Astrocytosis, microglia activation, oligodendrocyte degeneration, and pyknosis following acute spinal cord injury

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Abstract

Glial activation and degeneration are important outcomes in the pathophysiology of acute brain and spinal cord injury (SCI). Our main goal was to investigate the pattern of glial activation and degeneration during secondary degeneration in both gray matter (GM) and white matter (WM) following SCI. Adult rats were deeply anesthetized and injected with 20 nmol of N-methyl-d-aspartate (NMDA) into the ventral horn of rat spinal cord (SC) on T7. Animals were perfused after survival times of 1, 3, and 7 days. Ten-micrometer sections were submitted to immunocytochemistry for activated macrophages/microglia, astrocytes, oligodendrocytes, and myelin. Astrocyte activation was more intense in the vacuolated white matter than in gray matter and was first noticed in this former region. Microglial activation was more intense in the gray matter and was clear by 24 h following NMDA injection. Both astrocytosis and microglial activation were more intense in the later survival times. Conspicuous WM vacuolation was present mainly at the 3-day survival time and decreased by 7 days after the primary damage. Quantitative analysis revealed an increase in the number of pyknotic bodies mainly at the 7-day survival time in both ventral and lateral white matter. These pyknotic bodies were frequently found inside white matter vacuoles like for degenerating oligodendrocytes. These results suggest a differential pattern of astrocytosis and microglia activation for white and gray matter following SCI. This phenomenon can be related to the different pathological outcomes for this two SC regions following acute injury.

Keywords: Rat; Spinal cord; Glia; White matter; Apoptosis; Myelin; Vacuolation; Pyknosis

Introduction

Both experimental studies and clinical observations have contributed to the idea that the pathophysiology of spinal cord injury (SCI) can be divided in three major temporal outcomes: acute, subacute, and late phases (McDonald et al., 2002; Schwab and Bartholdi, 1996; Tator, 1995). The pathological mechanisms underlying them are either a direct consequence of the primary trauma or a destructive cascade of events called secondary degeneration (Tator, 1995; Tator and Koyanagi, 1997; Yamaura et al., 2002).

Secondary degeneration is a group of destructive events that can impact on cells that are not affected or only marginally affected by the primary pathological insult (McDonald and Sadowsky, 2002; Tator and Fehlings, 1991; Tator and Koyanagi, 1997). In the case of the central nervous system (CNS), neurons and their axons that are not affected or only partially affected by the primary pathological insult may degenerate later due to the mechanisms of secondary degeneration if not rescued by therapeutic intervention. In acute CNS diseases, a primary insult in the gray matter (GM) can spread into white matter (WM) or vice versa, significantly increasing the axonal damage with significant functional...
deficit (McDonald and Sadowsky, 2002; Schwab and Bartholdi, 1996; Tator and Koyanagi, 1997; Yamaura et al., 2002).

The mechanisms of secondary degeneration are not fully understood, but many factors can be involved (Becker et al., 2003). From these factors, inflammation and glutamate-mediated excitotoxicity are believed to be of crucial importance. The role played by glial cells during CNS secondary degeneration is not fully understood (Kreutzberg, 1996; Streit, 2000; Sykova, 2001). Microglia and astrocytes could contribute to excitotoxicity by releasing excitatory neurotoxins such as quinolinic acid and glutamate following brain and spinal cord trauma (Blight et al., 1995, 1997; Giulian et al., 1993; Hermann et al., 2001; Obrenovitch, 2001; Popovich et al., 1994, 2002).

There is strong experimental evidence that oligodendrocyte degeneration can be a very important pathological outcome, which can give rise to significant functional deficit following WM damage in both experimental and human diseases (Fowler et al., 2003; Irving et al., 1997; Li et al., 1999a,b; Matute et al., 2001; McDonald et al., 1998; Stirling and Jander, 1996; Tator and Koyanagi, 1997; Yamaura et al., 2002). Nevertheless, few authors have addressed the outcome, which can give rise to significant functional deficit (McDonald and Sadowsky, 2002; Schwab and Bartholdi, 1996; Tator and Koyanagi, 1997; Yamaura et al., 2002).

