Injection of the sciatic nerve with TMEV: A new model for peripheral nerve demyelination

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Abstract

Demyelination of the human peripheral nervous system (PNS) can be caused by diverse mechanisms including viral infection. Despite association of several viruses with the development of peripheral demyelination, animal models of the condition have been limited to disease that is either autoimmune or genetic in origin. We describe here a model of PNS demyelination based on direct injection of sciatic nerves of mice with the cardiovirus, Theiler’s murine encephalomyelitis virus (TMEV). Sciatic nerves of FVB mice develop inflammatory cell infiltration following TMEV injection. Schwann cells and macrophages are infected with TMEV. Viral replication is observed initially in the sciatic nerves and subsequently the spinal cord. Sciatic nerves are demyelinated by day 5 post-inoculation (p.i.). Injecting sciatic nerves of scid mice resulted in increased levels of virus recovered from the sciatic nerve and spinal cord relative to FVB mice. Demyelination also occurred in scid mice and by 12 days p.i., hindlimbs were paralyzed. This new model of virus-induced peripheral demyelination may be used to dissect processes involved in protection of the PNS from viral insult and to study the early phases of lesion development.

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Introduction

Infectious agents are implicated in the development of human peripheral demyelinating diseases such as Guillain–Barre syndrome (Bitan et al., 2004; Takahashi et al., 2005; Tababella and Nowzari, 2005; Ponticelli and Campise, 2005; Koga et al., 2005; Izurieta et al., 2005). Diverse viral infections have been associated with peripheral demyelinating disease including those caused by cytomegalovirus (Tababella and Nowzari, 2005), Epstein–Barr virus (Bitan et al., 2004; Takahashi et al., 2005; Koga et al., 2005), human immunodeficiency virus-1 (Brannagan and Zhou, 2003), and varicella zoster virus (Roccatagliata et al., 2001). Some cases of peripheral demyelination have also been associated with viral vaccinations (Izurieta et al., 2005; Khamaisi et al., 2004). These reports cumulatively suggest that infection and/or the resulting immune response to infection may be responsible for inducing peripheral demyelinating disease in humans but despite this strong inference, models used to study this condition involve only autoimmune or genetic etiologies (Van Rijk et al., 2003; Moon et al., 2006; Zhu et al., 2001; Bour-Jordan et al., 2005; Meekins et al., 2004). Although current animal models recapitulate certain forms of peripheral nervous system (PNS) myelin loss (Van Rijk et al., 2003; Moon et al., 2006; Zhu et al., 2001; Bour-Jordan et al., 2005; Meekins et al., 2004), none of them describe virus-induced demyelination of PNS. This prompted us to explore the possibility of establishing a murine model to mimic this human condition.

Intracerebral (i.c.) inoculation of mice with Theiler’s murine encephalomyelitis virus (TMEV) is commonly used to study viral demyelination and remyelination of the central nervous system (CNS) (Drescher et al., 1998; Drescher et al., 2000a,b; Dal Canto and Lipton, 1975; Lipton, 1975; Rodriguez, 1993; Pena Rossi et al., 1997; Rodriguez et al., 1995; Rodriguez et al., 1994a,b; Rodriguez and David, 1985). Because the pathologic changes and subsequent clinical signs of the disease in mice are
similar to those observed in humans with multiple sclerosis (MS), TMEV infection of mice is also used to model a hypothetical viral etiology of MS (Dal Canto and Lipton, 1975; Lipton, 1975). Demyelination is induced by TMEV-mediated damage to CNS white matter (Lipton, 1975); chronic demyelination is attributed to the development of autoimmune responses to several myelin protein epitopes (Miller et al., 1997a,b). Strains of TMEV belonging to the TO subgroup (BeAn and Daniel’s) induce acute encephalitis followed by demyelination (Lipton, 1975). The primary host determinant of demyelination is the murine haplotype: H-2b, H-2d. mice are resistant to TMEV-induced demyelination (Rodriguez et al., 1993). Resistance or susceptibility to demyelination can also be altered by deletion or depletion of CD8+ T cells (Rodriguez et al., 1997) or CD4+ T cells (Rodriguez et al., 1997; Rodriguez and Sriram, 1988), or by modulating a number of immune system components (Rodriguez et al., 1995; Fiette et al., 1995; Murray et al., 1998; Paya et al., 1990).

