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A Comparative Analysis of Lesion Development and Intraspinal Inflammation in Four Strains of Mice Following Spinal Contusion Injury

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Abstract

Susceptibility to neuroinflammatory disease is influenced in part by genetics. Recent data indicate that survival of traumatized neurons is strain-dependent and influenced by polygenic loci that control resistance/susceptibility to experimental autoimmune encephalomyelitis (EAE), a model of CNS autoimmune disease. Here, we describe patterns of neurodegeneration and intraparenchymal inflammation after traumatic spinal cord injury (SCI) in mice known to exhibit varying degrees of EAE susceptibility [EAE-resistant (r) or EAE-susceptible (s) mice]. Spinal cords from C57BL/6 (EAE-s), C57BL/10 (EAE-r), BALB/c (EAE-r) and B10.PL (EAE-s) mice were prepared for stereological and immunohistochemical analysis at 6 hrs, 3, 7, 14, 28 or 42 days following mid-thoracic (T9) spinal contusion injury. In general, genetic predisposition to EAE predicted the magnitude of intraparenchymal inflammation but not lesion size/length or locomotor recovery. Specifically, microglia/macrophage activation, recruitment of neutrophils and lymphocytes and *de novo* synthesis of MHC class II was greatest in C57BL/6 mice and least in BALB/c mice at all times examined. However, lesion volume and axial spread of neurodegeneration were similar in C57BL/6 and BALB/c mice and were significantly greater than in C57BL/10 or B10.PL mice. Strains with marked intraspinal inflammation also developed the most intense lesion fibrosis. Thus, strain-dependent neuroinflammation was observed after SCI but without a consistent relationship to EAE susceptibility or lesion progression. Only in C57BL/6 mice was the magnitude of intraspinal inflammation predictive of secondary neurodegeneration, functional recovery or fibrosis.

Indexing Terms

strain differences; mouse; neuroinflammation; T cell; macrophage; wound healing; spinal cord injury

Rapidly emerging data on mouse genetics and genetic engineering have caused mice to become a preferred model for studying mechanisms of delayed neurodegeneration and

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plasticity/repair after traumatic spinal cord injury (Jones et al., 2002; Beattie et al., 2002; Simonen et al., 2003; Kim et al., 2003; Zheng et al., 2003; Demjen et al., 2004). Using mice, we and others have revealed a pivotal role for the immune system in both degenerative and repair processes after SCI (Bartholdi and Schwab, 1997; Jones et al., 2002; Ma et al., 2002; Noble et al., 2002; Gonzalez et al., 2003; Wells et al., 2003; Ma et al., 2004; Kerr and Patterson, 2004; Okada et al., 2004). Still, the degree to which a specific strain of mouse can or should be used to study neuroinflammation after SCI is unknown. Indeed, just as different strains of mice display distinct vulnerabilities to neuronal excitotoxicity (Schauwecker and Steward, 1997; Steward et al., 1999), we should expect that immunological influences on neuron/glia survival and tissue repair will be strain-dependent.

A recent comparative analysis of C57BL/6 and 129X1/SvJ mice showed that endogenous cellular repair/axonal regeneration following SCI were improved in 129X1/SvJ mice and was associated with an attenuated inflammatory response relative to C57BL/6 mice (Ma et al., 2004). Multiple undefined genes are responsible for impaired leukocyte recruitment in 129X1/SvJ mice (White et al., 2002). Similarly, poorly defined genetic determinants are responsible for strain-dependent cytokine and chemokine production in C57BL/6 and BALB/c mice (Charles et al., 1999; White et al., 2002; Duleu et al., 2004).

Experimental autoimmune encephalomyelitis (EAE) is a complex polygenic inflammatory disease of the CNS (Bernard, 1976; Tuohy et al., 1988; Olsson et al., 2000). In EAE, disease susceptibility is associated in part with major histocompatibility complex (MHC) loci (H-2 complex) (Linthicum and Frelinger, 1982; Fritz et al., 1985). BALB/c mice (H-2^d) are considered to be EAE-resistant while C57BL/6 (H-2^b) are EAE susceptible (Bernard, 1976; Tuohy et al., 1988). Other non-MHC-related genes also influence the onset and progression of disease. For example, in EAE susceptible mice, production of pro-inflammatory cytokines is preferentially upregulated in the CNS whereas anti-inflammatory profiles predominate in resistant strains (Maron et al., 1999).

Recent data indicate that neuronal survival in models of traumatic neurodegeneration and glutamate-mediated excitotoxicity is inversely related to a mouse or rat's susceptibility to EAE (Kipnis et al., 2001). Specifically, neuron survival is enhanced in EAE-resistant strains but is exacerbated in EAE-susceptible animals. The cellular and molecular mechanisms underlying this effect are unknown but undoubtedly are related to gene (strain)-dependent regulation of immune function in the context of neuronal injury.

Using three inbred and one congenic mouse strain with varying degrees of EAE susceptibility, we compared intraspinal inflammation and corresponding changes in lesion morphometry (e.g., myelin sparing, lesion length and volume) induced by a traumatic spinal contusion injury. Of these four strains, C57BL/6 are most commonly used for SCI research and served as a comparator for all other strains. The data reveal significant inter-strain differences in post-traumatic inflammation and lesion development with C57BL/6 mice showing the most robust inflammatory response and the largest and longest contusion lesions. For other strains, genetic susceptibility to EAE did not consistently predict the magnitude of intraspinal inflammation, the extent of secondary neurodegeneration or functional recovery. However, in all mice exhibiting robust inflammatory responses (all but BALB/c mice), dense lesion fibrosis was apparent.