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Animals were deeply anesthetized with subcutaneous injection of Hypnorm/Hypnovel (midazolam 3.38 mg kg$^{-1}$, fentanyl citrate 0.21 mg kg$^{-1}$, fluanisone 6.75 mg kg$^{-1}$) and held in a stereotaxic frame. A homeothermic blanket unit was used to maintain body temperature. A rectal thermometer was used to monitor the animal temperature during surgery. Spinal processes of T6 and T8 were fixed in the frame, and T7 spinal process was removed. After cutting the dura, 20 nmol of N-methyl-D-aspartate (NMDA) (Sigma, USA) in 0.25 μl of sterile saline was injected into the left ventral horn over a period of 2 min using a finely drawn glass capillary needle ($N = 3–5$ per time point). The capillary was left for a further 2 min before being slowly withdrawn. Control animals (two to three per time point) were injected with the same volume of N-methyl-D-aspartate (NMDA) in sterile saline or vehicle only. We used the following stereotaxic coordinates: 0.9 mm lateral to posterior median sulcus and depth 1.5 mm from the pial surface in the dorsoventral axis. To identify the injection site, a small quantity of colanyl blue was added to both NMDA and vehicle solution. After surgery, animals were allowed to rest with water and food ad libitum during the survival times 1, 3, and 7 days.

Perfusion and histological analysis

After survival times 1, 3, and 7 days, animals were deeply anesthetized with an overdose of pentobarbitone (Sagatal) and transcardially perfused with heparinized saline followed by periodate–lysine–2% paraformaldehyde (PLP) or Bouins fixative depending on the immunocytochemistry to be performed. Spinal cords were dissected after perfusion and postfixed for 4–6 h in PLP or 1 week in Bouins fixative. PLP-perfused and postfixed tissue was cryoprotected in 30% sucrose for 24–48 h, embedded in OCT, and frozen in liquid nitrogen and isopentane. Both coronal and longitudinal 10-μm sections were cut on a cryostat; some 50-μm sections were also stained by cresyl violet staining, which allows visualization of cell body loss in the lesion area. After postfixation, Bouins fixative-perfused tissue was embedded in wax. Wax-embedded spinal cords were sectioned on a microtome at 10 μm thick along the whole extension of the ventrodorsal axis. Sections were mounted onto electrostatically charged slides in distilled water at 45°C. On average, 50 slides containing three sections each were obtained. The whole spinal cord for all experimental animals was longitudinally sectioned along the ventrodorsal axis. PLP frozen sections were

Material and methods

Experimental animals

Male adult Wistar rats were obtained from Harlan-Olac (Bicester, UK). All animals were housed under standard conditions with pelleted food and water available ad libitum. All experimental procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 under the UK Home Office license, and all efforts were made to avoid animal suffering and distress.

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mounted onto gelatinized slides. For immunohistochemical and histopathological analyses, one section (per antibody per animal) was chosen from three different sectioning levels: ventral WM (VWM), lateral WM (LWM), and dorsal WM (DWM) per antibody per animal. Using this procedure, we were able to obtain sections labeled by all antibodies used for all experimental animals. For OX42 immunohistochemistry, which did not work in wax-embedded sections (Bouins fixative), we used PLP-fixed coronal sections. For this analysis using coronal sections, we have evaluated the lesion over 7 mm along both rostral and caudal directions.

**Histopathological analysis and immunocytochemistry**

**Visualization of the lesion area**

The lesion area was visualized in 50-µm sections stained with cresyl violet for animals perfused with PLP or at 10-µm sections stained by hematoxylin and eosin (HE). The site of NMDA injection was recognized by the presence of colonym blue in the tissue and by the loss of cell bodies, as expected following an excitotoxic lesion (Dusart et al., 1991; Marty et al., 1991).