Natural TMEV infections of mice are spread via an oral/fecal route of transmission (Theiler, 1940; Theiler and Gard, 1940). A likely route of infection following ingestion of the virus involves the peripheral nerves of the gut (Ren and Racaniello, 1992) whereby transport of virus to the CNS could then establish a chronic demyelinating disease characterized by inflammation and destruction of the myelin sheaths surrounding the axons. We therefore used the sciatic nerve in the leg (a relatively accessible peripheral nerve) as an example of the PNS to determine whether TMEV injection directly into the nerve would induce peripheral demyelination. Following direct inoculation of sciatic nerves of female FVB mice with TMEV, we observed demyelination coincident with viral replication within the nerve. Virus subsequently spread into the CNS. In immunodeficient scid mice, increased viral replication and subsequent paralysis indicated a role for the adaptive immune response in controlling PNS virus infection. Because the precise site of initial virus inoculation is known, this model can be used to study the earliest stages of lesion development.

Results

**TMEV is detected in sciatic nerves following direct injection with virus**

Initial demyelination is due to virus-induced damage in the model of TMEV-induced CNS demyelination (Dal Canto and Lipton, 1975). To test whether TMEV could be detectable in sciatic nerves following inoculation, FVB mice were inoculated with $1 \times 10^4$ pfu/sciatic nerve (described in Materials and methods). Briefly, the thigh musculature of the mouse was separated to expose the sciatic nerve, into which virus was injected directly using a 30 gauge needle. Injection did not occur through the muscle itself. Mice were killed, the nerve dissected, and then immunostaining of OCT-embedded sciatic nerves was performed using a polyclonal TMEV antiserum. At 3 days post-inoculation (p.i.), viral protein was detected in sciatic nerves of virus-injected (Fig. 1A), but not HBSS-injected (control) mice (Fig. 1B). Earlier in infection, virus positive cells were rarely detected in the sciatic nerve (data not shown). To test whether we were detecting residual protein from the virus inoculum, an equivalent amount of UV-inactivated TMEV was prepared and injected as before into sciatic nerves of FVB mice. UV-inactivation of virus was confirmed by the inability of the virus to form plaques on an L2 cell monolayer (data not shown). Examination of the injected sciatic nerve by immunohistochemistry for the presence of virus 3 days p.i. showed that viral protein was not detected in the sciatic nerve of mice inoculated with UV-inactivated virus (Fig. 1C).

To identify the cells associated with TMEV in the sciatic nerve, we performed immunofluorescent staining on TMEV-infected sciatic nerves from female FVB mice. A polyclonal antiserum to TMEV was used, in combination with antibodies specific for either macrophages or myelinating (Schwann) cells. Markers of these cell types were chosen as macrophages have been reported as a reservoir of TMEV in the CNS model of demyelination (Clatch et al., 1990), and direct infection of Schwann cells would support the ability of TMEV to induce demyelination of the PNS. At 5 days post-infection, the time-point where we observed maximal immunoreactivity for virus, virus co-localized with markers for myelinated cells (Figs. 1D–F), indicating that Schwann cells are infected following intracisternal nerve injection. This was not observed in control mice; only myelin immunoreactivity was observed in the uninfected (contralateral) sciatic nerves (Fig. 1G). To demonstrate the specificity of the anti-myelin antibody, brain tissue from a normal mouse was used as a control (Figs. 1H–I). The cortex, an area of the brain rich in myelin (that is, a white matter area) showed intense immunofluorescence (Fig. 1H). An adjacent gray matter region which contains minimal numbers of myelin-producing cells (the hippocampus) was devoid of staining (Fig. 1H). Macrophages in the sciatic nerve of virus-injected animals were also observed to be infected (data not shown). Together, the immunostaining data suggest that TMEV can productively infect the sciatic nerve of mice by this route of infection.