MATERIALS AND METHODS

Spinal cord injuries

All surgical and postoperative care procedures were performed in accordance with The Ohio State University Institutional Animal Care and Use Committee. Age- and weight-matched

female C57BL/6 (n=33), BALB/c (n=30), C57BL/10 (n=30) and B10.PL (n=28) mice (~20g) were obtained from Harlan (Indianapolis, Indiana) or Jackson Laboratories (Bar Harbor, Maine; B10.PL mice only). All mice were anesthetized with ketamine (80mg/kg)/xylazine (10mg/kg) prior to receiving a partial vertebral laminectomy at T₉. After laminectomy, mice received a moderate spinal contusion injury (0.5 mm displacement) using the OSU electromechanical spinal contusion injury device as described previously (Jakeman et al., 2000; Jones et al., 2002; Sroga et al., 2003). Postoperatively, animals received prophylactic antibiotics (1mg/kg Gentacin/s.c.) for 5 days and 2 cc Ringer's solution (s.c.) to prevent dehydration. Bladders were voided manually twice per day for the duration of the study. Urine pH was monitored weekly throughout the study and animals were observed daily for signs of infection or abnormal wound healing at the site of surgery. There were no overt signs of infection in any mice.

Tissue processing

Mice were anesthetized (as above) then euthanized by intracardiac perfusion at 0, 6 hours, 3, 7, 14, 28, and 42 days post injury (dpi; n=3–9/time point/strain). All mice were perfused intracardially with 100 ml of 0.1M PBS (pH 7.4) followed by 100 ml of 4% paraformaldehyde. Perfused spinal cords were post-fixed via immersion in 4% paraformaldehyde for 2 hours then were rinsed and stored overnight at 4°C in 0.2M PB (pH 7.4). The next day, tissues were cryoprotected in 20% sucrose for 48 hours. Spinal cords were blocked into 1 cm segments centered on the impact site then were embedded in OCT compound (VWR International). Transverse serial sections (10µm) were cut through each 1 cm block using a Microm cryostat (HM 505 E) and collected on SuperFrost+ slides (Fisher Scientific, Fair Lawn, NJ) then stored at –20°C until use. Each slide contained sections from each strain to ensure uniformity of staining between strains.

Immunohistochemistry

Frozen sections were air-dried for 2 hours then for an additional 2 hours on a slide warmer (37°C). After rinsing (0.2M PB; 1 min), sections were overlaid with blocking serum for 1 hour at 4°C then serum was aspirated and primary antibody was incubated overnight at 4°C in humidified chambers. The next day, sections were rinsed x 3 with 0.5M Tris-buffer (pH 7.6) and biotinylated secondary antibody was incubated overnight at 4°C. Labeled sections were rinsed x3 with 0.5M TB then incubated in 6% H₂O₂/methanol for 15 min to quench endogenous peroxidases. Bound antibody was visualized using Elite-ABC reagent (Vector Laboratories, Burlingame CA) and DAB as a substrate (Vector Laboratories, Burlingame CA). Labeled sections were dehydrated through ascending alcohols, cleared in xylene then coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). Antibody specificity has been previously documented for all antibodies used in this study (see Table 1) using western blot or immunoprecipitation assays. The M1/70 clone precipitates proteins of 190kDa (CD11b) and 105kDa (CD18) from spleen (a source of macrophages) and purified macrophages (Springer et al., 1979). These proteins comprise the CD11b/CD18 (Mac-1) membrane complex. The 17A2 clone precipitates the 21 and 26kDa polypeptides and the 45 and 35kDa δ and γ chains of the T-cell receptor-CD3 complex (Miescher et al., 1990). The polyclonal rabbit anti-fibronectin antibody labels a single 230kDa protein via western blot (Kleba et al., 2002). The M5/114 antibody immunoprecipitates 35kDa, 31kDa and 28 kDa proteins corresponding to the α and β chains of I-A and I-E (MHC class II) (Bhattacharya et al., 1981). The 7/4 clone labels neutrophils from most strains of mice except BALB/c mice (Hirsch et al., 1983). Consistent with this observation, we were unable to detect 7/4 labeling in the uninjured or injured spinal cord or peripheral blood of BALB/c mice (see Results). Another neutrophil specific clone, Ly-6G (Gr-1), labels a 14–17kDa band by western blot analysis and specifically labels neutrophils in all strains (Jutila et al., 1988). Data in Figure 5 (see below) confirmed positive neutrophil labeling in each strain. To confirm specificity of

the anti-CD4 (KT174) and CD8 (YTS105.18) antibody clones, we labeled lymphoid tissues (spleen, lymph node) and naïve and injured spinal cord from nude mice (mice lacking T lymphocytes). No CD3, CD4 or CD8 labeling was present in any of these tissues (data not shown).

To ensure consistency of these protocols over time and between individual lots, specificity of labeling is always confirmed on positive control tissues. For these (and past) studies, sections of lymph node and spleen were cut from each strain providing a positive control for staining of leukocyte antigens. Additional controls for staining specificity included incubating injured spinal cord sections and positive control sections with substrate alone, ABC complex alone or secondary antibody/no primary antibody. We have previously characterized the optimal dilutions for each of the antibodies described in the context of the naïve and injured mouse spinal cord (Jakeman et al., 2000; Jones et al., 2002; Sroga et al., 2003).

Photomicrographs were captured at 1300×1030 pixels using a Zeiss Axiocam digital camera fitted to a Zeiss Axioplan 2 Imaging Microscope. Confocal images were created using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Inc.). All images were imported into Adobe Photoshop CS (Adobe Systems, Inc., San Jose, CA) where background illumination was subtracted from all images and contrast enhancement was selectively applied to help recreate the tissue as viewed through the microscope. For confocal analysis, up to 10 sequential optical sections were collected over an optical scanning plane of 10–14 μm then were stacked to create the final image. Stacked images were imported into Adobe Photoshop CS and contrast enhanced or the RGB channel was split to reveal the staining patterns of the individual fluorophores.