**Immunocytochemistry**

**Antibodies**

To evaluate the pattern of activation and degeneration of glial cells in both GM and WM, we performed a number of immunocytochemical procedures. For OX42 immunocytochemistry, the fixative was 4% paraformaldehyde. Bouins fixative was used for the other antibodies.

Activated macrophages/microglia were labeled using the antibody OX42 (1:2000, Serotec). This antibody recognizes the C3b receptor of complement, present on both activated and resting macrophages/microglia (Robinson et al., 1986). Astrocytes were labeled using an antibody against the glial fibrillary acid protein (GFAP, Dako, 1:2000). Oligodendrocytes were revealed thorough CNPase immunocytochemistry (Chemicon, 1:100), an enzyme highly expressed in the cytoplasm of oligodendrocytes and Schwann cells (Sprinkle, 1989). Based on previous reports that, after brain trauma and ischemia, oligodendrocytes become TAU-1-positive (Irving et al., 1996a,b, 2001), we used the TAU-1 antibody (1:500, Chemicon) to label dephosphorylated epitopes on pathological oligodendrocytes.

**Labeling protocol**

Slide-mounted sections were removed from the freezer, kept in an oven at 37°C for 30 min, and rinsed once in 0.1 M phosphate buffer saline (PBS) for 5 min. The wax sections were first deparaffinized in gradients of xylene and absolute alcohol. To improve labeling intensity, sections were then pretreated in 0.2 M boric acid (pH 9.0) previously heated to 65°C for 25 min. This temperature was maintained constant over the period of the pretreatment. Sections were further allowed to cool for about 20 min and incubated under constant agitation in 1% hydrogen peroxide solution in methanol for 20 min. Sections were then rinsed in 0.05% PBS/Tween (Sigma) solution for 3 min (three times) and incubated with 10% normal horse (OX42, MBP, TAU-1) or normal goat (GFAP) serum in PBS for 30 min. Without further rinsing, sections were then incubated with the primary antibody diluted as previously mentioned in PBS for 2 h, rinsed in PBS/Tween solution for 3 min (3 times), and incubated with the biotinylated horse antimouse (OX42, MBP, TAU-1) or goat antirabbit (GFAP) secondary antibody (Vector Laboratories) diluted at 1:100 or 1:200, respectively, in PBS for 1 h. For the TAU-1 antibody, the incubation time for the primary antibody was 24 h. As a negative control, normal horse or normal goat serum rather the primary antibody was used. Sections were rinsed again for 3 min (three times) and incubated in the complex avidin–biotin–peroxidase (ABC Kit, Vector Laboratories) for 45 min. Sections were then rinsed four times (3 min each rinse) and DAB reacted according to protocol published elsewhere (Schnell et al., 1999). After DAB reaction, sections were rinsed three times (3 min each) in 0.1 M phosphate buffer, dehydrated using alcohols and xylene, and covered-slipped. Some sections were also counterstained with hematoxylin.

**Pyknosis quantitation**

We noted significant number of pyknotic bodies in the WM of NMDA-injected animals. To quantify this finding, we counted the number of pyknotic cells visualized by hematoxylin and eosin staining. We considered the absolute number of pyknotic cells in three sections per animal per time point (N = 9). Counts were performed using sections from the three regions of spinal cord WM described below.

**Topographical analysis**

To evaluate the regional effects of NMDA injection, we performed both qualitative and quantitative analysis (counts of pyknotic bodies) in four regions of the rat spinal cord: ventral WM (VWM), lateral WM (LWM), dorsal WM (DWM), and central GM.

