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Authors
Libbey, Jane E
Lane, Thomas E
Fujinami, Robert S

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Axonal Pathology and Demyelination in Viral Models of Multiple Sclerosis

Jane E. Libbey, M.S., **Thomas E. Lane, Ph.D.**, and **Robert S. Fujinami, Ph.D.**

Department of Pathology, University of Utah, 15 North Medical Drive East, 2600A EEJMRB, Salt Lake City, UT 84112, USA

Abstract

Multiple sclerosis (MS) is an immune-mediated inflammatory demyelinating disease of the central nervous system (CNS). Monozygotic twin studies suggest that while there is a genetic contribution, genetics alone cannot be the sole determining factor in the development of MS. As the rates of MS are increasing, particularly among women, environmental factors such as viral infections are coming to the foreground as potential agents in triggering disease in genetically susceptible individuals. This review highlights pathological aspects related to two pre-clinical viral models for MS; data are consistent between these two models as experimental infection of susceptible mice can induce axonal degeneration associated with demyelination. These data are consistent with observations in MS that axonal damage or Wallerian degeneration is occurring within the CNS contributing to the disability and disease severity. Such early damage, where axonal damage is primary to secondary demyelination, could set the stage for more extensive immune mediated demyelination arising later.

Introduction

Multiple sclerosis (MS) is an immune mediated inflammatory disease of the central nervous system (CNS). It is characterized by multifocal regions of inflammation and myelin destruction accompanied by significant neuronal degeneration and axonal loss, which is part of the disease process. Approximately 2–2.5 million people are afflicted by this disease world-wide with about 400,000 cases in North America. Most individuals initially present with clinically isolated syndrome (CIS) that may be monofocal or multifocal in nature; CIS attacks are generally characterized by perturbations in vision and sensory disorders (Miller *et al*., 2012). Typically, MS runs a protracted clinical course lasting over several decades with episodes of exacerbation followed by variable periods of remission. As neurons and axons are lost, the disease course moves from a relapsing-remitting form to a more progressive clinical course. Available evidence indicates that the cause of MS is multifactorial and includes the genetic background of the individual. Clinical data supports the role of genetics based upon concordance rates of acquiring MS between monozygotic

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Corresponding Author: Robert S. Fujinami, Ph.D. (Robert.Fujinami@hsc.utah.edu).

Disclosure

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twins. However, in studying discordant monozygotic twins Baranzini *et al*. (2010) could not identify a genetic difference that could account for susceptibility in one of the twins. While genetics, particularly an association with human leukocyte antigen DR B*1501 [major histocompatibility complex (MHC) class II], plays a role in the disease, it is believed that environmental factors such as viral infections contribute to either initiation of the disease and/or exacerbations. Environmental factors are considered to play an important role in the development of MS in genetically susceptible individuals based on epidemiological studies (Kurtzke, 1993). These findings warrant the use of infectious disease models for examining the molecular and cellular mechanisms contributing to the neuroinflammation and demyelination characteristics of MS.

Viruses such as measles virus, human T cell leukemia virus type-1, human coronaviruses, herpes simplex virus type-1, human herpesvirus-6, and Epstein-Barr virus have been associated with MS, although no clear causal role has been firmly established (Friedman *et al.*, 1999; Johnson, 1994; Kennedy and Steiner, 1994; Lincoln *et al.*, 2008; Lipton *et al.*, 2007; McCoy *et al.*, 2006; Olson *et al.*, 2005; Pugliatti *et al.*, 2008; Tompkins *et al.*, 2002; Tsunoda and Fujinami, 2002; Wu and Perlman, 1999). The development of animal models in which the clinical and histological pathology is similar to that observed in the majority of MS patients is imperative in order to attempt to better understand the underlying pathological mechanisms contributing to MS. Several excellent rodent models of MS have been developed which meet the necessary criteria. The classical experimental animal model for MS is experimental autoimmune (allergic) encephalomyelitis (EAE). This is an induced autoimmune disease initiated in susceptible animals through injection of myelin antigen in adjuvant. More recently spontaneous EAE disease models have been described (Pollinger *et al.*, 2009).

Viral models of human demyelinating disease are important tools for studying the pathogenesis of immune mediated mechanisms of disease. Many of these viral models of MS have a strong MHC component, similar to MS, which plays an essential role in dictating susceptibility to disease. An example of this is persistent infection of genetically susceptible mice with the neurotropic JHM strain of mouse hepatitis virus (JHMV). This virus is a positive-sense single-stranded RNA virus that is a member of the *Coronaviridae* family. Susceptible strains of mice persistently infected with JHMV have ongoing axonal damage and demyelination mediated by inflammatory T cells and macrophages/microglial cells (Lane and Buchmeier, 1997; Lane *et al.*, 2000; Liu *et al.*, 2001; Wu and Perlman, 1999). Infection of susceptible strains of mice with JHMV results in an acute encephalomyelitis followed by a chronic demyelinating disease. Similar to MS, components of the immune system, e.g., T cells and macrophages, are considered important in amplifying the severity of white matter damage (Lane *et al.*, 2000; Wu and Perlman, 1999; Wu *et al.*, 2000). Clinically, mice develop hind-limb paralysis and this correlates with the severity of neuroinflammation and demyelination.