Quantitative analysis of myelin sparing, lesion volume and lesion length

Eriochrome cyanine (EC) staining was used to visualize intact myelin and distinguish it from gray matter and lesioned tissue. Serial sections cut over the rostrocaudal extent of the lesion were incubated in EC for 30 minutes at room temperature, washed in dH₂O then differentiated in 5% iron alum followed by borax-ferricyanide for 5–10 minutes. The injury epicenter was defined qualitatively as the spinal cord section with the least amount of myelin sparing. To calculate the area of spared myelin, images of three EC-stained sections (epicenter and sections immediately rostral and caudal) were digitized using a Zeiss Axioplan 2 Imaging microscope (Carl Zeiss Inc., North America) and an MCID Elite image analysis system (Imaging Research; St. Catharines, Ontario, Canada). For each strain, spared myelin at the impact site was expressed relative to the area of white matter measured 3 mm rostral to the site of injury (Olby and Blakemore, 1996; Rabchevsky et al., 2001). This within-animal normalization was necessary to eliminate fixation-dependent artifacts in spinal cord cross-sectional area (CSA) that can occur between specimens. Grossly visible (light microscopy of EC stain) myelin pathology was absent from rostral sections and quantitative analysis of rostral CSA was $1.3\text{--}1.4\text{ mm}^2$ in all strains ($p > 0.05$). Area of spared myelin was not normalized to CSA at the impact site since the latter was significantly different between strains at 42 dpi (ANOVA; $p < 0.05$). Had this normalization been performed, strains with smaller CSAs after SCI (e.g., C57BL/6) would yield an artificially large proportion of spared myelin.

Lesion volume was calculated using the Cavalieri method (Howard and Reed, 1998). Briefly, a point grid of known area was randomly overlaid onto printed images of transverse spinal cord sections cut at 200 μm intervals over the rostro-caudal extent of the lesion. Areas devoid of EC staining or exhibiting clear pathology were considered lesioned tissue (except for normal or residual gray matter at rostral/caudal lesion poles). Lesion volume was calculated according to the formula: $(V = T \cdot a/p \cdot \sum p)$ where T equals the distance between

sections, a/p equals calculated area per point and $\sum p$ equals the sum of points counted across all sections. Lesion length was determined by calculating the distance between the rostral and caudal-most sections devoid of visible lesion or abnormal tissue architecture.

Quantitative analysis of immunohistochemistry

Sections cut at the impact site (epicenter) and adjacent sections immediately rostral/caudal to the epicenter were analyzed from each animal/group then were averaged. Microglia/macrophage activation was quantified as described previously (Popovich et al., 1997; Popovich et al., 1999; Popovich et al., 2003). Briefly, coronal spinal cord sections labeled with M1/70 (anti-Cd11b) or M5/114.15.2 (anti-MHCII) antibodies were digitized then quantified using the MCID Elite system. Within each section, areas of positive immunoreactivity were expressed relative to the total sample area. Data are expressed as a proportional area (PA). Although PA measurements cannot differentiate more cells from larger cells expressing more antigen, both variables would increase PA measures (Popovich et al., 1997). More importantly, this approach can be standardized between users in contrast to cell counting which is not consistent or feasible due to microglia/macrophage clustering. A similar approach was used to quantify M1/70 (anti-CD11b) labeling rostral to the injury epicenter (see Fig. 7). Briefly, the dorsal columns were outlined on high-power digitized images and the PA of labeled microglia was measured as described above. Because lesion lengths were different between strains, tissues were analyzed ~2mm rostral to the rostral boundary of each lesion where visible myelin damage was absent (~3.5mm rostral to impact site).

Unlike microglia/macrophages, infiltrating lymphocytes exhibit uniform sizes and shapes with individual cells exhibiting distinct nuclear profiles. Consequently, CD4 and CD8+ lymphocytes were manually counted throughout the entire cross-section at high-power (40x) in sections cut at the impact site (3 sections/animal). Only cells exhibiting clear membrane and nuclear labeling (cresyl violet counterstain) within the parenchyma (not cells trapped in meninges or perivascular spaces) were counted. To ensure that individual cells were not double-counted on adjacent sections, profile counts were performed on sections separated by 200 μ m (average T cell diameter <20 μ m). Identical parameters were used to quantify neutrophils.

Statistics

Immunohistochemical and morphometric data were evaluated using one or two-way analysis of variance (strain and time post injury as the two factors) followed by Bonferroni post-hoc analysis. Results were considered statistically significant at $p < 0.05$. All data points represent group mean $\pm$ SEM.

RESULTS

Histological and Morphometric Analysis of the Lesion Site

Consistent with a previous report by Inman et al. (Inman et al., 2002), we observed lesion cavities in all strains during the first week post-injury. Pockets of red blood cells (RBC) were found throughout these cavities at 6 hours, revealing that acute primary trauma has a similar effect in all strains (i.e., microvascular injury and petechial hemorrhage) resulting in acute lesions of similar average length (mean lesion length in C57BL/6= 2.2mm; B10.PL=2.0mm; C57BL/10=2.6mm; BALB/c=2.6mm; $p > 0.05$). However, by 3 dpi RBC deposits persisted only in BALB/c mice (Fig. 1) – an effect that was associated with an attenuated CNS macrophage response relative to other strains (Fig. 1; also see Fig. 6 below).

After 7 days, the impact site was defined by a rim of spared white matter surrounding a centralized zone of dynamic tissue reconstruction. Specifically, although small cysts could still be seen in all strains out to 14 dpi (shown for C57BL/10 in Fig. 2A,B), a fibrous tissue matrix dominated the lesion site and was found within and surrounding former regions of cavitation and necrosis. After 14 dpi, this fibrous tissue appeared densely packed in all strains except BALB/c mice where the impact site appeared less cellular giving the newly formed matrix a “loose” appearance (with persistence of small cysts) (C57BL/6 – BALB/c comparison in Fig. 2C,D). Strain-dependent differences in fibrous tissue deposition were clearly visible by differential interference contrast (DIC) optics in EC-stained sections (C,D) and were confirmed qualitatively using anti-fibronectin antibodies (Figs. 2E,F).