**Statistical analysis**

Average and standard deviation were calculated for all counts. Comparisons between different groups were assessed by analysis of variance (ANOVA)–Newman-Keuls with significance level at 0.05%. All statistical analyses were performed using the Software BioEstat 2.0 (Sociedade Civil Mamirauá/CNPQ-Brazil).
Results

Excitotoxic damage, vacuolation, and pyknosis of white matter

Injection of 20 nmol of NMDA into the ventral horn of rat spinal cord induced conspicuous excitotoxic damage, characterized by an intense inflammatory response, edema, and loss of motor neuron cell bodies as previously described (Dusart et al., 1991; Marty et al., 1991). Pallor and necrosis were present in the VWM at 1 day after NMDA injection (data not shown). At this survival time, VWM presented loss of HE staining characterizing the pallor. Necrosis was present even in the more ventral part of VWM and affected part of the LWM (not illustrated) predominantly at 3 to 7 days following NMDA injection. There was an increase in the VWM necrotic area at these later time points, in which WM cavitation was present (data not shown). DWM did not present necrosis.

Vacuolation occurred in both GM and WM, but WM was clearly more affected. In the VWM, a small number of vacuoles were present at 1 day after NMDA injection but not in the control animals (Figs. 1A–B). Vacuolation was virtually absent in the LWM and DWM at the same survival time (not illustrated). The number of vacuoles increased considerably by 3 days following NMDA injection, mainly in the caudal parts of the rat SC (Fig. 1C). Some vacuolation occurred in the LWM and was rare in the DWM. The number of vacuoles decreased considerably in the VWM at 7 days following NMDA injection (Fig. 1D). The contralateral sides of VWM and LWM presented a significant number of vacuoles only at the 3-day survival time (data not shown).

Glial activation

In both GM and WM of vehicle injected animals, OX42-positive cells presented small cell bodies and slender processes with characteristics of nonactivated microglia (Figs. 2A–B). In the animals injected with NMDA, these cells had a more intense labeling by presenting two major morphological profiles with morphological characteristics of microglial activation from 1 day after injection: cells with hypertrophic cell bodies with short pseudopodic processes and round cells presenting morphology of activated macrophages (Figs. 2C–D). The latter morphology could not be distinguished from that labeled by the ED1 antibody (used in a concomitant study) confirming that OX42-positive microglial cells become activated macrophages in pathological conditions. The round OX42-positive cells were present only in NMDA-injected animals. Nevertheless, using only morphological criteria and immunohistochemistry, it cannot be excluded that part of these cells are not blood-borne macrophages. These morphological findings were similar to the LWM and occurred in a lesser extent in the DWM.

Astrocytes activation was negligible in the ventral GM at 1 day following NMDA injection (Fig. 3B), but a number of activated astrocytes were present in the VWM at the same survival time (Fig. 4B). Astrocytes in this latter region displayed hypertrophic cell bodies with shorter and thicker processes when compared to astrocytes...
in the former one. The number of activated astrocytes in both GM and WM increased considerably at 3 and 7 days following NMDA injection (Figs. 3C–D and 4C–D). The astrocyte activation phenomenon in the WM started faster and occurred with more intensity than in the GM (Figs. 3 and 4). Astrocyte death occurred from 3 days after NMDA injections at the lesion epicenter in the VWM and adjacent areas (not illustrated). GFAP-positive debris

Fig. 2. Microglia activation following NMDA injection into the ventral horn of rat spinal cord. Camera lucida drawing of a coronal section from a vehicle-injected animal (A) and corresponding photomicrograph with emphasis in the ventral horn (B). The central canal is visible at the left top of B. Morphological aspect of resting microglia (arrow in B). Camera lucida drawing of a section obtained from the VWM of an NMDA-injected animal 24 h after injection (C) and the corresponding photomicrograph (D) about 60 μm caudal to the injection site. There is a conspicuous activation of microglia in the ventral horn (arrow in D). The inset illustrates the WM region from which sections have been taken. Scale bars: A and C = 300 μm; B and D = 50 μm.