**Replicating virus can be isolated from sciatic nerves**

We isolated sciatic nerve tissue from FVB mice 5 days p.i. and assayed for infectious virus. At 5 days p.i., the adaptive antiviral immune system has been triggered in mice when inoculated i.c. with TMEV (Lindsay et al., 1991; Lindsay and Rodriguez, 1989). Nerve tissue was homogenized, frozen and thawed, then titrated on L2 cell monolayers. Titers of $5.0 \pm 0.7 \times 10^4$ TCID$_{50}$ ($n=3$) per mg nerve tissue were detected at this time-point. At an average weight of 31 mg per dissected sciatic nerve, the total infectious viral load in the dissected nerves at 5 days p.i. averaged $1.55 \times 10^9 \pm 2.2 \times 10^8$, an approximate 100-fold increase in titer over that which was inoculated. Earlier in the infection (day 3 p.i.), sciatic nerve titers were lower ($9.1 \times 10^4 \pm 0.5 \times 10^4$; $n=3$), supporting the conclusion that replication was occurring in the sciatic nerve. Prior to day 3, virus was rarely isolated from the sciatic nerve.
By days 7–9 post-injection, viral load in the sciatic nerve was below the level of detection of the assay, which is about $3 \times 10^3$ per mg. As similar results were obtained in SJL/J mice (data not shown), the results observed are unlikely to be solely a function of the genetic background of the mice.

It has been established that TMEV replicates to higher titers in the CNS of scid mice, which lack functional B and T cells (Bosma et al., 1983), than in immunocompetent mice following i.c. inoculation (Drescher et al., 1999; Njenga et al., 2004). We hypothesized that TMEV would similarly generate higher titers in sciatic nerve tissue of scid mice if the adaptive immune response was involved in protection of the sciatic nerve. Scid mice were inoculated with $1 \times 10^4$ pfu per sciatic nerve, then killed 5 days later. We observed an increase of more than 4 logs in viral titer in sciatic nerves of the scid mice ($3.2 \pm 5.1 \times 10^9$ TCID<sub>50</sub>/mg nerve tissue) relative to immunocompetent FVB mice. These results indicated that the adaptive immune response plays a critical role in protection of mice inoculated with TMEV via the sciatic nerve.

**Inflammatory cells infiltrate TMEV-injected sciatic nerves**

Inflammatory cells are not detected in normal PNS or CNS (Male et al., 1992; Male et al., 1990a,b; Wekerle et al., 1986) but damage to either the PNS or CNS can culminate in the recruitment of inflammatory and/or immune cells to the site of injury (Male et al., 1990a, 1992). Lymphocytic infiltration of the brain in the TMEV model of CNS demyelination is apparent by 3–5 days p.i. (Lindsay and Rodriguez, 1989; Lindsley et al., 1988); the tissue is infiltrated by T and B cells as well as macrophages (Lindsley et al., 1988).

Immunohistochemical staining was used to examine whether immune cells were recruited during TMEV replication in the sciatic nerve. Sciatic nerves were dissected from FVB
mice 5 days after TMEV injection was embedded in OCT as described previously (Drescher et al., 2004). Inflammatory cells were not observed in un.injected sciatic nerves from control, unmanipulated mice (data not shown) but macrophages (Fig. 2A) and T cells (Fig. 2B) were detectable in TMEV-inoculated nerve tissue. HBSS-injected (mock-inoculated) sciatic nerve tissue was also negative for inflammatory cells at the same time point (Figs. 2C, D). B cells were not detected in any sample (data not shown). The absence of immune cells in HBSS-inoculated sciatic nerves indicates that the recruitment of inflammatory cells to virus-containing sciatic nerve tissue was not attributable to the physical insult of the injection process but occurred in response to a specific, sustained viral insult to the sciatic nerve.

