of data on brain-derived TMEV-WW infections (2) with the results reported here suggests that the amount of viral RNA present in white matter cells throughout brain-derived virus infection was 1.5 to 2 times less than that found in cells throughout tissue culture-adapted virus infection. A comparison of grain counts from tissue culture-adapted TMEV-infected CNS tissues indicated that the number of infected cells and the amount of viral RNA per cell remained relatively constant from 20 to 78 dpi despite the increasing intensity of spinal cord lesions.

Tissue culture-adapted TMEV-WW routinely caused larger and more necrotizing spinal cord lesions than those reported to have been caused by brain-derived TMEV-WW (2, 13). For example, tissue culture-adapted virus has produced lesions which extended from the lateral edge of the posterior horn to the anterior median sulcus and which were sometimes gliotic. Brain-derived virus has been found to regularly produce demyelination, with conservation of axons (2, 6; W. G. Stroop, personal observation). In contrast, we found that the demyelination produced by tissue culture-adapted TMEV-WW appeared to be due to necrosis of both myelin sheaths and axons.

The demyelination observed during chronic brain-derived TO or TO-like TMEV infections has been proposed to have an immunopathological basis (10, 11), although it is unclear to which antigens the immune response is directed. The hybridization studies reported here demonstrated viral RNA in white matter cells throughout tissue culture-adapted virus infection, but did not allow definition of which cell type contained the RNA. Recently, Dal Canto and Lipton (4) demonstrated the presence of viral antigens predominately in macrophages during the chronic phase by ultrastructural immunohistochemical techniques. These authors suggested that TMEV persisted mainly in macrophages, although other CNS cells contained lower levels of viral antigens. These observations, the results reported here, and our previous findings on the location of viral RNA in CNS tissue (2, 18) together suggest that TMEVs may infect a variety of CNS cells, but replicate in them at various efficiencies. Once macrophages are infected or activated (or both), they apparently play a direct role in myelin degradation. However, since Lipton and Dal Canto did not find an abundance of viral antigen on myelin sheaths or within oligodendrocytes, it is unclear why macrophages selectively strip myelin and do not attack other antigen-containing CNS cells. It is possible that limited lysis of oligodendrocytes might provide the local antigen stimulus for continued myelin-specific phagocytosis. Clearly, definition of the antigens that elicit the immune attack are critical to understanding the pathogenesis of demyelination.

The three strains of TMEV examined by in

situ hybridization differ strikingly in their ability to induce CNS disease. The highly lethal TMEV-GDVII replicates exclusively in neurons (18); brain-derived TMEV-WW is moderately lethal and replicates initially both in neurons and in spinal cord white matter cells, but persists only in spinal cord white matter cells (2); tissue culture-adapted TMEV-WW produces no acute mortality (9, 12, results reported here) and appears to replicate nearly exclusively in spinal cord white matter cells. Differences among virus cell receptors may explain the apparently specific tissue tropisms among the TMEVs we have examined. Relative differences between the abilities of the strain to replicate in specific cells may play a role in the ultimate degree and type of demyelination produced.

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