Fig. 3. Activation of astrocytes in the central gray matter of rat spinal cord following NMDA injection into the ventral horn. Contralateral side (A) and NMDA-injected animals at 1 day (B), 3 days (C), and 7 days (D) following injection. The astrocyte activation is more conspicuous at the later survival times (arrows in C and D). The sections were counterstained with hematoxylin. The inset illustrates a region in the limit between central gray matter and lateral white matter regions from which sections have been taken. Gray matter (GM) and white matter (WM) scale bar = 50 μm.
was present in greater amounts at 3 days following NMDA injection but was completely removed at the 7-day survival time (not illustrated). In the LWM, the pattern of astrocytes activation was similar to that described for the VWM, while in the DWM, astrocytes were less hypertrophic.

Degenerating oligodendrocytes were present in the VWM and LWM up to 6 mm from the lesion epicenter in the rat spinal cord at 3 days after NMDA injection (Fig. 5B). These pathological glial cells were frequently found inside WM vacuoles (Fig. 5B) in a pattern similar to that found for pyknotic cells (Fig. 5C). Swollen oligodendro-
cytes were also found at 7 days following the primary injury (not illustrated). In the present model of SCI, only axons were labeled by the TAU-1 antibody. Oligodendrocytes have not been labeled by this antibody, as previously reported in other pathological conditions (Irving et al., 2001).

Pyknosis in the white matter

Pyknotic cells were present in the WM of the NMDA-injected animals but not in the control ones (Figs. 5C–D). These pyknotic cells were frequently found inside vacuoles even in WM regions more than 7 mm distal from the lesion epicenter (Fig. 5C). Quantitative analysis revealed an increase in the number of pyknotic bodies mainly in the caudal parts of SC (Student $t$ test, $P < 0.05$), as a function of survival time (Table 1 and Fig. 6). VWM and LWM presented the higher number of pyknotic profiles mainly at 7 days following NMDA injection (Fig. 6). Differences between groups were assessed by ANOVA–Newman-Keuls, and the results are shown in Table 1.

Discussion

The excitotoxic model of SCI

The injection of NMDA induced intense neuronal degeneration and acute inflammation in the ventral horn and central GM of rat spinal cord. The histopathological findings were similar to those previously reported after both traumatic and excitotoxic damage to spinal cord (Carlson et al., 1998; Dusart et al., 1991; Marty et al., 1991). Both VWM and LWM presented progressive necrosis, cavitation, glial activation, and axonal damage, which are common findings in several animal models of SCI (Guth et al., 1999; Tator, 1995; Zhang and Guth, 1997) as well as following SCI in humans (Davis and Khangure, 1994; Tator and Koyanagi, 1997). The possible role of acute inflammation on axonal damage in the present model of SCI will be addressed in a separate paper. In the present study, we dealt with glial activation and degeneration comparing the results in both GM and WM.

### Table 1

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<tr>
<th>White matter region</th>
<th>Average number of pyknotic cells (±SD) per section per animal*</th>
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<td>VWM</td>
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<td>Caudal</td>
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SD = standard deviation.

* Values are averages of the number of pyknotic cells per section per animal (±SD). Sections were counted for each white matter region per animal.

* ANOVA–Newman-Keuls ($P < 0.05$).