Different mechanisms have been proposed to explain JHMV-induced demyelination. Several studies suggest that demyelination in JHMV-infected mice involves immunopathologic responses against viral antigens expressed in infected tissues even though infectious virus cannot be isolated (Glass and Lane, 2003a; 2003b; Haring *et al.*,

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2001; Haring and Perlman, 2003; Kim and Perlman, 2005). Although virus-specific antibodies are considered important in suppressing viral recrudescence, antibodies do not have a prominent role in promoting demyelination (Ramakrishna *et al.*, 2002, 2003). JHMV infection of immunosuppressed or immunodeficient mice results in very high levels of virus within the CNS and mice succumb. However, demyelination is not a prominent feature, indicating that infection of oligodendrocytes in this model does not directly result in an increase in demyelination (Lane *et al.*, 2000; Wu *et al.*, 2000). Infiltrating immune cells, e.g., T cells and macrophages/microglial cells, are critical in amplifying white matter damage in this model. Interestingly, adoptive transfer of splenocytes from immunocompetent mice immunized with JHMV into immunodeficient animals infected intracranially with JHMV results in robust demyelination that correlates with accumulation of activated T cells and macrophages within the CNS (Glass and Lane, 2003a; 2003b). Data suggests that demyelination in JHMV-infected mice is not the result of epitope spreading and induction of an immune response against neuroantigens, as has been reported to occur during Theiler's virus-induced demyelination (Miller *et al.*, 1997). However, adoptive transfer of T cells from JHMV-infected rats to naïve recipients results in demyelination (Watanabe *et al.*, 1983). Whether a similar response occurs in JHMV-infected mice and what the contributions are to demyelination is not clear at this time.

Another complementary viral model to JHMV infection is Theiler's murine encephalomyelitis virus (TMEV, a positive-sense single-stranded RNA virus that is a member of the *Picornaviridae* family) infection of genetically susceptible mice which produces an inflammatory CNS demyelinating disease. Infection of mice with TMEV has provided important insight into the immunopathogenesis of demyelination (Olson *et al.*, 2005; Tompkins *et al.*, 2002; Tsunoda and Fujinami, 2002). Persistent infection of susceptible mice such as SJL/J mice with TMEV results in an immune-mediated demyelinating disease with virus persisting in macrophages and glial cells in the CNS of infected mice. Infection with either JHMV or TMEV results in a neurologic disease that is similar both clinically and histologically to the human demyelinating disease MS, including axonal and neuronal damage. This ties in with the hypothesis that an environmental agent such as viral infection may be a contributing cause to and an initiating factor in exacerbations of MS in genetically susceptible individuals. The JHMV and TMEV models offer an excellent opportunity in which to study both the underlying immunopathological mechanisms that may drive axonal damage and demyelination in MS patients as well as developing novel therapeutic methods for promoting axonal preservation and remyelination.

Several groups have reported axonal damage occurring early during the course of MS. Damaged axons were found in regions around plaques in white matter without demyelination (Kuhlmann *et al.*, 2002), in remyelinating lesions, and in normal-appearing white matter (NAWM) (Bitsch *et al.*, 2000; Bjartmar *et al.*, 2001), in addition to active demyelinating lesions. Magnetic resonance spectroscopy (MRS) analyses have also found gray matter lesions (Sharma *et al.*, 2001), cortical damage, and axonal injury in MS patients (Bjartmar *et al.*, 2001; Lovas *et al.*, 2000; Matthews *et al.*, 1998). Axonal damage has also been observed in JHMV-induced demyelination (Dandekar *et al.*, 2001; Das Sarma *et al.*, 2009), as well as in TMEV-infected mice (McGavern *et al.*, 1999; Tsunoda and Fujinami,

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1999; Tsunoda *et al.*, 2003). Axonal damage in MS is believed to occur secondarily after the destruction of the myelin sheath or loss of oligodendrocytes. However recent data from viral models also suggest that axonal damage could occur independently of demyelination and be a primary event in the demyelinating process.

Axonal damage and loss can take the form of Wallerian degeneration characterized by the anterograde degeneration of axons, generally caused by injury to the upstream or proximal part of the axon or by death of the neuron body (Kuhn *et al.*, 1988). Wallerian degeneration is being recognized as a common event. Various groups using magnetic resonance imaging (Henry *et al.*, 2003; Rocca *et al.*, 2013; Sawlani *et al.*, 1997; Simon *et al.*, 2000; Werring *et al.*, 2000), MRS (De Stefano *et al.*, 1999), and autopsy or biopsy (Adams and Kubik, 1952; Singh *et al.*, 2013) have reported Wallerian degeneration and/or axonal degeneration.

In MS, the primary target is believed to be either the oligodendrocytes, the myelin forming cells of the CNS, or myelin itself. Although axonal degeneration has been demonstrated in both MS and EAE, axonal damage is thought to occur secondarily to myelin damage. In this case, the lesion would develop from the myelin (outside) to the axon (inside) (Figure 1, Outside-In model) (Tsunoda and Fujinami, 2002; Zipp and Aktas, 2006). In this Outside-In model, myelin and/or oligodendrocytes could be damaged by different ways: direct virus infection, T cells, autoantibodies, and/or macrophages, or a combination. However, using both TMEV and JHMV infection of the CNS as pre-clinical models for MS (Lane and Buchmeier, 1997; Tsunoda and Fujinami, 1999), we have found that neuronal loss (TMEV) and axonal damage (TMEV and JHMV) precedes the development of demyelination with microglial cell activation (Tsunoda *et al.*, 2003; Greenberg *et al.*, 2014). Through the use of these models, there is strong support for demyelination occurring secondarily to axonal degeneration. Here, one of the first events would be an early loss of the axon (inside) leading to secondary loss of the myelin (outside) (Figure 1, Inside-Out model).