By 42 dpi, lesion cavities were absent from all strains (except BALB/c where small cavities persisted). Instead, a centralized core of densely packed connective tissue containing numerous inflammatory foci was surrounded by a visible rim of (presumably) spared white matter (Fig. 3A–D). Quantitative analysis of spared myelin, lesion length and lesion volume revealed significant inter-strain differences but without a direct correlation to EAE susceptibility. Specifically, myelin was reduced to similar degrees in C57BL/6 (EAE-s) and BALB/c mice (EAE-r) (Fig. 3E). Stereological analysis of the lesion at and beyond the impact site revealed larger and longer lesions in C57BL/6 and BALB/c mice compared to C57BL/10 (EAE-r) and B10.PL (EAE-s) mice at 42dpi (Fig. 3F&G; Fig. 4). In mice with smaller lesions and increased myelin sparing at the injury site (e.g., C57BL/10 and B10.PL), there was more rapid and complete spontaneous recovery of plantar stepping and coordinated locomotion than in either BALB/c or C57BL/6 mice. Locomotor deficits were most severe in C57BL/6 mice. (*Note: Spinal cords analyzed in this study were derived from mice used to develop a novel locomotor rating scale for SCI mice. Because those raw data were submitted in a separate report (Basso et al., 2005, in revision), only the general behavioral correlations provided above are included.*)

Neutrophils

After SCI in rats, maximal neutrophil infiltration occurs within a few hours after injury then decreases 24–48 hours later (Dusart and Schwab, 1994; Taoka et al., 1997). Acute neutrophil influx also has been described in SCI mice (Bartholdi and Schwab, 1997; Tjoa et al., 2003); however, a systematic analysis of neutrophil influx has not been described over extended post-injury intervals.

Using tissue sections cut adjacent to those used for morphometric analyses (above), neutrophils were labeled with Ly-6G (Gr-1) antibodies. In all strains, neutrophils were absent from uninjured spinal cord but infiltrated the lesion within 6 hrs post injury (Fig. 5A). Surprisingly, peak neutrophil numbers were found 3–14 dpi (Fig. 5A,B) with numbers decreasing over the next 4 weeks. Still, large numbers persisted in all strains out to 42 dpi (Fig. 5A). All strains exhibited similar kinetics of neutrophil infiltration; however, the magnitude of this response was smallest in BALB/c mice (Fig. 5A,D). In all strains, neutrophils were restricted to zones of tissue degeneration at early post-injury intervals and to zones of fibrosis at later survival times.

Because prolonged neutrophil influx was unexpected, we questioned the specificity of the Ly-6G antibody in our sections. Follow-up analyses with a different neutrophil-specific clone (7/4, Serotec; labeling not shown) together with light microscopic evaluation of semi-thin (1 μ m) resin-embedded sections (Fig. 5E,F) confirmed our initial observations.

Macrophage/Microglial (CNS macrophages) activation

Previously we showed that the magnitude, time course and spatial distribution of CNS macrophage activation is similar in SCI rats and C57BL/6 mice (Popovich et al., 1997; Sroga et al., 2003). Figure 6 confirms these observations in different strains of mice. However, the magnitude of CNS macrophage activation is consistently lowest in BALB/c mice. Specifically, macrophage activation was evident by 6 hours with prominent clusters of phagocytes occupying gray matter regions by 3 dpi (Fig. 6B&C). Based on previous data, this likely heralds the onset of monocyte infiltration (Popovich and Hickey, 2001). Within the lesion epicenter, CNS macrophage activation was maximal 7–14 dpi (only 7dpi shown in Fig. 6D&E), declined thereafter, but remained elevated above uninjured controls for up to 6 weeks (Fig 6F&G). Away from the impact site, CD11b+ microglia were visible throughout the white matter in all strains (C57BL/6 vs. BALB/c shown in Fig. 7). However, within axonal pathways undergoing Wallerian degeneration (e.g., gracile fasciculus), microglia/macrophage activation was markedly attenuated in BALB/c mice relative to other strains (Fig. 7A,F,G).

MHC class II

MHC class II glycoprotein expression is limited in the normal spinal cord (Hickey et al., 1985; Popovich et al., 1997). After SCI, *de novo* expression of MHC class II is increased on resident microglia and newly recruited hematogenous macrophages (Popovich and Hickey, 2001). In the current study we found that MHC class II+ cells were restricted to the dorsal roots and meninges or within a subset of perivascular spaces within the first 6 hours post-injury (data not shown). Parenchymal labeling (absent 0–6 hrs pi) was first observed by 3 dpi on small round cells infiltrating the epicenter (data not shown). Because surrounding parenchymal cells remained MHC class II-negative, these round cells were likely hematogenous leukocytes. These latter observations were consistent for all strains.

Between 3–14 dpi, a marked increase in MHC class II expression was seen in all strains but to the greatest extent in C57BL/6 mice (Fig. 8A,B). During this time, most MHC class II+ cells exhibit microglial morphology (Fig. 8D). After 7 dpi and coincident with the onset of tissue reorganization (based on the histological appearance of fibrosis and fibronectin deposition (see text above and Fig. 2), MHC class II expression increased in all strains but was largely restricted to newly organized zones of fibrosis. Foci of densely packed MHC class II+ perivascular cell clusters co-localized to fibrotic domains. These cell clusters were most prominent in C57BL/6 mice (Fig. 8E–H) and were absent from BALB/c mice. Previously, we showed that these cell clusters contained lymphocytes, monocytes and “fibrocytes” (cells with phenotypic and functional similarities to fibroblasts, leukocytes and stem cells (Sroga et al., 2003). Fibrocyte-rich clusters were not observed until 28 dpi, were more numerous by 42 dpi (Fig. 8E–H) and were always associated with blood vessels suggesting that they steadily infiltrate chronic contusion lesions (Sroga et al., 2003). Confocal microscopic analyses revealed that most cells in these clusters were MHC class II+ but only a subset co-expressed macrophage markers (e.g., CD11b) (Fig. 8F–H).