TMEV spreads from the sciatic nerve (PNS) to the spinal cord (CNS)

Studies utilizing poliovirus (Solomon and Willison, 2003; Sabin and Ward, 1941; Sabin, 1956), herpes simplex virus (Hemachudha et al., 2005; Faber et al., 2004; Song and Jia, 1999; Fujiki and Tashiro, 1997), and rabies virus (Hemachudha et al., 2005; Faber et al., 2004) have shown that virus can be transported to the CNS during viral replication following infection of the PNS. We therefore asked whether TMEV was also capable of spreading from the PNS to the CNS following direct injection of the sciatic nerve. At day 3 post-injection, no virus was detected in the spinal cords of either FVB or scid mice (sensitivity of plaque assay, 200 pfu/g tissue). Seven days following sciatic nerve injection of scid mice with TMEV, we detected 2.4±0.8×10⁶ pfu/g (n=3) TMEV in the spinal cord, while a hundred fold lower viral titers detected in the spinal cords (6.4±1.7×10³ pfu/g; n=3) of FVB mice again indicated a role of the adaptive immune system in suppressing TMEV replication in this system. The differences in viral titers between FVB and scid mice are similar to those that have been reported in the CNS following intracerebral injection with TMEV (Njenga et al., 1997).

We also determined whether mice were functionally impaired following sciatic nerve injection with virus. Scid mice first demonstrated hindlimb paralysis 12 days after sciatic nerve injection with TMEV, although FVB mice appeared showed no altered movements or activity levels at the same time-point relative to mock-infected control mice. Neither scid nor FVB mice were maintained longer than 16 days p.i. These results indicate that virus is transported from the PNS to the CNS following injection of TMEV into the sciatic nerve and that in the absence of a functional adaptive immune response, the virus induces sufficient damage to the nervous system which can result in functional alterations and eventually death.

PLP levels are altered and demyelination is induced following TMEV infection of the sciatic nerves

Injury to myelin-producing cells results in alterations in mRNA levels of genes involved in myelination (Gupta et al., 1991; Yaghootfam et al., 2005; Kawczak et al., 1998; Ozden...
If remyelination processes were invoked following virus insult, an increase in transcription of genes encoding myelin components should be detectable. However, if demyelination occurred in the absence of remyelination, a decrease in transcripts associated with the production of myelin proteins might be the result of either death and/or damage to myelin-producing cells. In the CNS model of TMEV-induced demyelination, myelin proteolipid protein (PLP) transcripts in spinal cord have been shown to be altered relatively within hours following TMEV-induced injury (Rodriguez et al., 1994a,b). We injected sciatic nerves of FVB mice with virus to test whether injection of TMEV into the sciatic nerve altered levels of PLP gene transcripts in the sciatic nerve. RNA was isolated from dissected sciatic nerves at various time-points after injection, and real-time RT-PCR performed utilizing primers and a dual-labeled fluorogenic probe for PLP. Real-time PCR was standardized to a housekeeping gene (glyceraldehyde-3-phosphate-dehydrogenase, GAPDH) (Medhurst et al., 2000). Data are expressed as the fold-increase in PLP mRNA level relative to the levels of PLP mRNA in un.injected (control) sciatic nerves. A significant increase in the level of PLP mRNA was observed within 2 h post-injection compared to uninfected controls (4.9±0.5-fold-increase over control; p=0.02). However, although there was an increase in PLP mRNA abundance over the following 24 h, the differences between the points were not significant (1.8±0.4-fold over baseline; p<0.2) (Fig. 3). By 72 h p.i., a 15.7±2.7-fold increase (p<0.032) in transcript levels had occurred in virus-injected sciatic nerve tissue relative to the level in uninjected control mice (Fig. 3). PLP mRNA levels then decreased to a 3.1±0.3-fold increase over control by day 5 p.i (p=0.02). To determine whether sustained inflammation was required for alterations in PLP mRNA levels, PLP mRNA abundance was also measured in sciatic nerves of mock-infected female FVB mice (injected with HBSS). No significant alterations in PLP mRNA levels were detected relative to the uninjected sciatic nerve over the course of the experiment (5 days; Fig. 3), results that indicated that putative transient inflammatory responses which may be induced by injecting the nerve with physiologic salt solution, are inadequate for PLP transcript levels to be altered in the sciatic nerve. Together, these results are consistent with the induction of myelin repair processes in the virus-injected sciatic nerves within hours of virus infection.