Imaging of Axonal Degeneration in the Ventral Spinal Cord in JHMV-infected Mice

Visualization of axonal damage within the spinal cord in pre-clinical viral models of MS has previously relied upon immunohistochemistry of fixed tissue. Recently, we employed our model of JHMV-induced demyelination to establish an alternative imaging system in order to provide *ex vivo* imaging of axons within the ventral murine spinal cord through use of two-photon (2P) microscopy. 2P microscopy allows for real-time visualization of cellular migration and intercellular interactions within intact organs (Germain *et al.*, 2012). 2P microscopy has been employed to characterize axonal degradation and immune cell dynamics in the dorsal spinal cord during demyelinating disease progression in mice with EAE, an autoimmune model of neuroinflammation and demyelination (Kim *et al.*, 2010; Nikic *et al.*, 2011; Nitsch *et al.*, 2004; Siffrin *et al.*, 2010). Using a novel ventral-side imaging preparation, we have now been able to visualize axons at defined times post-infection following intracranial infection of mice with JHMV (Greenberg *et al.*, 2014).

We used transgenic Thy1-YFP mice, which express yellow fluorescent protein (YFP) in a subset of medium-to-large caliber axons (Feng *et al.*, 2000; Kerschensteiner *et al.*, 2005), to

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study axonal pathology in the ventral spinal cord during the course of demyelination following JHMV infection. Lesions in the ventral spinal cord contained axons displaying a spectrum of 'focal axonal degeneration' [FAD, an established scale of axonal damage (Nikic *et al.*, 2011)] morphologies (Figure 2a). Long, continuous axons with no damage are defined as 'FAD stage 0′; FAD stage 1 axons contain focal swellings progressing along the length of the axon; and FAD stage 2 axons have gaps separating areas of YFP fluorescence. Analysis of FAD at various time points following JHMV infection revealed a progression of axonal pathology (Figure 2b), similar to studies examining FAD in the dorsal spinal cord following myelin oligodendrocyte glycoprotein immunization (Nikic *et al.*, 2011).

Although FAD reveals axonal damage and is associated with mitochondrial damage as well as demyelination (Nikic *et al.*, 2011), it is not clear if damaged axons are completely transected. Our findings, using immunofluorescence microscopy to visualize SMI-32 staining [which specifically detects a non-phosphorylated epitope in neurofilament H (Campbell and Morrison, 1989)] revealed that in areas where the axonal YFP signal was markedly diminished and fractured, SMI-32 staining often appeared punctate or absent, suggesting severely damaged axons. Additionally, we observed various stages of axonal damage along the length of a single axon (Figure 3a); including areas of intact YFP signal without SMI-32 staining (intact healthy axon), YFP signal concomitant with SMI-32 staining (intact but damaged axon), no YFP or SMI-32 signal (transected area of axon without continuous neurofilament), and SMI-32 staining without YFP signal (intact but damaged axon). The latter case indicates that, although a loss of YFP fluorescence correlates with varying degrees of axonal damage, it does not necessarily indicate complete axonal transection. We conclude that loss of YFP signal correlates with varying degrees of axonal damage, but not necessarily with an irreversibly transected axon. Whereas both FAD 1 and FAD 2 axons had areas with axonal damage, axons exhibiting loss of both YFP and SMI-32 signal at multiple loci were more common in FAD 2 axons (Figure 3b).

Axonal Degeneration Precedes Demyelination in TMEV Infection

Similar observations were seen in SJL/J mice infected with the Daniels (DA) strain of TMEV. In following the kinetics of the axonal damage in mice, we found using double-immunofluorescence confocal microscopy and using the SMI 311 cocktail of antibodies to stain non-phosphorylated, damaged axons, that SMI 311^+ axons were encased by myelin basic protein $(MBP)^+$ myelin in white matter of the spinal cord prior to the development of overt clinical disease following TMEV infection (Figure 4a–f). A similar pattern of distended or damaged axons with intact myelin sheaths has been observed in tissues from brains of MS patients (Suzuki *et al.*, 1969). As early as seven days after infection, MBP^+ myelin sheaths lacking SMI 312 labeled axons, of which SMI 312 is a pan-axonal neurofilament marker that binds normal axons, were observed. This suggests that the axons were lost or injured and thus became negative for SMI 312 reactivity (Figure 4g–j). These empty myelin sheaths were similar to empty myelin sheaths in NAWM found in MS brain tissue as described by Bjartmar and colleagues (Bjartmar *et al.*, 2001).

Since induction of axonal loss/injury in DA virus infection was not accompanied by demyelinating lesions, we investigated possible effector mechanisms that could initiate

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axonal injury during the acute phase and prior to the development of overt clinical disease. As direct virus infection of cells can cause tissue damage in the brain during TMEV infection, we tested whether virus persistence in neurons correlated with axonal injury in DA virus infection. Seven days after infection, DA virus antigens could be detected mainly in the gray matter of the brain (Tsunoda and Fujinami, 1996; 1999). We detected viral antigens in only a few neurons in the gray matter and no viral antigen containing cells were found in the white matter in the spinal cord (Figure 5a). At 14 days after infection, a small number of viral antigen positive cells were seen in the white matter (Figure 5b). A modest increase in viral antigen positive cells was seen 21 days after infection (Figure 5c). Numbers of cells containing virus antigen remained low during the chronic phase of disease (Figure 5d).