** ANOVA–Newman-Keuls ($**P < 0.01$).
Glial activation and damage to spinal cord

In this study, there was astrocyte and microglial activation following NMDA injection, mainly at the later survival times used (3–7 days). There was a conspicuous difference in the pattern of glial activation in the GM and WM. Astrocytes first appeared reactive in the VWM, and the degree of astrogliosis seemed to be higher in the GM than in the WM. Nevertheless, the microglial activation appeared to be higher in the GM than in the WM. These differences can be related to the different neuropathological outcomes in these two CNS regions. It has been shown that, in both experimental animals and human beings, the central GM first become necrotic a few hours following the primary injury, and the WM matter is affected later about 8 h after the primary damage (Schwab and Bartholdi, 1996; Tator, 1995; Tator and Fehlings, 1991; Tator and Koyanagi, 1997). Since it has been shown that WM does not contain NMDA receptors (Yam et al., 2000), direct activation of NMDA receptors in the WM would be an improbable cause of WM damage. NMDA receptors are present on the endothelial cells of GM vasculature (Krizbai et al., 1998), and posttraumatic ischemia is an important secondary mechanism of injury in both experimental animals and humans (Tator and Fehlings, 1991; Tator and Koyanagi, 1997). Damage to blood vessels in the GM can induce damage to the adjacent WM (McDonald and Sadowsky, 2002; Tator and Fehlings, 1991). In the present study, extensive hemorrhage has not been observed, but a reasonable possibility is that NMDA molecules can bind to receptors in the endothelial cells inducing blood–brain barrier breakdown, acute inflammation, and tissue damage, as previously reported (Bolton and Perry, 1998). The expansion of the primary necrotic area is a secondary pathological phenomenon involving a multitude of mechanisms including pathological release of excitatory amino acids, free radical formation, nitric oxide (NO) synthesis, and inflammation (Lynch and Dawson, 1994; Tator, 1995; Zhang et al., 1997). Zhang et al. (1997) used several anti-inflammatory approaches to unambiguously show that the progressive necrosis subsequent to traumatic SCI is initiated and maintained by inflammatory mechanisms. In this study, it was clear that different mechanisms contribute to both primary and secondary components of tissue injury depending on the SC region. Neuronal degeneration and activated macrophages/microglia in the GM could also release cytokines and other diffusible factors like nitric oxide, which can induce bystander damage to WM or potentiate deleterious tissue damage by pathological activation of non-NMDA receptors (Banati et al., 1993; Gehrmann et al., 1995; Hermann et al., 2001; Popovich et al., 2002). The earlier and preferential microglial activation in the GM suggests that the release of neurotoxins by microglia could induce GM damage in the first hours after SCI. Microglia are believed to contribute to secondary degeneration in a number of other pathological conditions such as ischemia and amyotrophic lateral sclerosis (Aronica et al., 2001; Catania et al., 2001; Raivich et al., 1999; Stoll and Jander, 1999). In these pathological disorders, microglia can synthesize harmful soluble factors including nitric oxide (NO), free radicals, proteolytic enzymes, arachidonic acid metabolites, tumor necrosis factor (TNF-α), and interleukin-1 (IL-1) (Bal-Price and Brown, 2001; Banati et al., 1993; Minghetti and Levi, 1998; Nathan, 1987; Raivich et al., 1999; Stoll and Jander, 1999). During cerebral ischemia, microglia have been reported to release NO and IL-1, which can have a role in the pathogenesis of secondary degeneration (Bal-Price and Brown, 2001; Banati et al., 1993; Gehrmann et al., 1995; Minghetti and Levi, 1998; Nathan, 1987; Raivich et al., 1999; Schwab and Bartholdi, 1996; Stoll and Jander, 1999). Similar findings have been reported following cerebral damage using excitotoxic models (Bal-Price and Brown, 2001; Love, 1999; Obrenovitch, 2001; Tahraoui et al., 2001). Recently, Hermann et al. (2001) reported that 60 pg of TNF-α or 32 μg of kainate alone did not induce significant SC damage, which could be found with a mixture of the two substances. Hermann et al. (2001) suggested that TNF-α could potentiate AMPA-mediated neurotoxicity during rat SCI. In this model, TNF-α might act on AMPA receptors on microglia, which would induce these glial cells to release more glutamate and TNF-α, thus exacerbating the lesion outcome. Other studies have shown that microglia and/or astrocytes can release glutamate or quinolinic acid in pathological conditions (Barger and Harmon, 1997; Dawson and Dawson, 1998; Hermann et al., 2001; Piani et al., 1992). In addition, it has been shown that treatment with minocycline, a second generation tetracycline, decreases microglia/macrophage activation and proliferation (Tikka and Koistinaho, 2001; Wells et al., 2003; Yrjanheikki et al., 1999), which reduced delayed oligodendrocyte death, attenuated axonal dieback, and improved functional outcome after SCI (Lee et al., 2003; Stirling et al., 2004). The effects of minocycline may be related to reduction of macrophage synthesis of NO or a direct effect of this drug as a Ca²⁺ chelator (Stirling et al., 2004).