Demyelination of the sciatic nerve is observed following injection with TMEV

As the observation that PLP mRNA levels were increased in TMEV-inoculated sciatic nerves was consistent with activation of a myelin repair mechanism in response to the viral insult, we performed histopathological analyses of sciatic nerves from virus-injected mice for evidence of demyelination. Osmicated

Fig. 4. Pathology of the sciatic nerve of virus-injected (B, C) and HBSS-injected (A) mice. HBSS-injected sciatic nerves are characterized by uniform, well-preserved myelin around the axons; infiltrating cells are absent (A). Mice injected with TMEV (B, C) show demyelination of the axons (thick white arrow; demyelination presents as paler circles versus the dark well-myelinated axons). In addition, the sciatic nerve contains cells which, due to their foamy appearance, appear to be macrophages (thin, white arrow) as well as myelin debris (thin black arrow). Sciatic nerves were osmicated, embedded in plastic, and 1 μm sections prepared. Slides were stained with 4% para-phenylenediamene (PPD) and examined by light microscopy. Original magnification ×1200.
plastic sections of sciatic nerves inoculated with TMEV 5 days previously were stained with para-phenylenediamene (PPD), then examined for evidence of myelin loss; HBSS-injected sciatic nerves served as controls. Sciatic nerves injected with HBSS appeared histologically normal, characterized by uniform, well-preserved myelin around the axons (Fig. 4A). However, extensive myelin loss was observed in sciatic nerves of TMEV-inoculated mice (Figs. 4B, C). Demyelination of sciatic nerves of TMEV-inoculated mice was distinguished by loss of myelin around the axons (thick, white arrows, Figs. 4B, C), by the presence of cellular debris (thin black arrows, Fig. 4C) and by cells that appear to be foamy macrophages, indicating that the macrophages are engaged in cleaning cellular debris (thin, white arrows, Fig. 4C). These results demonstrate that demyelination occurs as a result of the productive TMEV infection occurring in sciatic nerves following direct inoculation with virus.

Discussion

Results presented here describe a novel model of virus-induced peripheral demyelinating disease. Following inoculation of TMEV directly into the mouse sciatic nerve, infectious virus transits to the CNS. Detection of increased virus loads demonstrate productive virus replication occurs in inoculated nerves. Demyelination of the sciatic nerve was related to the presence of replicating virus for no demyelination was observed in mock-infected nerves. Demyelination could be the result of direct infection of myelinating cells and/or due the in resulting immune response. This model should have relevance for testing putative infectious etiologies of human nervous system diseases such as Guillain-Barre syndrome and multiple sclerosis, as well as for studies of PNS remyelination. In our model of virus-induced peripheral demyelinating disease, only virus-injected (not mock-infected) sciatic nerves were infiltrated by immune cells, indicating that immune cell recruitment to the sciatic nerve requires sustained, specific stimuli. Observations in scid mice demonstrated TMEV achieved higher viral titers than in immunocompetent FVB mice, suggesting a role for the adaptive immune system in virus suppression and likely, virus clearance. These results may be relevant to the human condition in instances of severely immunosuppressed individuals experiencing peripheral demyelinating diseases (Rao and Thomas, 2005; Hulgan et al., 2005; Ferrari et al., 2006). An advantage of the model presented herein is that the site of the initial damage is known, thus allowing for the study of the beginning phases (that is, minutes or hours after insult) of lesion development and repair—processes that are typically difficult to study in vivo. One can speculate that this model may also be applicable for studies of viruses that may not replicate well when injected into mice: direct injection of infectious virions into the sciatic nerve may induce a host response of interest and might also provide a productive microenvironment for the virus to replicate in an otherwise refractory host.