Axonal damage is a cardinal feature in experimental animal models of MS as well as in MS. Areas of axonal injury can be found in NAWM. Interestingly, we found no correlation between axonal injury and areas of perivascular cuffing, as detected by conventional myelin staining (Luxol fast blue staining). To explore further a possible link between axonal injury and inflammation, lectin staining and immunohistochemistry were used to identify activated microglial cells/macrophages and T cells, respectively. *Ricinus communis agglutinin* (RCA) I lectin staining was used to identify monocyte/macrophage lineage cell-types (Suzuki *et al.*, 1988; Tsunoda *et al.*, 1996; Tsunoda *et al.*, 1997). Seven days after DA virus infection, small round RCA I^+ cells were seen mostly in the gray matter of the spinal cord. In the anterior horn of the spinal cord, collections of RCA I^+ microglia were present around dying neurons (neuronophagia; Figure 6a, inset). Fourteen days after infection, RCA I^+ cells disappeared from the gray matter, but appeared as clusters in the white matter (Figure 6b). Glial stars made up of RCA I^+ microglial cells were present with SMI 311^+ axons that were swollen. By 21 days after infection, RCA I^+ microglial cells appeared larger and plumper, consistent with a more rounded phagocytic phenotype (Figure 6c). Macrophages infiltrating from the periphery were also present. During the chronic phase, RCA I^+ cells localized with the demyelinating lesions of the white matter (Figure 6d). T cells were identified by anti-CD3 staining. Seven days after TMEV infection, T cells were seen mainly in the gray matter of the spinal cord, with a small number of T cells present in the white matter. Fourteen days after infection, T cells were seen in association with glial stars and microglial cells within the ventral root exit zone (VREZ) (Figure 6e). Perivascular inflammation was not seen at 14 days post infection. Perivascular cuffs made up of T cells and macrophages were not detected in the white matter until the chronic phase of disease (Figure 6f). Axonal damage was occasionally accompanied by both microglia and T cells, but not by perivascular cuffing prior to the development of overt clinical disease.

Conclusion

Using two well-accepted viral models of MS, axonal damage is found to occur prior to myelin dissolution. We argue that the pathogenesis and initiation of MS is multifactorial where genetics as well as environmental factors (infections) may contribute to the disease initiation in some individuals. In those instances in which viral infection serves as a contributing factor, axonal injury could be a primary event with demyelination as a secondary consequence. The virus could be cleared, remain latent, persist, or manifest as an

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occult/abortive infection. Once the cascade of events is initiated, infectious virus does not need to be present and ensuing demyelination is driven by immunological mechanisms.

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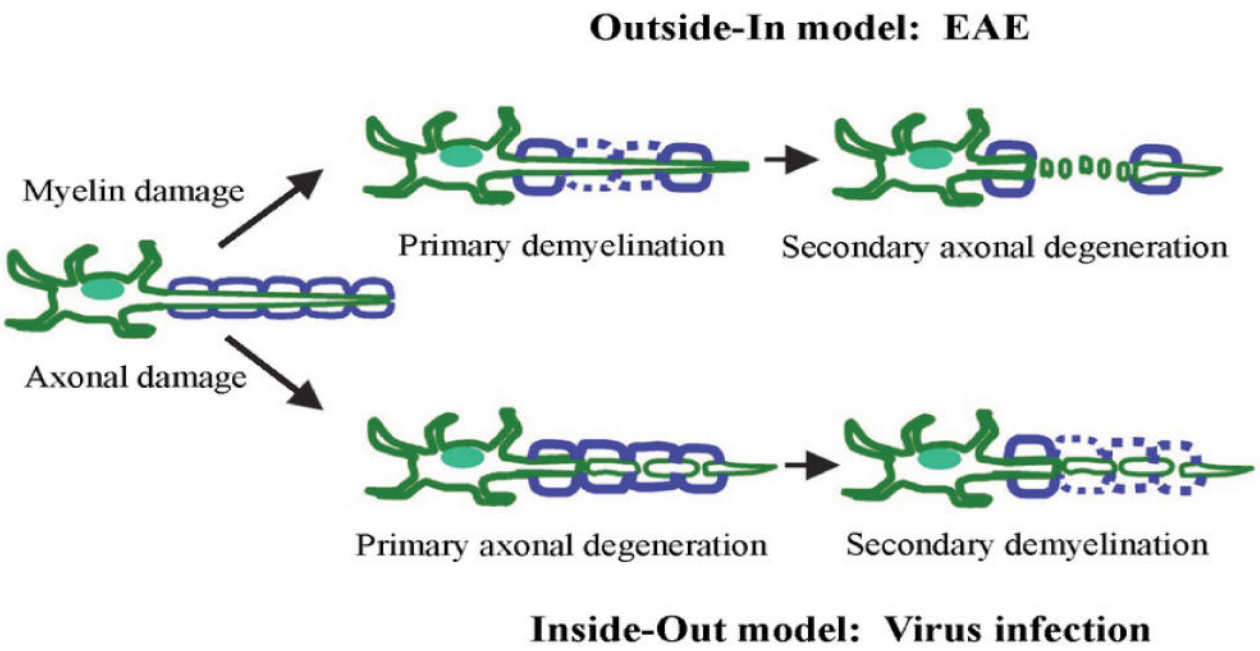

Figure 1.

Inside-Out and Outside-In models for multiple sclerosis. Virus infection can cause axonal damage that could lead to secondary demyelination with inflammation. In this context the inflammatory milieu could activate myelin specific T cells that then go on to attack myelin. The resulting myelin damage then could lead to additional axonal damage. This scenario would result in a cycle of increasing damage in the CNS with concomitant loss of function.

