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Quantitation of Spinal Cord Demyelination, Remyelination, Atrophy, and Axonal Loss in a Model of Progressive Neurologic Injury

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Abstract

Spinal cord pathology, such as demyelination and axonal loss, is a common feature in multiple models of central nervous system (CNS) injury and disease. Development of methods to quantify spinal cord pathology objectively would aid studies designed to establish mechanisms of damage, correlate pathology with neurologic function, and assess therapeutic interventions. In this study, we describe sensitive methods to objectively quantify spinal cord demyelination, remyelination, atrophy, and axonal loss following the initiation of a progressive inflammatory demyelinating disease with Theiler's murine encephalomyelitis virus (TMEV). Spinal cord demyelination, remyelination, and atrophy were quantified from representative 1- μ m-thick cross sections embedded in Araldite plastic using interactive image analysis. In addition, this study demonstrates novel, automated methodology to quantify axonal loss from areas of normal-appearing white matter, as a measure of secondary axonal injury following demyelination. These morphologic methods, which are applicable to various models of CNS injury, provide an innovative way to assess the benefits of therapeutic agents, to determine mechanisms of spinal cord damage, or to establish a correlation with sensitive measures of neurologic function.

Keywords

Theiler's virus; neuropathology; morphology; multiple sclerosis; myelin diseases

INTRODUCTION

Spinal cord disease and injury can result in extensive pathology that induces neurologic dysfunction. For example, the spinal cord inflammation observed in chronic relapsing experimental autoimmune encephalomyelitis (EAE; reviewed by Martin et al., 1992) and Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease (reviewed by Rodriguez et al., 1987b) results in primary destruction of the myelin and secondary axonal loss in the spinal cord white matter. In addition, axonal degeneration has recently

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been observed in proteolipid protein-deficient mice, demonstrating that secondary pathology can occur in the absence of inflammation (Griffiths et al., 1998). In fact, central nervous system (CNS) pathology has been observed in various animal models of autoimmune diseases, viral infections, toxic injury, ischemia, spinal cord contusion, neurodegenerative disorders, and myelin deficiencies. Pathologic changes can include inflammation, demyelination, axonal injury or loss, and neuronal dropout. This CNS damage undoubtedly contributes to the observed disruption in motor coordination. Therefore, objective and sensitive measures of this pathology would aid in establishing a correlation with measures of neurologic function or in determining the effectiveness of therapeutic agents.

Development of quantitative methods to determine the histopathologic consequences of spinal cord contusion validated qualitative morphologic observations and has allowed statistical comparisons between treatment groups (Iwasaki et al., 1985; Noble and Wrathall, 1985; Iizuka et al., 1986; Blight and Decrescito, 1986; Fehlings and Tator, 1995; Rosenberg and Wrathall, 1997). For example, Rosenberg and colleagues (1999) developed a semiquantitative axonal injury index (AII) that was sensitive to the effects of injury severity. This quantitative measure was recently used to demonstrate the effectiveness of a glutamate receptor antagonist in reducing tissue loss following spinal cord injury (Rosenberg et al., 1999). This illustrates the usefulness of developing methods for quantitative morphometry.

In contrast to the case with spinal cord contusion, it is often more difficult to assess the damaging effects of a multifocal inflammatory disorder in the CNS. Lesions can be spread throughout the brain and spinal cord and contain various pathologic features. These include edema, inflammation, macrophage infiltration, myelin ovoids, demyelination, axonal degeneration/loss, and remyelination. In this study, we used Daniel's strain of TMEV to induce a chronic progressive CNS inflammatory disease with neurologic injury and demonstrate reliable methods capable of quantifying spinal cord demyelination, remyelination, atrophy, and axonal loss. This model of CNS demyelination is a progressive multifocal inflammatory process and demonstrates each of the aforementioned pathologic features in the spinal cord white matter during the chronic phase of disease.

Infection of susceptible strains of mice with TMEV results in a biphasic disease characterized by an acute neuronal infection, followed by chronic immune-mediated demyelination (Lipton, 1975). In susceptible mice, the virus persists in glial cells and, therefore, CNS pathology is limited primarily to the spinal cord white matter. Mice resistant to TMEV infection also develop the same acute neuronal infection as susceptible mice; however, the virus is cleared following this acute phase (within 21 days) and no signs of progressive spinal cord pathology or neurologic deficits are observed. Susceptible mice show secondary injury to axons following demyelination, even though the virus is cleared from neurons during the acute phase of the disease.