Early work (Rustigian and Pappenheimer, 1949) demonstrated that injection of TMEV into the leg muscle resulted in flaccid paralysis, initially observed in the injected leg and subsequently in the uninfected leg. Naturally occurring TMEV infections occur via the fecal–oral route; in some cases virus transits to the CNS with subsequent paralysis (Theiler, 1940; Theiler and Gard, 1940). Such spread of virus from the alimentary tract to the CNS could be via peripheral nerves and/or blood (Ren and Racaniello, 1992). Although the receptor for TMEV remains unknown, one potential receptor for TMEV is P0, a protein structurally consistent with other picornavirus receptors (Libbey et al., 2001; Mendelsohn et al., 1989; Bernhardt et al., 1994) and thought to be confined to the PNS (Libbey et al., 2001). Schwann cells, the cell responsible for myelination of the PNS, are susceptible to infection by diverse viruses including TMEV (Watanabe et al., 2006; Jin et al., 2004; Tanimura et al., 2004; Levine et al., 2003; Nien et al., 1998; Libbey et al., 2001; Frankel et al., 1986). Based on the literature, there was no a priori reason to doubt that such peripheral infections indeed can and do occur naturally and could be modeled in experimental systems. Indeed, polio has been well-studied with regard to its transport from the periphery to the CNS (Ren and Racaniello, 1992; Ohka et al., 1998).

We hypothesize that following TMEV injection of the sciatic nerve, Schwann cells are infected and demyelination occurs through death of Schwann cells or impairment of their ability to produce myelin. Once Schwann cells are infected, replicating virus can be transported to the spinal cord. The mechanism of transport to the spinal cord has not been elucidated, but based on the literature axonal transport is one potential mechanism (Martinat et al., 1999). In contrast to the situation observed by others (Martinat et al., 1999) using the neurovirulent GDVII strain, immunocompetent mice in our studies did not experience overt clinical deficits (i.e., slowed movement, paralysis, death) associated with virus infection. The differences in clinical presentation between our studies and the previous studies could be attributed to either different cell types becoming infected in the spinal cord, or to altered viral loads. Despite the high degree of genetic identity between the GDVII and DA strains of TMEV (Pevear et al., 1988; Pevear et al., 1987), these TMEV strains preferentially infect different cell types in the CNS. GDVII localizes primarily in gray matter, while DA establishes infection primarily in white matter. Several reports ascribe the differences in virus localization in the mouse CNS to differences in receptor usage (Shah and Lipton, 2002; Jnaoui et al., 2002; Tsunoda et al., 2001; McCright et al., 1999; Zhou et al., 2000; Jnaoui and Michiels, 1999).

Previous work has shown that TMEV, strain GDVII, can be transported from the periphery to the CNS via fast axonal transport within 48 h of footpad inoculation (Martinat et al., 1999). Although the impact of the virus infection on myelin integrity was not explicitly addressed in this study (Martinat et al., 1999), it was noted that pathologic changes were reported in the gray matter of the spinal cord once virus was in the CNS, implying minimal involvement of white matter areas of the CNS and PNS. Strain GDVII is considered highly neuroviral and has not been shown to infect white matter areas of the CNS in vivo; a characteristic attributable to differences in receptor usage by the GDVII and TO viruses (Tsunoda et al., 2001; Jnaoui et al., 2002).

The majority of studies involving virus infection of the PNS have studied cases of human peripheral dysfunction (Ferrari et
was used for all experiments. was used as an indicator of inactivation. The same stock of virus cell monolayer to ensure inactivation; lack of cytopathic effect light for 10 min. An aliquot of the virus was assayed on an L2 TMEV was used. Undiluted virus stock was placed under a UV pfu/mL or TCID50/mL. In some experiments, UV-inactivated stored frozen in aliquots at 30% w/v sucrose, 100 mM NaCl, 20 mM Tris the virus was collected by overnight ultracentrifugation through centrifugation (Beckman SW28.1, 25,000×RPM, 8 °C, 30 min), freeze-thaw lysis. Following removal of cellular debris by previously described (Rodriguez et al., 1986), collected by an ultracentrifuge (Beckman SW28.1, 25,000×RPM, 8 °C, 30 min), the virus was collected by overnight ultracentrifugation through 30% w/v sucrose, 100 mM NaCl, 20 mM Tris–HCl pH 7.5, into a glycerol pad. Virus was suspended in Hank’s Balanced Salt solution (HBSS) containing 2×10^4 pfu of TMEV. Mock-infected, control animals were injected with 10 μL HBSS. Wounds were closed using surgical staples (MikRon® AUTO-CLIP® 9 mm, Becton Dickinson, Sparks, MD) and the wound site treated with triple anti-bacterial ointment. Acetaminophen was added to the drinking water (80 mg/5 mL) as a post-operative analgesia.