The earlier and more intense activation of astrocytes in the WM could also be related to the progressive necrosis of this CNS region commonly found after SCI. Astrocytes are the major glial cells of the CNS (Chanas-Sacre et al., 2000; Eng et al., 2000). During development and in the normal CNS, astrocytes play an important role in the maintenance of the CNS homeostasis (Anderson and Swanson, 2000; Raivich et al., 1999). These glial cells are present in two major morphological types: fibrillary and protoplasmatic astrocytes (Raivich et al., 1999). It has been reported that fibrillary astrocytes are easily labeled with GFAP immunocytochemistry and are predominantly present in the WM (Raivich et al., 1999). Protoplasmatic astrocytes are present in the GM and are not intensely labeled by GFAP (Raivich et al., 1999). In the present...
study, protoplasmic astrocytes were intensely labeled by GFAP immunohistochemistry. This was likely a consequence of the pretreatment of the sections used in the present investigation. Such a procedure clearly improves the intensity of GFAP labeling in both brain and spinal cord tissues (unpublished results). Our results also suggest a different activation pattern for both astrocyte populations. Fibrillar astrocytes seem to be earlier and more intensely activated than protoplasmic astrocytes, which can imply a major role for the former in WM pathology. In pathological conditions, reactive astrocytes display increased activity of various oxidative and lysosomal enzymes (Chao et al., 1996; Schwab and Bartholdi, 1996). Astrocytes can release quinolinic acid, IL-1, and NO, a mechanism that has been postulated to contribute to CNS damage induced by HIV-related neurotoxicity (Aschner, 1998; Blight et al., 1997; Dawson and Dawson, 1994, 1998; Dawson et al., 1993).

**Oligodendrocyte degeneration, vacuolation, and demyelination**

A direct role for NMDA receptors on the WM damage found in the present study would be unlikely as there are no NMDA receptors in glial cells, myelin, and axons (Agrawal and Fehlings, 1996; Garcia-Barcina and Matute, 1998; Yam et al., 2000). Nevertheless, glutamate released from degenerating neurons and glial cells in the GM or other sources in the WM could act on non-NMDA receptors, which are present in astrocytes, oligodendrocytes, and myelin (Agrawal and Fehlings, 1996; Li and Stys, 2000; Li et al., 1999b). In a number of recent papers, Stys et al. (Li and Stys, 2000; Li et al., 1999a, 2000), using an in vitro model of WM pathology, reported evidences for the role of non-NMDA receptors on the mechanisms of WM damage after trauma and ischemia (Li and Stys, 2000, 2001). These authors have shown that oligodendrocytes, astrocytes, and the myelin sheath, rather than the axonal cylinder, are preferentially damaged following injection of the glutamatergic agonists AMPA and kainate into the isolated dorsal columns of rat spinal cord. Demyelination and oligodendrocyte death were present in our study. It is possible that, in the ventral region of spinal cord, WM vacuolation, damage to myelin, and oligodendrocytes can be more important pathological events than damage to the axonal cylinder. Recently, Fowler et al. (2003) have demonstrated that AMPA induces structural damage to the cytoskeleton of axons in vivo, as well as neuronal and myelin damage, and that this occurs through AMPA receptor-mediated mechanisms.