T cells

Previously, we showed that peak T cell infiltration in SCI rats occurs 3–7 dpi but is delayed until ~7–14 dpi in mice (Popovich et al., 1997; Sroga et al., 2003; Jones et al., 2005). Here we extend those observations to include counts of different T cell subsets (CD3+, CD4+, CD8+) in multiple strains of mice over a period of 6 weeks following SCI. Similar to neutrophils, T cells were absent from uninjured spinal cord. After SCI, T cell infiltration was delayed until 14 dpi (Fig. 9A–C). Between 2–4 weeks, T cell numbers decreased then increased over the following two weeks reaching levels comparable or greater to those achieved at 14 dpi (Fig. 9). A similar biphasic T cell influx has been described in SCI rats

(Sroga et al., 2003). Although similar kinetics were evident for CD4+ and CD8+ T cells, approximately twice as many CD4+ cells were found at all times post-injury (Fig. 9B,C). In all strains except BALB/c, T cells were distributed within cell clusters associated with centralized zones of fibrosis (see Fig. 8) and were rarely found in spared white matter (Fig. 9D,E). Fewer T cells were present in BALB/c mice at all times (Fig. 9A–E).

DISCUSSION

Recent data suggests that vulnerability to immune-mediated CNS injury is genetically encoded and is related to EAE susceptibility (Kipnis et al., 2001). Consequently, we compared the evolution of secondary pathology and trauma-induced neuroinflammation in four strains of mice. Based on published data we expected to find greater intraparenchymal inflammation, larger lesions and impaired functional recovery in EAE-susceptible (EAE-s) mice (e.g., C57BL/6, B10.PL) compared to EAE-resistant (EAE-r) mice (e.g., BALB/c, C57BL/10). However, these variables correlated only in C57BL/6 mice.

Genetic susceptibility to EAE, secondary neurodegeneration and SCI

EAE is a model of autoimmune demyelination used to study human multiple sclerosis (MS). In EAE, CNS pathology is caused by macrophages and autoreactive T cells (Raine, 1984; Cross et al., 1990). The onset of neuroinflammation and subsequent neuropathology is regulated in part by genetic determinants which vary between rat and mouse strains (Fritz et al., 1985; Tuohy et al., 1988; Mendel et al., 1995). Recent data indicate that the magnitude of CNS pathology induced by excitotoxicity or trauma is attenuated in EAE-r mice and rats (Kipnis et al., 2001). In that study, Kipnis and colleagues showed that BALB/c mice (EAE-r) exhibited a T-cell dependent neuroprotective response whereas trauma- or glutamate-mediated neuronal cell loss was enhanced in C57BL/6 mice (EAE-s).

Here we show that post-traumatic inflammation was markedly reduced in BALB/c (EAE-r) mice compared to C57BL/6 (EAE-s) mice but that lesion size and length were similar between the two strains. In B10.PL mice, another EAE-s strain, profound intraspinal inflammation occurred after SCI but was associated with smaller contusion lesions and improved functional recovery relative to C57BL/6 and BALB/c mice. C57BL/10 mice (EAE-r) were similar to B10.PL mice. Thus, a correlation between EAE susceptibility and post-SCI inflammation and anatomical/functional recovery is only apparent in C57BL/6 mice.

Existing data suggest that strain variations in neuroinflammation are due to genetically-encoded differences in cytokine and chemokine synthesis (Charles et al., 1999; Ma et al., 2004). In C57BL/6 and BALB/c mice, basal chemokine and cytokine levels in lymphoid tissue are distinct. High levels of CXCL10 and IFN γ mRNA promote MHC class II expression and macrophage/T cell activation (i.e., Th1 immunity) in C57BL/6 mice. Conversely, the naive lymphoid microenvironment of BALB/c mice favors the production of cytokines and chemokines (e.g., TGF β ; IFN β) that induce Th2 immunity. Our current data support the preferential induction of Th2 immunity in SCI BALB/c mice, i.e., leukocyte numbers and MHC class II expression were dramatically reduced relative to all other strains. Although the functional implications of reduced neuroinflammation in BALB/c mice remain ambiguous, dramatic differences in RBC clearance and tissue repair (e.g., fibrosis) were evident (see Figs. 1&2).

Strain-dependent differences in CNS cytokine/chemokine signaling also may explain the attenuated microglia/macrophage response observed at and away from the injury site in BALB/c mice (see Figs. 6&7). In a number of rat and mouse SCI models, activated microglia/macrophages have been found within axonal pathways containing apoptotic

oligodendrocytes, sprouting/regenerating axons, remyelination, damaged and remodeling microvasculature and degenerating axons (Popovich et al., 1996; Zhang et al., 1996; Popovich et al., 1997; Shuman et al., 1997). In each of these studies, a “cause-effect” relationship was implied yet proof remains elusive. Still, there is no doubt that activated microglia/macrophages are functionally capable of and are ideally positioned to influence various secondary degenerative and repair processes. Based on data in Figs. 6&7, future studies should consider how macrophage-mediated outcome measures can be influenced by the strain being evaluated. For example, a recent study by Perrin et al. suggests that a deficiency in MCP-1, MIP-1 α and/or IL-1 β -mediated microglia/macrophage activation is responsible for delayed macrophage activation in Wallerian degenerating pathways of the spinal cord (Perrin et al., 2005). In that study, BALB/c mice were used. Our current data (Fig. 7) would suggest that the sluggish microglia/macrophage response and slow myelin clearance that they described is a strain-dependent phenomenon. It would be interesting to know the chemokine/cytokine cascades in each of the strains that we have examined and whether these molecules are responsible for the divergent CNS macrophage responses that we describe. This information could have significant implications for experimental SCI and our understanding of inflammatory/immune-mediated contributions to CNS injury and repair. For example, macrophages use CD11b/Mac-1 to phagocytose myelin (Slobodov et al., 2001). If SCI-associated induction of CD11b/Mac-1 is linked to discrete cytokine profiles, one would predict that site-selective blockade or injection of those cytokines would promote macrophage-mediated myelin clearance and increase feasibility of fostering axonal regeneration (Ousman and David, 2001; Perrin et al., 2005). Interestingly, in the Perrin et al. study, slow myelin clearance and attenuated macrophage activation were overcome in BALB/c mice by injecting MCP-1, MIP-1 α and/or IL-1 β (Perrin et al., 2005).