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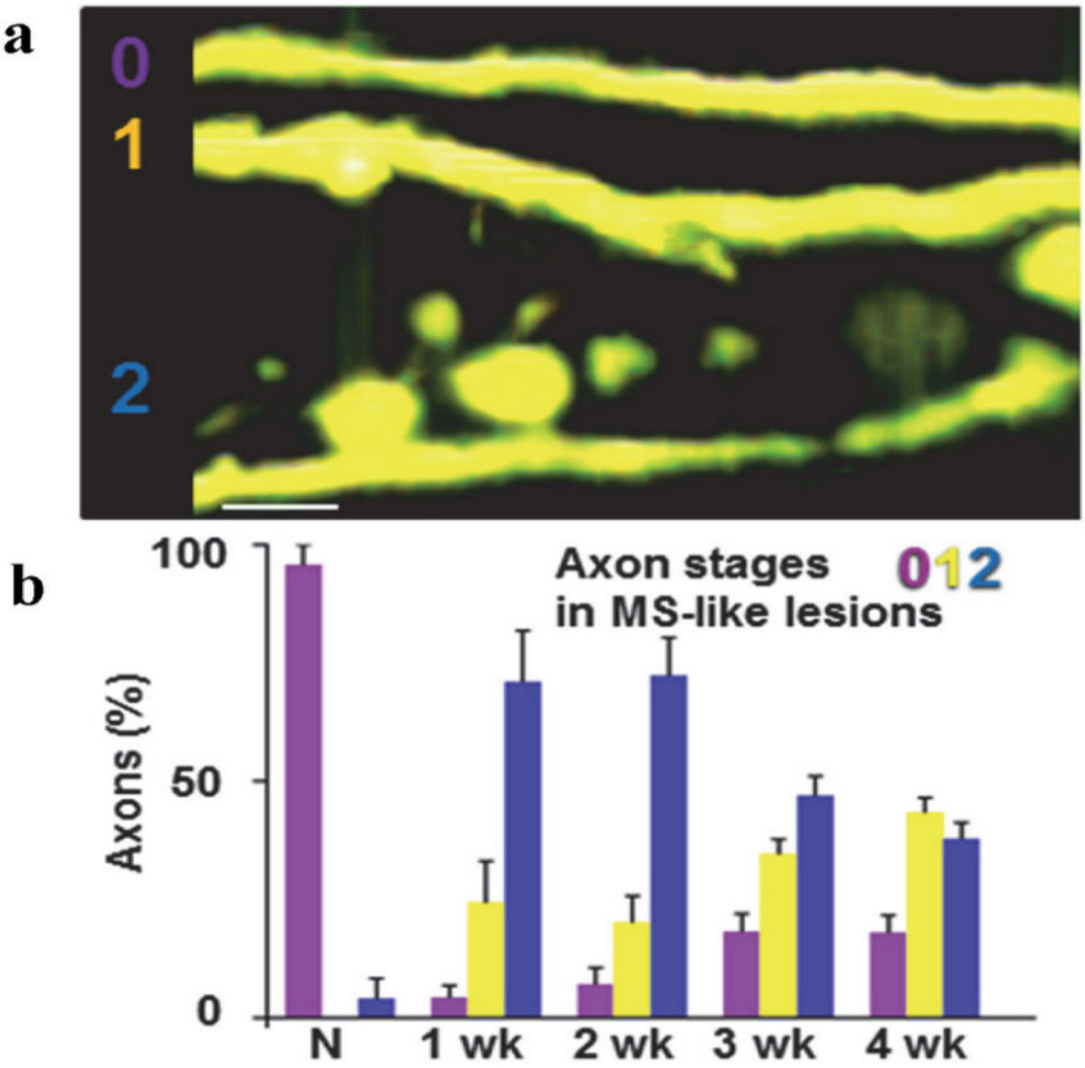

Figure 2.

Focal axonal degeneration occurs in the ventral side of JHMV-infected Thy1-YFP mouse spinal cords. (a) Representative image illustrating multiple stages of FAD (0, 1, 2, as indicated) in the ventral side of a Thy1-YFP (yellow) spinal cord 21 days following JHMV infection. Scale bar = 20 μm. (b) Proportions of axons (in % ± s.e.m.) displaying different FAD stages in normal spinal cord (N) and in lesioned regions 1–4 weeks (wk) after JHMV infection; differences at all time points compared to control are significant, $P < 0.001$ to 0.05.