WM vacuolation was a conspicuous finding mainly at 3 days following NMDA injection. Both VWM and LWM were affected, while DWM was virtually unaffected. Tissue vacuolation has been reported in other experimental conditions, such as ischemia (Kanellopoulos et al., 2000), trauma (Li et al., 1995), and hexachlorophene intoxication (Andreas, 1993; Hantzschel and Andreas, 2000). In the later experimental model of CNS injury, the histopathological events include cerebral edema, WM vacuolation, and oligodendrocyte injury (Andreas, 1993; Hantzschel and Andreas, 2000). The mechanisms by which vacuolation occurs are not known, but they can be related to axon and astrocyte swelling, as well as to edema inducing splitting of the myelin from the axolema (Pantoni et al., 1996; Yam et al., 1998). Vacuolation can be related to demyelination (Ludwin, 1987). Myelin vacuolation can be a direct consequence of myelin damage or appears as a secondary outcome after oligodendrocyte injury or may arise due to both factors during pathological conditions, such as in toxic and immune diseases, and viral-induced demyelination (Ludwin, 1987). In these circumstances, vacuolation generally represents separation between the myelin sheath and axolema, with subsequent fluid accumulation. In the model of cuprizone-induced demyelination, vacuolation occurs following oligodendrocyte damage (Ludwin, 1987). In these circumstances, osmotic alterations on oligodendrocytes can induce intramyelinic vacuoles with spatial periodicity (Ludwin, 1987). Nevertheless, in other pathological conditions, such as experimental allergic encephalomyelitis (EAE) and hexachlorophene-induced neurotoxicity, vacuolation can be the primary pathological event (Winchell et al., 1982). It is possible that some of the mechanisms previously discussed can be responsible for the intense WM vacuolation found in the present work. Vacuolation seems to be a reversible phenomenon, as the number of vacuoles decreased at 7 days after NMDA injection. Other authors (Andreas, 1993) have reported this vacuolation reversibility. It is not clear why the caudal part of rat spinal cord presented more vacuoles. Wallerian degeneration in the spinal cord region is a possible explanation.

Some previous studies reported that oligodendrocytes become positive for TAU-1 during brain acute disorders (Irving et al., 1996a,b, 1997, 2001). In the present study, we did not find TAU-1-positive oligodendrocytes in the WM of the injured rat SC.

A large number of pyknotic profiles were present in both VWM and LWM mainly at the later survival times and in the caudal part of rat spinal cord (Fig. 7). The condensed chromatin of the pyknotic cells is suggestive of apoptosis. Oligodendrocytes have been reported to become apoptotic at later survival times following SCI in both rodents and primates (Beattie et al., 2000; Casha et al., 2001; Crowe et al., 1997; Shuman et al., 1997; Stirling et al., 2004; Zehr et al., 2004). The pyknotic cells present at both VWM and LWM can be apoptotic oligodendrocytes. Degenerating oligodendrocytes as well as pyknotic bodies were found inside WM vacuoles (Fig. 5B), mainly at the later survival times. Nevertheless, microglial apoptosis has been reported after contusion SCI in rats (Shuman et al., 1997). Further studies using a specific double-labeling protocol for apoptotic cells (i.e., antiactive caspase 3 antibody) and oligodendrocytes, microglia, or astrocytes should be performed to
confirm which glial component is more or less susceptible to undergo apoptosis in the present model of SCI.

Conclusion

In this study, we used an excitotoxic model of acute SCI to comparatively investigate the pattern of glial activation and degeneration in both gray and white matter. We have demonstrated that the activation of WM astrocytes occurs earlier and more intensely than for GM astrocytes. These differences can influence the differential pathological outcome in the WM, which normally develop more intense necrosis and cavitation. We also suggest that microglial activation can be a pathological event more important for GM rather than for WM degeneration. A great number of pyknotic cells were found in the damaged WM, which can represent apoptosis of oligodendrocytes.

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References