All animal experiments were performed in accordance with guidelines of the Association for Accreditation and Assessment of Laboratory Animal Care (AAALAC), the Creighton University Institutional Animal Care and Use Committee (IACUC), and Federal regulations.

In summary, the results presented herein introduce a new murine model for studying pathogenic mechanisms of virus-mediated peripheral nerve demyelination. The ability to study lesion dynamics in the context of both myelin damage and repair is a unique character of this model. The model may additionally be utilized to study the roles of the immune system in limiting demyelination as well as repair damage to the PNS. A greater understanding of these processes could permit better treatment of conditions associated with viral infections of the PNS in humans.

Materials and methods

Virus

TMEV, strain DA, is a prototypic demyelinating strain of the TO subgroup; the disease observed in the CNS following infection with TMEV/DA has been extensively described (Drescher et al., 1998, 2000a,b; Dal Canto and Lipton, 1975; Lipton, 1975; Lipton and Dal Canto, 1976; Pena Rossi et al., 1991, 1997; Lipton and Dal Canto, 1977, 1979; Rodriguez and David, 1985). TMEV was propagated in BHK cells as previously described (Rodriguez et al., 1986), collected by freeze-thaw lysis. Following removal of cellular debris by centrifugation (Beckman SW28.1, 25,000 × RPM, 8 °C, 30 min), the virus was collected by overnight ultracentrifugation through 30% w/v sucrose, 100 mM NaCl, 20 mM Tris–HCl pH 7.5, into a glycerol pad. Virus was suspended in Hank’s Balanced Salt solution (HBSS), tittered on BHK monolayers (pfu/mL), and stored frozen in aliquots at −80 °C. Titers are measured either by pfu/mL or TCID50/mL. In some experiments, UV-inactivated TMEV was used. Undiluted virus stock was placed under a UV light for 10 min. An aliquot of the virus was assayed on an L2 cell monolayer to ensure inactivation; lack of cytopathic effect was used as an indicator of inactivation. The same stock of virus was used for all experiments.

Mice

Female mice (FVB or B6.CB17-Prkdc<sup>-scid</sup>/SzJ) were purchased from Jackson Laboratories (Bar Harbor ME) at 6 weeks of age. Mice were housed at 5 per cage in Thoren Maxi-Miser System cages (Hazleton, PA) and provided food and water ad libitum. The entire cage set including food and water was steam sterilized prior to use. Cages were changed weekly.

Injection of the sciatic nerve

Mice were anesthetized using IsoFlo (Abbott Laboratories, North Chicago, IL), the hindleg wetted with 95% ethanol, skin cut, and muscle moved to expose the sciatic nerve, per published methodology (Palmer et al., 2000). Mice were kept on a warming pad which aids in reduction of stress by helping the mouse to maintain proper body temperature during the procedure. A 30 gauge needle attached to a Hamilton syringe (Fisher Scientific, St. Louis, MO) was used to inject the sciatic nerve with 10 μL of virus diluent (Hanks’ Balanced Salt Solution, HBSS) containing 2×10<sup>4</sup> pfu of TMEV. Mock-infected, control animals were injected with 10 μL HBSS. Wounds were closed using surgical staples (MikRon® AUTO-CLIP® 9 mm, Becton Dickinson, Sparks, MD) and the wound site treated with triple anti-bacterial ointment. Acetaminophen was added to the drinking water (80 mg/5 mL) as a post-operative analgesia.