MATERIALS AND METHODS

Mice

SJL/J mice from The Jackson Laboratories (Bar Harbor, ME; $n = 15$) and C57BL/6 $\times$ 129/J β_2 -microglobulin-deficient mice, from R. Jaenisch of the Whitehead Institute (Cambridge,

MA; $n = 5$), were inoculated intracerebrally at 6–8 weeks of age with 2×10^6 PFU of the Daniel's strain of Theiler's virus in a 10 μ l volume. Age-matched control mice were intracerebrally sham-infected with 10 μ l of PBS. All mice analyzed in this study were at least 180 days postinjection.

Tissue Processing

All analyses involved the use of spinal cord tissue processed in the following manner. Mice were anesthetized with 10 mg of pentobarbital (ip) and perfused at 120 PSI via intracardiac puncture with 50 ml of Trump's fixative (phosphate-buffered 4% formaldehyde with 1% glutaraldehyde, pH 7.2). The spinal cords were removed, sectioned coronally into 1-mm blocks, postfixed with 1% osmium tetroxide (in 0.2 M phosphate buffer) for 1.5 hr, and embedded in Araldite (Polysciences, Warrington, PA). One-micrometer-thick cross sections were cut from the number of blocks specified in Results.

Quantitation of Spinal Cord Demyelination/Remyelination

To calculate total white matter, demyelination, and remyelination areas from 10–12 spinal cord cross sections per mouse, a Zeiss interactive digital analysis system (ZIDAS) and camera lucida attached to a Zeiss photomicroscope (Carl Zeiss Inc., Thornwood, NY) were used. Areas were defined and traced as described in Results. Total areas of lesions, Schwann cell remyelination, or oligodendrocyte remyelination were calculated by summing all respective areas traced on each of the 10–12 spinal cord sections per mouse. The percentage of spinal cord lesion area per mouse was obtained by dividing the total area of lesions by the total area of white matter sampled and multiplying by 100. The percentage of spinal cord remyelination per mouse was obtained by dividing the total area of remyelination (both Schwann cell and oligodendrocyte) by the total area of lesions and multiplying by 100.

Spinal Cord Atrophy

Areas for spinal cord atrophy assessment were calculated from 15 spinal cord sections per mouse using an Olympus AX70 microscope (1.25 $\times$ objective) fitted with a SPOT color digital camera to digitize spinal cord images. Areas shown in Figure 3 were manually traced and calculated using a program written from the KS400 image analysis software (Kontron Elektronik GmbH, Munich, Germany). Sections were classified into cervical, thoracic, lumbar, and sacral groups (C1–C7, C8–T11, T12/13–L3, L4–S1) based on location within the spinal cord. Areas were averaged and used to calculate standard errors. Statistical comparisons between areas were performed using unpaired Student's *t*-tests ($P < 0.05$).

Myelinated Axonal Area Distributions

To calculate myelinated axon area frequencies, a 1- μ m section was cut from the 1-mm block corresponding to T6 in each animal. To ensure an identical intensity of myelin labeling, all 1- μ m T6 sections used in the experiment were stained with the same batch of 4% paraphenylenediamine for exactly 20 min. An Olympus AX70 microscope (60 $\times$ oil objective) fitted with a SPOT color digital camera was used to digitize spinal cord images using the sampling scheme described for Figure 4. This resulted in the sampling of approximately 145,000 μ m² of white matter and an average of approximately 24,700 axons

per mouse. A program written from the KS400 image analysis software (Kontron Elektronik GmbH) was used to automate the calculation of axonal areas as described in the results and for Figure 5. To eliminate the majority of small regions that did not correspond to axons, areas less than $0.09 \mu\text{m}^2$ were excluded from the analysis. Myelinated axonal area frequencies were divided into three categories to facilitate comparisons: 0–4 μm^2 (small fibers), 4–10 μm^2 (medium fibers), and greater than 10 μm^2 (large fibers).