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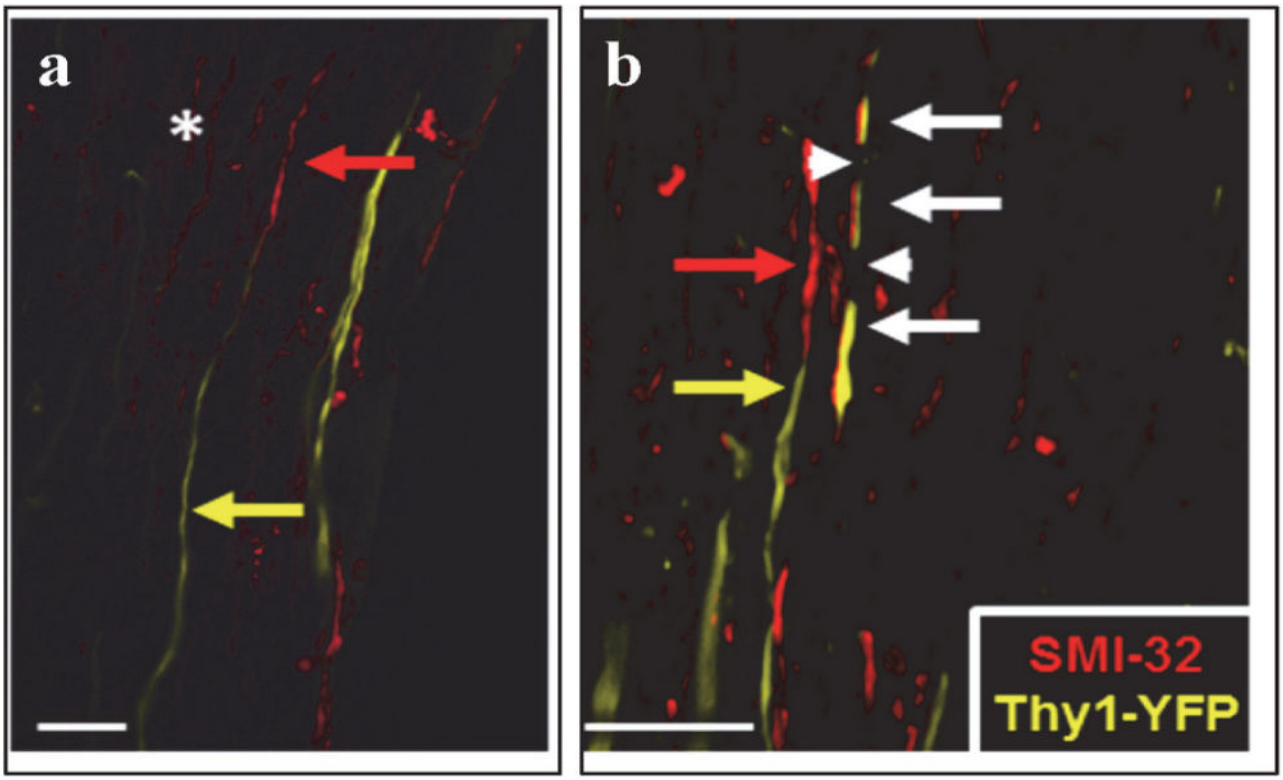

Figure 3.

Loss of YFP signal correlates with axonal damage and occasionally complete axonal bisection. (a) Loss of YFP signal does not necessarily indicate axonal bisection. Fluorescence image shows axonal regions without damage (yellow arrow) and damaged axonal regions (SMI-32^{+}) without YFP signal (red arrow). White asterisk represents lesion with multiple SMI-32^{+} axons. (b) Example of complete axonal transections (white arrowheads) adjacent to SMI-32^{+} lesions (white arrows). Scale bars = 20 μm.

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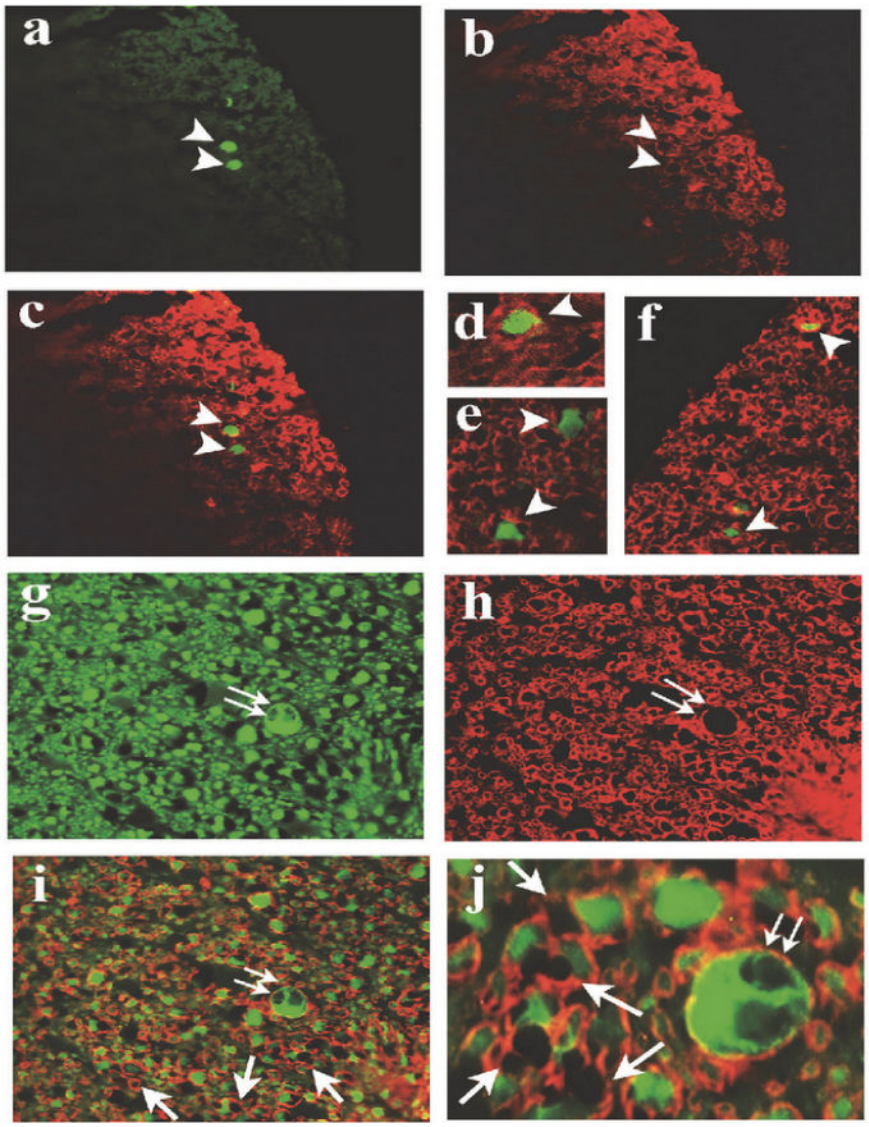

Figure 4.