While this type of approach might be ideal for fostering axon regeneration through defined axon pathways (e.g., dorsal columns), these same pro-inflammatory stimuli and effector mechanisms could evoke the release of degradative proteases or oxidants causing collateral tissue damage (i.e., secondary neuronal/glial cell death). Based solely on our correlative evaluation of macrophage/microglial activation and morphometric indices of lesion size/length (see Figs. 3&6), inflammatory-mediated secondary injury may only be prevalent in select strains (e.g., C57BL/6 mice). It could also be argued that because spinal contusion evokes large lesions in BALB/c mice but without a robust inflammatory response, that this is an ideal strain in which to evaluate the potential of therapies that promote immune effector functions. Obviously, additional work is needed to define the molecular cascades that regulate and promote intraspinal inflammation. However, based on our current data, this endeavor will become exponentially more difficult. Indeed, we must now consider what strain should be used in order to maximize our ability to detect a specific inflammatory-mediated “cause-effect” relationship.

Prolonged neutrophil influx after SCI in mice

In SCI rats, peak neutrophil number and effector potential occurs within the first 48 hours post-injury (Taoka et al., 1997). An early peak of neutrophil influx has also been described within 24 hours of SCI in mice (although later times have not been studied) (Noble et al., 2002; Tjoa et al., 2003). Here, we find that neutrophils enter the injured mouse spinal cord within 6 hours but reach peak numbers by ~14dpi. Interestingly, intraspinal neutrophils persist in all strains for up to 6 weeks post injury (latest time examined). The failure to resolve a neutrophil response in chronically injured spinal cord suggests that normal apoptotic control mechanisms are impaired or that their recruitment to the injury site is protracted. After mouse (and rat) SCI, mechanisms that regulate neutrophil recruitment and maintenance are not well-defined. Studies are needed to determine if Mcl-1 (an anti-apoptotic protein of the bcl-2 family) and/or cytokines capable of inhibiting neutrophil

apoptosis (e.g., GM-CSF, TNF α) are up-regulated in chronically injured mouse spinal cord (Opferman et al., 2003).

The functional implications of neutrophil infiltration are unclear. Neutrophil infiltration into injured or infected tissue typically signals the onset of the inflammatory phase of wound healing. By eliminating pathogens and debriding injured and nearby healthy tissue, neutrophils play a pivotal role in restoring tissue homeostasis. However, these same neutrophil effector functions are associated with the release of proteolytic enzymes and oxidative molecules (Campbell et al., 1982; Dallegri and Ottonello, 1997). Not surprisingly, blocking neutrophil activation/entry into injured rat spinal cord improves anatomical and functional recovery (Tonai et al., 2001; Bao et al., 2004). However, recent data in EAE suggests that neutrophils have immune-regulatory functions. Through the release of nitric oxide -- a potent oxidant and putative neurotoxin (Dawson et al., 1991; Chao et al., 1992; Bal-Price and Brown, 2001) -- neutrophils can suppress pathogenic T-cell reactions (Zehntner et al., 2005). In mouse contusion lesions, neutrophils and T-cells co-localize within fibrotic zones. Whether this represents a regulatory interaction after SCI is unknown.

Strain-dependent fibrosis after SCI

The injured mouse spinal cord is histologically distinct from that of other mammals. Instead of developing necrotic lesion cavities encased by reactive astroglia, mice develop fibrous connective tissue domains throughout the injury site (Zhang et al., 1996; Kuhn and Wrathall, 1998; Steward et al., 1999; Jakeman et al., 2000). This fibrotic lesion is reminiscent of an aberrant cutaneous wound healing response associated with chronic inflammation (Turck et al., 1987; Gharaee-Kermani and Phan, 2001). With the exception of BALB/c mice, chronic cellular inflammation with histological evidence of fibrosis was present in all strains that we examined.

BALB/c mice have been described as “fibrosis resistant” whereas C57BL/6 mice are “fibrosis prone” (Kolb et al., 2002). Although these designations are not absolute (Shi et al., 1997), strain-specific differences in immune function can influence matrix production. For example, “fibrosis-prone” mice produce higher levels of pro-inflammatory and pro-fibrotic mediators like TGF- β (Baecher-Allan and Barth, 1993; Franko et al., 1997; Seabrook et al., 1998). Strain-dependent cytokine production may also be responsible for regulating the amount and type of cell(s) expressing MHC class II.

Previously we showed that CNS macrophages are the predominant MHC class II expressing cells in the injured rat spinal cord (Popovich et al., 1997; Popovich and Hickey, 2001). In SCI mice, “fibrocytes” also express MHC class II. Fibrocytes are a novel hematogenous cell (Bucala et al., 1994; Abe et al., 2001) that we have previously shown express MHC class II within the newly formed “fibrotic domains” of injured mouse spinal cord (Sroga et al., 2003). Similar to macrophages and dendritic cells, fibrocytes produce collagen and fibronectin, express MHC class II, release cytokines/chemokines and prime naïve T cell activation via antigen presentation (Abe et al., 2001). Because T cells are required for fibrocyte differentiation (Abe et al., 2001), it is tempting to speculate that the extracellular matrix/fibrotic scar that dominates the injured mouse spinal cord results from T cell/fibrocyte interactions. This hypothesis is further supported by the observation that T cell infiltration, MHC II⁺ cell cluster formation (indicative of fibrocyte influx) and fibrosis is diminutive in BALB/c mice. If fibrosis is T-cell/fibrocyte-dependent, T-cell inhibition/depletion could reduce fibrosis and improve the ability of injured axons to grow/sprout through the non-permissive environment of the injured C57BL/6 mouse spinal cord (Inman and Steward, 2003; Ma et al., 2004). Thus, fibrosis represents a potential readout of how genetically encoded differences in post-traumatic inflammation could influence repair strategies after SCI.