RESULTS

Spinal Cord Pathology Observed Following TMEV Infection

Intracerebral injection of TMEV into susceptible strains of mice results in pathologic abnormalities similar to those observed in human multiple sclerosis (MS; Lipton and Dal Canto, 1976; Lehrich et al., 1976; Dal Canto and Lipton, 1977, 1979; Rodriguez et al., 1987b). For example, compared to a group of sham-infected controls (Fig. 1A,B), susceptible SJL/J mice show prominent spinal cord demyelination and macrophage infiltration at 192 days postinfection (Fig. 1C,D). Demyelinating lesions are observed throughout the cervical and thoracic cord and contribute to the neurologic deficits, which include disruptions in motor coordination, hindlimb paralysis, spasticity, and incontinence (Lipton and Dal Canto, 1976; McGavern et al., 1998). Spontaneous myelin repair is minimal in the SJL/J strain. In addition, we have demonstrated that axon fibers are significantly disrupted in these chronic lesions (Rivera-Quinones et al., 1998). Thus, TMEV-infected SJL/J mice serve as the prototypic strain for the assessment of strategies to inhibit demyelination, prevent secondary injury to axons and neurologic deficits, or promote CNS remyelination.

In contrast to the case in SJL/J mice, we have previously reported that C57BL/6 $\times$ 129/J mice of a resistant background deficient in MHC class I (β_2 -microglobulin-deficient mice) (Fiette et al., 1993; Pullen et al., 1993; Rodriguez et al., 1993) have extensive spontaneous remyelination following demyelination (Fig. 1E,F; Miller et al., 1995). CNS fibers are remyelinated by both oligodendrocytes and Schwann cells. Additionally, spinal cord lesions are comparable in size and located in anatomically similar regions compared to susceptible SJL/J mice at 180 days postinfection; however, these mice have relatively preserved axon fibers and minimal neurologic deficits at this time point (Rivera-Quinones et al., 1998).

Quantitation of Spinal Cord Demyelination and Remyelination

We have developed methodology to quantify the amount of spinal cord demyelination and remyelination in susceptible mice using plastic-embedded cross sections stained with 4% paraphenylenediamine to visualize myelin. To obtain a representative sampling of the entire spinal cord, 1- μm cross sections were cut from every third serial 1-mm block. This resulted in the generation of 10–12 cross sections that represent samples from the cervical, thoracic, lumbar, and sacral spinal cord. In animal models where pathology is focused to specific anatomical regions or is limited in severity, the cross sectional sampling should be adjusted accordingly. A 1- μm cross section from every 1-mm serial block increases the accuracy of the methodology when pathology is minimal. We used an interactive camera lucida attached

to a photomicroscope to calculate total white matter, lesion, oligodendrocyte remyelination, and Schwann cell remyelination areas (Fig. 2).

Lesion areas—Figure 2 illustrates the methodology used to quantify the aforementioned areas. From each of the 10–12 cross sections (Fig. 2A), the total area of white matter was outlined (Fig. 2B, red outline). Lesion areas, defined as regions of white matter with demyelination or remyelination, were then traced using a 10× objective (Fig. 2C, blue outline). Regions with demyelination often contain macrophage infiltration, inflammation, and little or no paraphenylenediamine stain (Fig. 1D). In mice with extensive myelin repair, the original boundaries of the demyelinated lesions must be determined. Because oligodendrocyte-mediated remyelination results in thinner myelin sheaths, original areas of demyelination were identified as having a lighter myelin stain. Lesions that contain Schwann cell remyelination were defined as having increased space between individual axons. An example of oligodendrocyte- vs. Schwann cell-mediated remyelination is shown in Figure 1F. The larger lesion outlined in Figure 2C is almost completely remyelinated; however, notice that the paraphenylenediamine stain is lighter than in the adjacent, preserved myelin. By summing the lesion areas containing primary demyelination with or without remyelination (oligodendrocyte or Schwann cell), a measure of total lesion load can be determined.

Remyelination areas—Following assessment of total white matter and lesion areas, regions with remyelination were traced (Fig. 2D, green outline). These regions were defined as having either Schwann cell or oligodendrocyte remyelination (Fig. 2E) and can be outlined together or independently. Oligodendrocytes are able to remyelinate multiple axon fibers, and this type of remyelination results in thin myelin sheaths compared to normally myelinated axons (Fig. 2E, blue outline). Thinly myelinated axons remain tightly compacted. In contrast, Schwann cells can remyelinate only one axon fiber. This results in thicker myelin sheaths and increased space between axon fibers compared to fibers with oligodendrocyte remyelination (Fig. 2E, red outline). In addition, Schwann cell bodies and nuclei can be observed adjacent to the axons they have remyelinated. To accurately trace areas of remyelination within lesions, a 25× objective was used.