Double labeling of axons in the white matter of the spinal cord 2 or 3 weeks after DA virus infection with one label detecting MBP^+ myelin (rhodamine, red). (a–f) Injured axons were detected with antibody against nonphosphorylated neurofilament protein (NFP) (fluorescein isothiocyanate, green). Axonal injury (a) (green, arrowhead) was detected in MBP^+ NAWM (b) (red). (c–f) Merged images demonstrated injured axons wrapped with MBP^+ myelin sheath. (g) Phosphorylated NFP-labeling (green) visualized not only normal axons but also distended axons (double arrows) that were wrapped with MBP^+ myelin sheath (red) (h). (i, j) Merged images demonstrated that some myelin sheaths lacked axonal staining (empty myelin, arrow) among intact myelinated axons. Reprinted, with permission (Elsevier), from Tsunoda *et al*., *Am J Pathol* 162(4):1259–1269, 2003.

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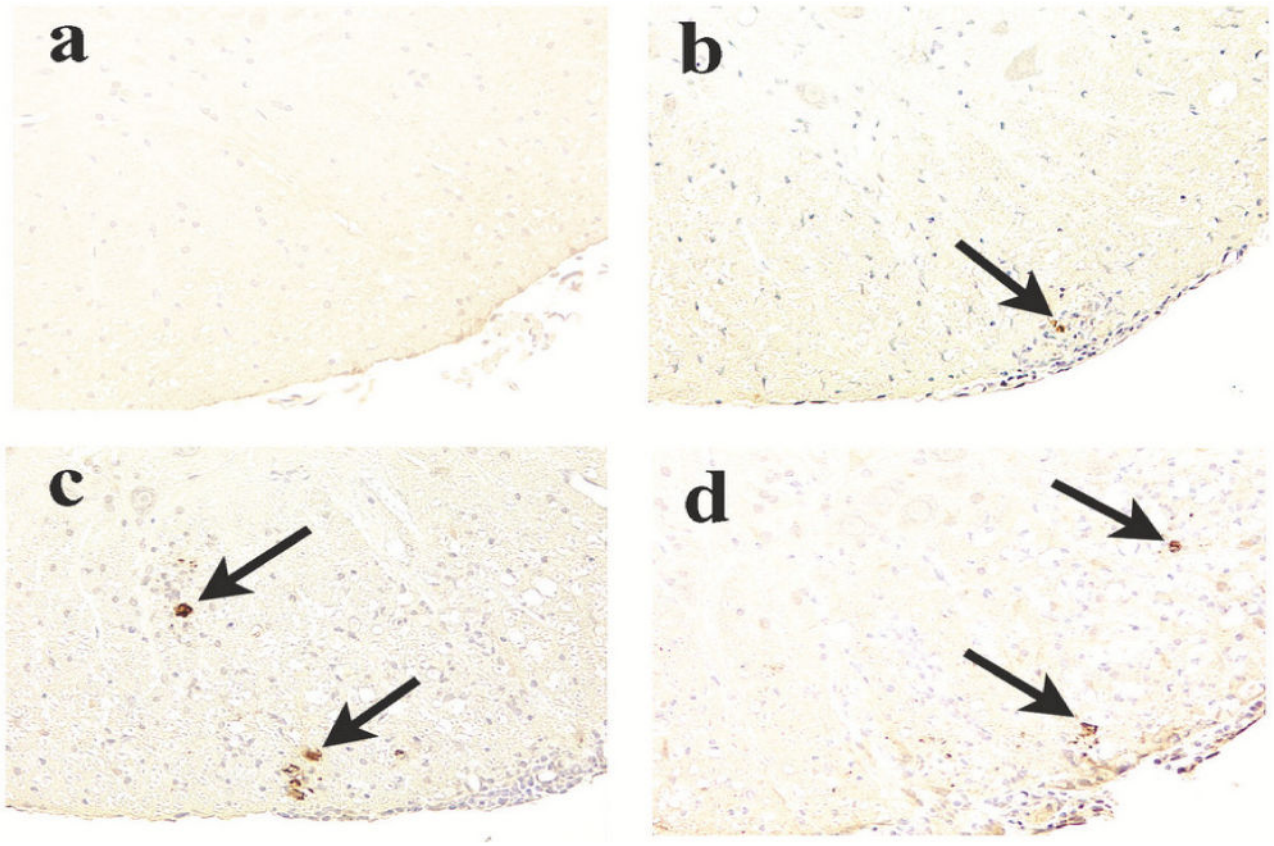

Figure 5.

Time-course study of DA virus persistence. (a) One week after infection virus antigen was not detected in the spinal cord. (b) A small number of virus antigen^{+} cells (arrow) were detected in the VREZ, 2 weeks after infection. (c) A slight increase of virus antigen^{+} cells was noted at 3 weeks after infection. (d) DA virus persistence remained at low levels during the chronic phase. Modified and reprinted, with permission (Elsevier), from Tsunoda *et al*., *Am J Pathol* 162(4):1259–1269, 2003.