Conclusions

Only in C57BL/6 mice did the magnitude of spinal cord inflammation correlate with lesion size and impaired functional recovery. However, in most strains (except BALB/c), a robust inflammatory response correlated with the formation of dense fibrosis at and nearby the site of injury. Because robust inflammation and fibrosis developed without consistently predicting anatomical and functional deficits in all strains (see Table 2) we must consider how undefined genetic determinants (specific to each strain) will influence our model systems. For example, if inflammation influences secondary degeneration after SCI, one would predict that C57BL/6 mice would be ideal for studying this relationship. However, an inflammatory response of similar magnitude and composition was noted in B10.PL and C57BL/10 mice. This could indicate that the molecular control of inflammation is distinct in these strains or that the effector potential of leukocytes and glia in those strains is biased towards neuroprotection. Finally, the diminutive neuroinflammatory reaction in BALB/c mice could prove useful in defining the efficacy of proinflammatory therapies. Indeed, low levels of post-traumatic neuroinflammation in BALB/c mice paralleled by large lesions and poor functional recovery would improve our ability to see a treatment effect if one exists. As we move forward using mice to study SCI, strain must be considered in defining the diversity of immune-mediated injury/repair mechanisms.

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Fig. 1.

Comparison of C57BL/6 and BALB/c lesion epicenters 3 days after SCI. Removal of extravasated red blood cells (RBCs) is impaired in BALB/c mice and is associated with an attenuated macrophage response. EC-stained cross-sections from the impact site of a C57BL/6 (A) and BALB/c (B) mouse at 3 dpi reveal large necrotic cavities surrounded by a rim of spared white matter. In BALB/c mice (B,C,E,F), RBC deposits persist at 3 dpi (E; differential interference contrast (DIC) optics). Conversely, RBC deposits are not found in C57BL/6 (D). Immunohistochemical stains for macrophages (Mac-1; F, G;) show fewer activated macrophages at the impact site in BALB/c mice (F) compared to other strains (G; C57BL/6 mouse). Box in B illustrates region shown in high-power in C. Scale = A&B (157 μ m); C (20 μ m); D–G (15 μ m).

Fig. 2.

Acute necrotic lesion cavities undergo tissue remodeling and become invested with a fibronectin-rich extracellular matrix. By 14 dpi in all strains, large necrotic lesion cavities were absent. However, small macrophage-filled cysts persisted (arrows in A; higher power in B; shown for C57BL/10 mice; A,B). By 14 dpi in most mice, a densely packed cellular matrix (shown in C,D at 42 dpi) fills necrotic cavities. The magnitude of this response was greatest for C57BL/6 mice (C, E) and least for BALB/c mice (D,F). Note the visible difference in tissue compaction (C,D; DIC optics of EC-stained tissue) and fibronectin staining (E,F) between C57BL/6 and BALB/c mice at 42dpi. Scale = A (55 μ m), B–D (14 μ m), E,F (110 μ m).

Fig. 3. Morphometric analysis of contused spinal cord 42 dpi from C57BL/6 (A), B10.PL (B), C57BL/10 (C), and BALB/c (D) mice. Quantitative analyses of myelin sparing (E), lesion volume (F) and lesion length (G) were determined from EC stained sections as described in Material/Methods. * $p < 0.05$ vs. B10.PL and/or C57BL/10 mice ($n = 5-9$ mice/strain). Scale = 200 μ m.

Fig. 4.

Three-dimensional (3D) reconstructions of contusion lesions from randomly chosen mice with the shortest/smallest (C57BL/10; A) and longest/largest lesions (C57BL/6; B) at 42dpi (gray = outline of spinal cord; red = degenerated/abnormal tissue as defined by light microscopic analysis of EC/cresyl violet-stained tissues). Most of the differences in lesion volume and length between strains can be attributed to longer lesion extensions in the dorsal columns (see Fig. 3 and arrows in Fig. 4). However, larger primary lesions are also visible at the impact site (delimited by line under 3D image of lesion). Scale = 475 μ m.

Fig. 5.

Prolonged intraspinal neutrophil infiltration is found in all mice after SCI (A) but is significantly less in BALB/c mice ($n=3-8$ mice/time/strain; *** $p<0.001$; ** $p<0.01$ vs. C57BL/6). Neutrophils were revealed using Ly-6G antibodies [shown in B&C for B10.PL mice (low and high-power, respectively) and in D for BALB/c mice; 14 dpi]. Note the characteristic polymorphonuclear morphology of Ly-6G-stained cells (arrows in C&D; cresyl violet counter-stain). This morphology is also apparent in resin-embedded semi-thin ($1\mu\text{m}$) sections from C57BL/6 mice at 14 (E) and 28 (F) dpi (neutrophils delineated by arrows in E,F and shown at high-power in inserts). Also, note the numerous lipid-laden macrophages distributed throughout the section that give the large cells surrounding neutrophils a “spotted” appearance. Scale = B ($100\mu\text{m}$), C&D ($25\mu\text{m}$), E&F ($12\mu\text{m}$).

Fig. 6.