After outlining all regions of interest, total areas can be calculated for each mouse as shown in Table I, which shows data obtained using the aforementioned methodology to analyze chronically infected SJL/J and class I-deficient mice. Areas of total white matter, lesions, oligodendrocyte remyelination, and Schwann cell remyelination were used to calculate percentages of spinal cord lesions and remyelination. The percentages of spinal cord lesion areas were comparable in SJL/J (mean = 11.67 ± 2.06) and class I-deficient (mean = 11.49 ± 2.91) mice and ranged from 5% to 19%. However, the percentage of spinal cord remyelination was significantly increased in class I-deficient mice (mean = 58.16 ± 8.19) compared to SJL/J mice (mean = 7.97 ± 2.67). We have roughly estimated that 0.25 mm² of remyelination can range from approximately 25,700 axons remyelinated by oligodendrocytes to 8,400 axons remyelinated by Schwann cells. The remyelination data illustrate that the methodology is sensitive and accurate in detecting differences in size of lesion areas and extent of remyelination between two strains. Repeated measures of

remyelination areas from a mouse with extensive myelin repair revealed comparable values, differing only by 1.5%. In addition, differences between lesions areas can also be detected with similar accuracy.

Detection of Spinal Cord Atrophy

In patients with multiple sclerosis (MS), magnetic resonance imaging (MRI) has demonstrated that spinal cord atrophy occurs during the course of disease and correlates well with disability (Kidd et al., 1993, 1996; Filippi et al., 1996; Losseff et al., 1996; Stevenson et al., 1998; Lycklama a Nijeholt et al., 1998). This atrophy is presumed to be an indicator of axonal loss. Spinal cord atrophy can also occur in animal models of MS, such as during the chronic stage of TMEV infection, and is likely the result of demyelination and axonal loss. Therefore, we have developed measures for the quantitative assessment of spinal cord atrophy; emerging literature suggests the need to evaluate therapeutic interventions that prevent axonal injury.

Figure 3 illustrates the methodology used to assess spinal cord atrophy. To obtain an accurate representation of the cord, 1- μ m sections were cut from every other 1-mm serial block. Sections (approximately 15 per mouse) were then incubated in a stain (4% paraphenylenediamine) to visualize myelin and distinguish between the gray and white matter. These sections were used to trace the following areas manually from a digitized image using image analysis software: total cord area; posterior column area; gray matter area; and lateral, anterolateral, and anterior column area (Fig. 3). Areas can be compared between individual mice at specific levels of the cord or grouped together to allow comparisons of extended spinal cord regions.

Table II shows a comparison of extended spinal cord regions in 192-day-infected and sham-infected SJL/J mice. Spinal cord areas were compared at C1–C7, C8–T11, T12/13–L3, and L4–S1. A minimal amount of pathology is observed in the gray matter or dorsal columns of chronically infected SJL/J mice, so measurement of these two regions serves as a strong internal control. We have previously demonstrated that only 3% of the demyelinating lesions were in the posterior columns of SJL/J mice infected for 180 days, whereas 30%, 37%, and 30% of the lesions were in the lateral, anterolateral, and anterior columns, respectively (Rivera-Quinones et al., 1998). Compared to sham-infected mice, no reductions in the dorsal column or gray matter areas were observed at any level of the spinal cord examined in 192-day-infected SJL/J mice. In contrast, statistically significant reductions were observed in total cord and lateral/anterior column areas at several levels. This was expected, because extensive demyelination and axonal pathology are observed in the lateral and anterior columns of chronically infected SJL/J mice. These results demonstrate that spinal cord atrophy occurs following chronic demyelination and that the aforementioned methodology can be used to quantify the amount of atrophy. Assessment of atrophy by this method is also specific; reductions in spinal cord areas were observed only in anatomical regions with chronic pathology and did not occur in resistant C57BL/10 mice lacking demyelination (data not shown).