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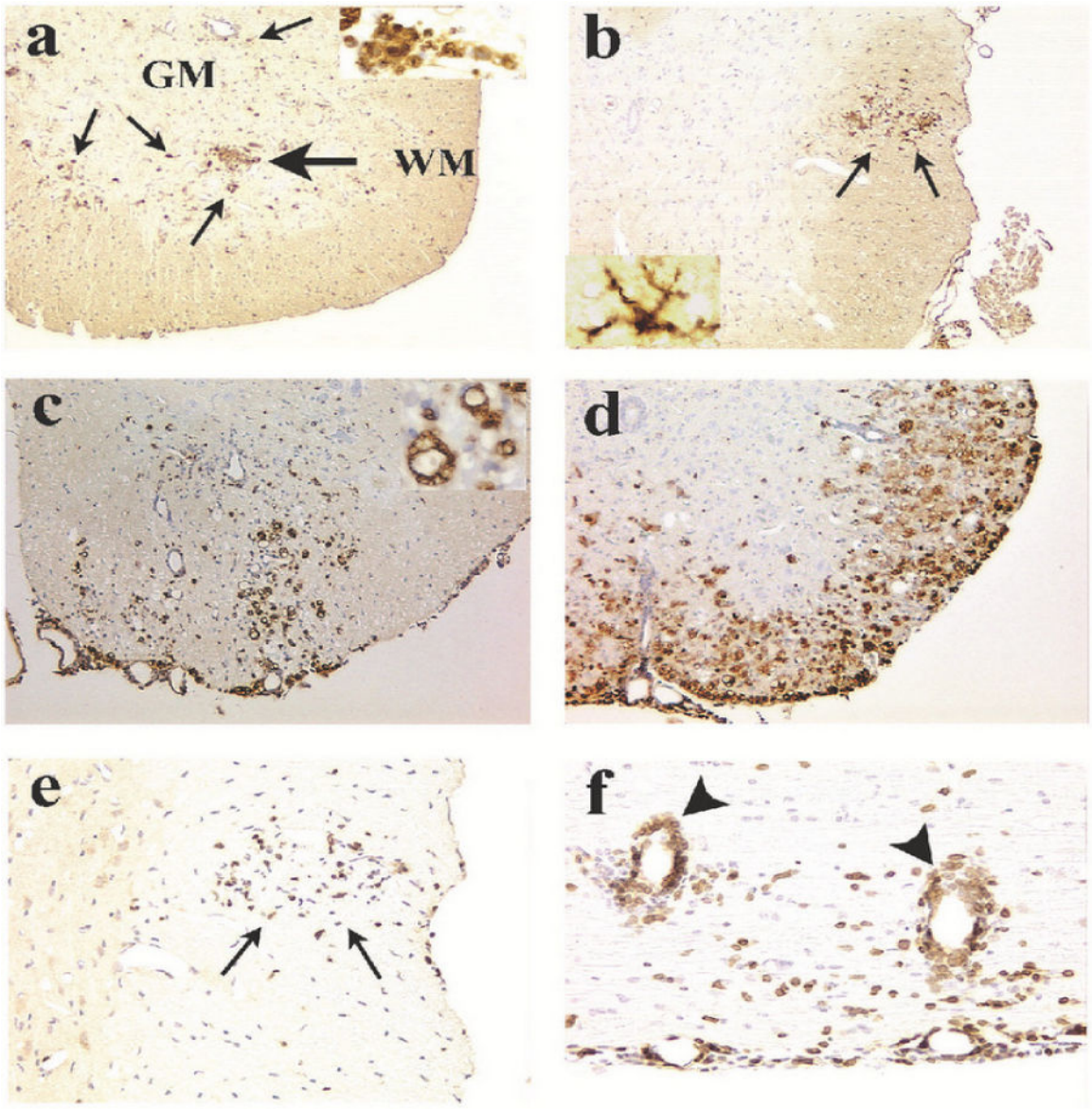

Figure 6.

Kinetics of microglia/macrophage (a–d) and T cell (e–f) infiltration in the spinal cord in DA virus infection. (a) During the acute phase, 1 week after infection, RCA I^+ microglia were detected in the gray matter (GM) (arrow), but not in the white matter (WM). In the anterior horn, RCA I^+ cells marked the positions of dead neurons (neuronophagia, large arrow, inset). (b) Two weeks after infection a cluster of RCA I^+ cells (glial star, arrow), which had dendritic processes (inset), was detected in the white matter. (c) Three weeks after infection RCA I^+ cells developed into rounded phagocytes (inset) and some RCA I^+ cells appeared to infiltrate into the parenchyma from the perivascular space and meninges. (d) During the chronic phase, a large number of RCA I^+ cells were seen in demyelinating lesion of the white matter. (e) A consecutive section of (b) showed that $CD3^+$ T cells (arrow) also infiltrated sporadically into the white matter. No perivascular cuffing was detected at this stage. (f) Typical perivascular cuffings (arrowhead) and meningitis with $CD3^+$ T cells were

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seen during the chronic phase, 1 month after infection. (a–d) RCA I lectin histochemistry; (e, f) anti-CD3 immunohistochemistry. Reprinted, with permission (Elsevier), from Tsunoda *et al*., *Am J Pathol* 162(4):1259–1269, 2003.