Time-course of CNS macrophage activation is similar between all strains but is dramatically reduced in magnitude in BALB/c mice (A; $n=3-6/\text{time/strain}$ except 42 dpi C57BL/6 where $n=9$; *, **, *** $p<0.05$, 0.01 or 0.001, respectively vs. C57BL/6). Macrophage activation in acutely injured spinal cord (3dpi) is most prevalent within central spinal cord gray matter (shown for C57BL/6 (B) and BALB/c (C) mice). Peak macrophage activation occurs 7–14dpi with large rounded CD11b+ macrophages occupying most of the cross-sectional area at the impact site (only 7dpi shown; D&E). By 42dpi, an intense CNS macrophage response persists in all strains except BALB/c mice [C57BL/6 (F); BALB/c (G)]. Scale = B–G (400 μm), high-power inserts of boxed regions (100 μm).

Fig. 7.

Activated microglia delineate boundaries of axonal pathways undergoing Wallerian degeneration in all strains except BALB/c. Quantitative analysis of CNS macrophage activation (see Materials & Methods) within dorsal columns revealed marked macrophage activation in all strains except BALB/c mice ($n=6-9/\text{strain}$; 42 dpi; *** $p<0.001$ vs. C57BL/6). Low-power images of C57BL/6 (B) and BALB/c (C) mice taken ~ 3.5 mm rostral to the impact site. Adjacent sections stained for myelin (D&E) or anti-CD11b (F&G) reveal the strict delineation of the macrophage response within the gracile fasciculus (wedge-shaped region indicated by dotted lines in EC-stained sections in D,E). Scale = B&C (250 μm), D&E (85 μm), F&G (70 μm).

Fig. 8.

MHC class II expression is increased as a function of time post-injury on microglia/macrophages and “fibrocytes”. MHC class II expression increased during the first week post injury (epicenter sections shown for C57BL/6 and BALB/c mice at 7 dpi; B&C, respectively) peaking 14–42 dpi in all strains but to the greatest extent in C57BL/6 mice (A; $n=3-6/\text{time/strain}$ except 42 dpi in C57BL/6 mice where $n=9$; *, *** $p<0.05$ or 0.001 , C57BL/6 vs. all other strains). At 7–14 dpi, most MHC II expression is found on cells with microglial/macrophage morphology (arrows; D). By 28–42 dpi, perivascular MHC class II+ clusters formed within zones of fibrosis in all strains except BALB/c mice (shown for C57BL/6 in E; *=vessel profiles). Confocal microscopic analysis (merged image in H) of CD11b (microglia/macrophages; AlexaFluor 546/red; F) and MHCII labeling (Oregon Green 488; G) shows that most MHC class II+ cell clusters contain few macrophages. Asterisks placed in blood vessel profiles provided for orientation between images. Scale = B,C (250 μm); D&E (20 μm), F–H (40 μm).

Fig. 9.

The time-course and phenotype of infiltrating T cells is similar in all strains after SCI (cell counts of anti-CD3 labeled sections; A). However, the magnitude of this response is highest in C57BL/6 mice and lowest in BALB/c mice (A–G). Quantitative analysis of T-cell subsets shows that the CD4:CD8 ratio of infiltrating T-cells is ~ 2:1 at all times post-injury (B,C). Boxed regions in D&E shown at high power in F&G, reveal CD3+ cell morphology at 42 dpi in C57BL/6 or BALB/c mice. Scale = D&E (130 μ m), F,G (30 μ m).

Table 1

Antibodies

Antibody Clone	Source	Working dilution	Immunogen	Vendor/Lot No.	Reference
CD4 (KT174) Murine CD4 cell surface antigen	Rat	1:200/2% rabbit serum	T cell clone C5	Serotec/010	(Natsume et al., 1999)
CD8 (YTS105.18) Murine CD8 alpha chain	Rat	1:800/2% rabbit serum	Mouse spleen cells	Serotec/0599	(Qin et al., 1990)
CD3 (17A2) T cell receptor associated CD3 complex	Rat	1:400 0.2MPB	$\gamma\delta$ TCR-positive T-T hybridoma D1	BD Pharmingen/M070025	(Miescher et al., 1989)
CD11b (M1/70) Murine CD11b cell surface antigen (Mac-1)	Rat	1:200/2% rabbit serum	T cell enriched splenocytes from B10 mice	Serotec/1299	(Springer et al., 1979)
Ly-6G (Gr-1) (RB6-8C5)	Rat	1:200/5% rabbit serum	Not reported (Developed by RL Coffman at DNAX)	Caltag/07020604	(Hestdal et al., 1991)
7/4 40kDa antigen on neutrophils	Rat	1:500 0.2M PB	Cultured bone marrow cells	Serotec/M0301	(Hirsch et al. 1983)
MHCII (M5/114.15.2) α -mouse I-A/I-E	Rat	1:3000/2% rabbit serum	Activated C57BL/6 mouse spleen cells	BD Pharmingen/M060704	(Bhattacharya et al., 1981)
Fibronectin	Rabbit	1:4000/5% goat serum	Purified human fibronectin	Sigma/10K4834	(Peterszegi et al., 2002)

Table 2

Summary analysis of morphometric and immunohistochemical analyses

Strain	EAE	Myelin Sparing	Lesion volume	Lesion length	Fibrosis	PMNs	Macrophage/Microglia	MHCII	T cells
C57BL/6 (H-2 ^b)	+	3	1	1	1	1	1	1	1
B10.PL (H-2 ^u)	+	1	3	4	2	1	3	2	2
C57BL/10 (H-2 ^b)	-	1	2	3	3	2	2	2	2
BALB/c (H-2 ^d)	-	2	1	2	4	3	4	4	4

Numbers designate a qualitative ranking of measured value on a scale of 1 (*most/target*) to 4 (*least/smallest*). Strains with similar quantitative data received the same rank. Designations of mice as (+ = EAE susceptible) or (- = EAE resistant) are based on the predominant phenotype described in the literature. It should be noted that EAE can be induced in Balb/C mice under the appropriate conditions (Munoz and Mackay, 1984; Shaw et al., 1992).