Automated Quantitation of Myelinated Axonal Areas From the Normal-Appearing White Matter

Axonal injury has been assessed from fields of normally myelinated axons in patients with MS (Davie et al., 1995, 1997; Narayanan et al., 1997; Stefano et al., 1997, 1998; Fu et al., 1998). Axonal loss can also occur in animal models of CNS disease and injury. For example, axonal pathology has been found in rodents following experimental autoimmune encephalomyelitis (EAE) (Raine and Cross, 1989) and TMEV-induced demyelinating disease (Rivera-Quinones et al., 1998). However, because descriptive pathology does not provide an accurate quantitative assessment of the number and caliber of fibers that remain, sensitive quantitative measures are needed to determine accurately the severity of axonal loss.

Axonal injury and degeneration can be an ongoing process during multifocal inflammatory demyelination and usually occurs at multiple levels of the spinal cord. Analysis of fields of myelinated axons from normal-appearing white matter provides a means to determine the severity of axonal loss with great accuracy at various time points following disease onset. Axon fibers can be lost following direct injury to the projecting neuronal cell body or following secondary injury to demyelinated axolemma. In either case, it is possible to calculate axonal fiber loss from fields of normally myelinated axons. For example, transection of a descending fiber tract at the T8 level of the spinal cord will result in one less myelinated fiber at the T6 level. The timing of this degeneration must be considered, insofar as injured or transected CNS axons are not lost immediately from a field of myelinated axons. Factors such as the type (motor vs. sensory), caliber, and length of the injured fiber in addition to the source of injury (demyelination, axonal transection, or direct neuronal damage) all affect the timing of degeneration. It is therefore essential to conduct a time course of axonal loss in individual models of CNS injury and disease before devising treatment strategies to prevent this type of pathology.

We have developed sensitive automated methodology to calculate myelinated axonal area frequencies. An example of this methodology is shown in Figures 4 and 5. One-micrometer-thick cross sections were cut from plastic-embedded 1-mm spinal cord blocks corresponding to T6. One-micrometer cross sections from any level of the cord can be used for this analysis; however, T6 was selected because it is the smallest thoracic spinal cord section and has a high white matter to gray matter ratio. Sections from each animal were stained with 4% paraphenylenediamine to visualize myelin. Fields (using a 60× objective) were then digitized from the spinal cord white matter of each cross section using the sampling scheme shown in Figure 4. Fields were collected in a clockwise manner around the cord to obtain a representative sampling of the posterior, lateral, anterolateral, and anterior columns. Images were centered between the gray matter and the meningeal surface. It is important to capture regions with minimal pathology, because automated calculation of axonal area frequencies is impossible in regions with demyelination or other extensive pathology.

After collecting the digitized fields, image analysis software was used to segment the gray values (145–255) corresponding to the axoplasm in the image. An example of the technique is shown in Figure 5. The frequency histogram in Figure 5B represents the number of pixels at each gray value (from 0 to 255) for a typical spinal cord image, shown in Figure 5A. After

segmenting the portion of the gray value frequency curve that corresponds to the axoplasm in the image (145–255; Fig. 5C,D, red), the result is the binary image shown in Figure 5E. From this image, regions corresponding to vasculature, cell bodies (glial or inflammatory), longitudinal axons, or pathology must be manually excluded. The program then uses the binary image to quantify the number and area (mm^2) of each white region in the field. These regions correspond to myelinated axons.

After calculation of myelinated axon areas from each of the fields shown in Figure 4, the data can be represented as relative frequency distributions by dividing the number of axons in any given size category by the total number of axons sampled (Fig. 5F). Data can also be represented as the absolute number of axons sampled in a given size category per unit area. Relative frequency distributions can be used to calculate the percentage of axon fibers within a specified range. For example, Table 3 shows a comparison of myelinated axon area frequencies from the lateral and anterior columns (Fig. 4, samples B–H) for a group of sham-infected and 192-day-infected SJL/J mice. The relative frequency of small fibers was comparable in 192-day-infected SJL/J mice (mean = 93.0 ± 0.5) compared to controls (mean = 89.4 ± 0.3). In contrast, the frequency of medium-sized and large fibers were reduced in 192-day-infected mice (medium-sized fiber mean = 5.62 ± 0.33 ; large fiber mean = 1.38 ± 0.14) compared to controls (medium-sized fiber mean = 6.98 ± 0.21 ; large fiber mean = 3.62 ± 0.13). These data demonstrate that medium-sized and large myelinated axon fibers are lost preferentially in chronically infected SJL/J mice and that this methodology can be used to quantify the loss.