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Tissue collection

Mice were killed with an injection of 100 μL sodium pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) i.p. Mice were then perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) (Drescher et al., 2000a,b). Sciatic nerves and spinal columns were dissected and placed in 4% paraformaldehyde in PBS at room temperature. In some cases, tissues were snap frozen under dry ice for RNA extraction or virus tittering, or embedded in OCT Frozen Embedding Media (Miles, Inc., Elkhart, IN) for immunostaining.

Immunostaining

Six micron thick sections of tissue embedded in OCT Frozen Embedding Media (Miles, Inc.) were cut and fixed in chilled acetone. Sections were stained with monoclonal antibodies to CD4 (BD Pharmingen, San Diego, CA), CD8 (BD Pharmingen), F4/80 (Chemicon, Temecula, CA) or a polyclonal antisera to TMEV using the avidin–biotin complex (ABC) immunoperoxidase technique (Vector Laboratories, Inc., Burlingame, CA) as described previously (Drescher et al., 1999). Development was performed using the DAB Substrate Kit (Vector Laboratories, Inc.). Slides were lightly counterstained with Mayer’s hematoxylin (Sigma), dehydrated, and mounted with Permount (Fisher Scientific).

Additional slides were stained using immunofluorescence. OCT-embedded sections were stained with primary antibodies to TMEV, and a monoclonal antibody to either F4/80 (Chemicon) or myelin (Clone HPC-7; Biosource, Saco, ME). Visualization was performed using Alexa-Fluor-labeled secondary antibodies (Molecular Probes/Invitrogen, Carlsbad, CA).
which were visualized at a wavelength of either 488 or 568. Coverslips were applied using Vectashield Mounting Media with DAPI (Vector Labs).

**Viral infectivity**

Sciatic nerves and spinal cords were dissected from mice at various times post-infection and tissue was stored at −80 °C until used. Plaque assays were performed on spinal cord tissue samples as described previously (Patrick et al., 1990) while titers in sciatic nerve tissue were expressed as TCD50/g nerve tissue (Tracy et al., 2002). In each case, a minimum of three samples were assayed per time point.

**Real-time PCR**

RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) from snap frozen sciatic nerves at various times p.i. per the manufacturer’s instructions. Real-time PCR was performed using dual-labeled fluorogenic TaqMan probes (Medhurst et al., 2000; Khademi et al., 2004). The reporter dye 6-carboxy-fluorescein (FAM) is attached to the 5′ end of the probe, and 6-carboxy-tetramethyl-rhodamine (TAMRA) to the 3′ end of the probe. Primers and probes were obtained/produced from Applied Biosystem’s Assays-by-Design Service. The primers/probes used are as follows: PLP: forward (5′→3′): CGC TGT CAG GCA GAT CTT TG; PLP: reverse (5′→3′): TGC GCT CAG GCC CTT G; PLP probe (5′→3′): ACT ACA AGA CCA CCA CTA GC. Assays and data analysis were performed using the ABI 7000 SDS Sequence Detection System (Applied Biosystems); five replicates of each sample were run. All data were reported as fold-increase (or decrease) compared to control. Relative changes in mRNA levels were determined using the difference in the cycle threshold value (ΔCt method) (Medhurst et al., 2000; Khademi et al., 2004).

**Sciatic nerve morphology**

Sciatic nerves were stored in 4% paraformaldehyde for at least 1 week after perfusion prior to processing the samples for plastic embedding. Samples were osmicated and embedded in Epon 812 plastic (Electron Microscopy Sciences, Hatfield, PA) as described previously (Drescher et al., 2000a,b). One micron sections were cut, stained with 4% PPD and cover-slipped. Sections were examined by light microscopy.

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**References**


Fiette, L., Aubert, C., Muller, U., Huang, S., Auget, M., Brahic, M., Bureau, J.F., 1995. Theiler’s virus infection of 129 Sv mice that lack the interferon α/β or interferon γ receptors. J. Exp. Med. 181, 2069–2076.