Examining areas of normal-appearing white matter should reflect the degree of axonal loss in the demyelinated lesions. This approach is superior to examining axonal loss in the lesions because of the difficulty in distinguishing demyelinated axons from glial processes on a 1- μm section. In addition, inflammation, macrophage infiltration, and edema in the demyelinated lesions make it impossible to segment and quantify axon fibers automatically. The myelinated fibers in the normal-appearing white matter are easy to quantify using imaging technology, and the data are accurate and reproducible.

DISCUSSION

In this study, we demonstrated quantitative methods to measure spinal cord white matter pathology in a chronic, multifocal inflammatory disorder. These methods will provide a powerful means for quantifying white matter pathology in the study of CNS demyelinating (autoimmune, toxic, infectious), dysmyelinating, and degenerative disorders. We have routinely used the quantitative measures of demyelination and remyelination described in this study to assess the role of therapeutic agents in the treatment of TMEV-induced demyelinating disease. For example, we have demonstrated the benefit of immunosuppression (Rodriguez and Lindsley, 1992) and antibodies directed against CNS antigens (Rodriguez et al., 1987a, 1996; Rodriguez and Lennon, 1990; Miller et al., 1994; Asakura et al., 1995) in enhancing remyelination. We have also identified strains of mice that have spontaneous remyelination following TMEV-induced demyelinating disease (Miller et al., 1995; Miller and Rodriguez, 1996). It should also be possible to quantify spinal cord pathology in additional animal models of disease, such as amyotrophic lateral

sclerosis, EAE, and other virus-induced demyelinating diseases. In addition, quantification of axonal loss and spinal cord atrophy may be useful in the study of spinal cord injury and mutant mice with myelin deficiencies. Following spinal cord contusions, significant primary and secondary axonal loss can occur in a very short period of time. This axonal loss will ultimately lead to spinal cord atrophy and paralysis. Therefore, a sensitive measure of spinal cord atrophy, such as the one described in the study, may be of use in the design of treatment strategies to preserve axon fibers. Axonal degeneration observed in myelin-deficient mice (Griffiths et al., 1998) might also be quantifiable using the described measures of spinal cord atrophy.

Recent MS literature has focused on the underlying pathology that ultimately leads to progressive motor dysfunction in patients (reviewed by Matthews et al., 1998). A greater emphasis is now being placed on the significance of permanent axonal damage during the course of inflammatory demyelination (Ferguson et al., 1997; Trapp et al., 1998). To this end, MRI and magnetic resonance spectroscopy (MRS) technologies are now being used to noninvasively determine not only the inflammatory lesion load but also reductions in cross-sectional spinal cord areas (Kidd et al., 1993, 1996; Filippi et al., 1996; Losseff et al., 1996; Stevenson et al., 1998; Lycklama a Nijeholt et al., 1998) and biochemical markers, such as N-acetyl groups (primarily N-acetylaspartate; NAA; Davie et al., 1995, 1997; Narayanan et al., 1997; Stefano et al., 1997, 1998; Fu et al., 1998). Insofar as reductions in cross-sectional spinal cord areas and NAA are presumed to be markers of axonal loss, noninvasive measures of these parameters will significantly advance our ability to determine how successful treatments of MS affect axonal preservation. Recent studies assessing the therapeutic benefit of recombinant interferon- β 1a in MS patients have utilized MRI to determine that the numbers of active lesions were lower in treatment groups than in controls (Anonymous, 1998). Future studies now have the ability to utilize improved imaging technology to evaluate impact on the accepted indicators of axonal loss.

Quantitative assessment of CNS pathology in MS patients highlights the usefulness of sensitive methodology in determining the factors that contribute to neurologic dysfunction and the therapies that will ultimately have the greatest clinical benefit. However, the methods described in this study are superior to imaging technology when studying animal models of CNS disease and injury, because it is possible to prove definitively what pathologic variables (demyelination, remyelination, axonal loss) change over the course of time and correlate best with measures of neurologic function. Therefore, these sensitive measures of pathology from CNS tissue will be of greater use to animal researchers primarily working with rodent models. In addition, the availability of numerous transgenic and knockout mice provides researchers with the ability to use these sensitive measures to determine the factors that contribute to pathology and neurologic deficits in various models of disease. We now have the ability to assess treatment protocols that affect individual or multiple pathologic variables. For example, combinatorial treatment strategies can be designed to limit demyelination and axonal loss while enhancing endogenous remyelination.

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Fig. 1.

Demyelination and remyelination are observed in the spinal cord white matter following TMEV-induced demyelinating disease. **A,B:** Spinal cords from uninfected mice or resistant, infected C57BL/6J mice show no signs of spinal cord demyelination. Note the normal-appearing white matter and intact myelin sheaths. **C,D:** In contrast, chronically infected susceptible SJL/J mice show extensive demyelination. Note the two defined spinal cord lesions (C) and the presence of macrophages (asterisks) and myelin debris (arrows; D). The field in D is almost completely devoid of intact myelin sheaths. **E,F:** Chronically infected class I-deficient mice also have demyelination; however, the lesions frequently show signs of myelin repair. The lesion encircled in E has almost complete remyelination. F illustrates a higher magnification field containing both oligodendrocyte and Schwann cell remyelination. The top half of the field contains mostly Schwann cell remyelination, whereas the bottom half of the field contains mostly oligodendrocyte remyelination.

Fig. 2.

Methodology used to quantify spinal cord lesions and remyelination. Spinal cord sections were stained with 4% paraphenylenediamine to visualize myelin (**A**), and total areas of white matter were outlined first (**B**, red outline). Afterwards, sections were examined for the presence of lesions (**C**, blue outline) and remyelination (**D**, green outline). Both areas were manually outlined and calculated by an automated quantification system. A 10× objective was used to quantify lesion areas accurately. Lesions containing remyelination (**D**) were analyzed at 25× and subdivided into regions containing either Schwann cell (**E**, red outline) or oligodendrocyte (**E**, blue outline) remyelination. **E** is an enlarged field captured from the area outlined in green in **D**.

Fig. 3. Methodology used to quantify spinal cord atrophy. Total cord area, posterior column area, gray matter area, and lateral/anterolateral/anterior column area were traced. Areas (mm^2) were automatically calculated for 15 spinal cord sections per mouse.

Fig. 4.

Sampling scheme used for the calculation of T6 myelinated axonal frequencies. Eight $18,071\text{-}\mu\text{m}^2$ fields (A–H) from the normal-appearing white matter were captured in distinct, clockwise anatomical regions: one in the posterior columns (A), two in the lateral columns (B,H), two in the anterolateral columns (C,G), and three in the anterior columns (D–F). All boxes are drawn to scale.

Fig. 5.

Methodology used to calculate myelinated axonal area frequencies. From digitized 60 $\times$ myelinated fields (**A**), image analysis software was used to calculate gray value frequency histograms where 0 corresponds to black and 255 corresponds to white (**B**). The gray values corresponding to the axoplasm (145–255; **C,D**, red) were segmented from the original image (**A**) to generate a binary image (**E**). The process of segmentation results in the conversion of axoplasm (145–255) to white regions. All unwanted gray values (0–144) are converted to black. The resultant binary image (**E**) was used for the automatic calculation of the number and area of white regions (axons) in the field following manual exclusion of unwanted areas. Axonal area frequency distributions can be plotted for each mouse (**F**). **F** illustrates a frequency distribution for axons of less than 5 μm^2 .

TABLE I

Quantitation of Spinal Cord Lesions and Remyelination *

Strain	Mouse number	Total white matter area (mm ²)	Area of lesions (mm ²)	Area of CNS remyelination (mm ²)	Area of PNS remyelination (mm ²)	Lesion area (%)	CNS and PNS remyelination area (%)
SJL/J	1	5.45	0.29	0.01	0.00	5.29	2.89
SJL/J	2	5.88	0.57	0.07	0.00	9.68	11.50
SJL/J	3	5.71	0.93	0.16	0.00	16.31	16.78
SJL/J	4	9.10	1.01	0.04	0.00	11.12	4.07
SJL/J	5	5.93	0.95	0.04	0.00	15.97	4.62
B6 × 129 β ₂ m	1	11.86	0.82	0.20	0.11	6.89	37.81
B6 × 129 β ₂ m	2	9.51	0.67	0.32	0.27	7.01	87.80
B6 × 129 β ₂ m	3	11.61	2.26	0.64	0.53	19.51	51.67
B6 × 129 β ₂ m	4	12.12	2.14	0.66	0.54	17.66	55.94
B6 × 129 β ₂ m	5	13.24	0.85	0.29	0.19	6.40	57.60

* Areas of total white matter, lesions, CNS remyelination, and PNS remyelination were assessed from 10–12 spinal cord cross sections in chronically infected SJL/J and class I-deficient (B6 × 129 β₂m) mice as described in Materials and Methods and Results. Percentage white matter with lesions and percentage remyelination were calculated from these areas. Data are represented as areas or percentages per mouse.

TABLE II

Quantitation of Spinal Cord Atrophy[‡]

Strain	Spinal cord level	Total cord area (mm ²)	Lateral and anterior column area (mm ²)	Dorsal column area (mm ²)	Gray matter area (mm ²)
SiL/J PBS	C1-C7	3.51 ± 0.09	1.71 ± 0.05	0.37 ± 0.01	1.43 ± 0.04
SiL/J TMEV	C1-C7	3.12 ± 0.11 [*]	1.37 ± 0.06 [*]	0.38 ± 0.03	1.37 ± 0.07
SiL/J PBS	C8-T11	2.06 ± 0.07	1.14 ± 0.04	0.23 ± 0.01	0.68 ± 0.03
SiL/J TMEV	C8-T11	1.64 ± 0.04 [*]	0.82 ± 0.02 [*]	0.21 ± 0.01	0.61 ± 0.01
SiL/J PBS	T12/13-L3	2.60 ± 0.09	1.21 ± 0.04	0.25 ± 0.01	1.14 ± 0.05
SiL/J TMEV	T12/13-L3	2.44 ± 0.10	1.04 ± 0.04 [*]	0.25 ± 0.01	1.16 ± 0.06
SiL/J PBS	L4-S1	1.19 ± 0.11	0.48 ± 0.05	0.08 ± 0.01	0.63 ± 0.06
SiL/J TMEV	L4-S1	1.09 ± 0.07	0.41 ± 0.03	0.07 ± 0.00	0.61 ± 0.04

[‡] Spinal cord areas were compared at four levels of the cord in sham (PBS)-infected (n = 7) and TMEV-infected (n = 7) SiL/J mice at 192 days postinjection as described in Methods and Results. Data are represented as areas ± SEM.

^{*} Statistically significant by Student's *t*-test (*P* < 0.05) compared to PBS controls.

TABLE III

Quantitation of Myelinated Axon Fiber Areas From the Lateral, Anterolateral, and Anterior Columns of Thoracic Cross Sections *

Strain	Total no. of fibers sampled	Relative frequency (%)		
		Small fibers	Medium-sized fibers	Large fibers
SJL/J PBS	21,109	90.26	6.32	3.42
SJL/J PBS	21,122	89.23	7.17	3.60
SJL/J PBS	21,233	89.83	6.73	3.45
SJL/J PBS	18,065	88.95	7.55	3.50
SJL/J PBS	19,022	88.73	7.16	4.11
SJL/J TMEV	17,383	91.62	6.55	1.82
SJL/J TMEV	18,864	94.01	4.88	1.11
SJL/J TMEV	20,429	92.67	5.89	1.44
SJL/J TMEV	20,029	92.57	5.96	1.47
SJL/J TMEV	16,210	94.12	4.83	1.05

* Myelinated axon fiber frequencies (Fig. 4, samples B–H) were compared between sham (PBS)-infected and TMEV-infected SJL/J mice at 192 days postinjection. Axons were divided into three size categories: small ($0\text{--}4\ \mu\text{m}^2$), medium-sized ($4\text{--}10\ \mu\text{m}^2$), and large (greater than $10\ \mu\text{m}^2$). Relative frequencies were calculated by dividing the number of axon fibers in each size category by the total number of axon fibers sampled. Frequencies are represented as percentages per mouse.