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Morphological characteristics of lectin labeled microglial cells in the ischemic rat spinal cord

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Injury to the spinal cord leads to cellular changes not only in the affected neurons but also in adjacent resident microglial cells. This study examines the microglial response to transient spinal cord ischemia induced by 8, 10 and 12 min of aorta cross-clamping linked with blood volume reduction. The microglial cells visualized by *Griffonia simplicifolia* B_4-isolectin (GSA I-B_4-HRP) were studied in vibratome sections from the lumbosacral spinal cord segments (L_3-S_2). A distinct type of morphological transformation of microglia characterized by both, increased lectin-binding and altered cell shape, was observed in all spinal cords subjected to transient ischemia. Our results show, that the activation of resident microglia, represented by their morphological transformation occurs in a graded fashion in response to different degrees of ischemic attack within 2–3 days of postischemic reperfusion. The response of microglia was, in addition, closely related to the neurological outcome examined at the end of the post-ischemic reperfusion period (1–7 days). We show that lectin labeling can serve for the continuous study of microglial morphological transformation under conditions of transient spinal cord ischemia.

Key words: spinal cord, transient ischemia, lectin histochemistry, microglia.

Introduction

The non-neuronal microglial cells that originate from monocytic/mesodermal cells are functionally related to peripheral tissue macrophages and other cells of the monocyte lineage. Like macrophages in other tissues, the normal, resting microglia appear to participate in the immune surveillance of the nervous system (Gehrman et al., 1995). However, microglial cells that invade the central nervous system during early embryonic development are quite regularly spread throughout the adult nervous tissue and thus concurrently form a population of resident cells that are separated from peripheral blood macrophages. Microglia respond quickly to neuronal damage and have possible robust effects on neurons and neuroglia. Microglial-neuronal interactions thus play

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an important role in the maintenance of neuronal microenvironment homeostasis (Bruce–Keller, 1999). A consistent feature of microglial cells is their graded activation by undergoing characteristic changes that are generally stereotypical under various pathological conditions. The rapid transformation of microglia from the resting to the activated state has been clearly shown in the selective vulnerable areas of the brain in response to different degrees of ischemic neuronal injury (Morioka et al., 1991; Gehrmann et al., 1992; 1995; Kato et al., 1994, 1995; Saganová et al., 2003). Microglial response precedes the neuronal injury by means of early morphological changes in the cell shape, release of a variety of cytotoxic agents (Giulian, 1993; Giulian et al., 1993) and a differential expression of immunologically important surface molecules (Streit et al., 1989; Morioka et al., 1992; Finsen et al., 1993). The "activation" of the resting interstitial microglial cells, manifested by their morphological changes,appears as cellular response in the spinal cord following peripheral nerve injury (Gehrmann et al., 1991; Colburn et. al., 1999), nociceptive stimulation (Molander et. al., 1997), tonic noxious stimulus (Fu et al., 1999), middle cerebral artery occlusion (Wu & Ling, 1998), and spinal cord injury (Watanabe et al., 1999). Observation supports the assumption that the various morphological appearances of microglia can be correlated with distinct neuronal functional states.

Although there is evidence that the lectin staining (Streit, 1990) is suitable for examination of the microglial response in ischemic brain region, there is only limited data on microglial response as well as on the possible lectin reaction in an ischemic spinal cord (Saganová, 1999). The purpose of this study was to examine the changes in microglial morphology as a response to transient spinal cord ischemia. Microglial activation and morphological changes in this glial pool were compared with the motor deficit and evaluated in relation to the different periods of ischemic insult and postischemic reperfusion.

Material and methods

Experiments were performed on 24 adult male Wistar rats. The animals were randomly divided into separate groups. In group 1 ($n = 14$) the rats underwent spinal cord ischemia by balloon catheter occlusion of the thoracic aorta for 8, 10 or 12 minutes with controlled mean proximal arterial pressure (MPAP) at 40 mm Hg. All surgical procedures apart from inflation of the balloon catheter were performed in the sham control group 2 ($n = 5$). Unoperated animals of group 3 ($n = 5$) served for evaluation of the effect of isolectin concentration (1/50) in the lectin histochemistry procedure.

General preparation

The methods for producing normothermic transient spinal ischemia have previously been described in detail (Coston et al., 1983; Marsala & Yaksh, 1994; Taira & Marsala, 1996). Briefly, all rats were initially anesthetized with 4% halothane in room air. After induction, rats were maintained with 1% to 1.5% halothane in air/O_2 mixture delivered by an inhalation mask. Body temperature was monitored with a rectal probe inserted 8 cm into the rectum and maintained between 37.2 °C and 37.5 °C. The tail artery was cannulated with a 22-gauge polytetrafluoroethylene catheter for monitoring the mean distal arterial pressure (MDAP) and for an intra-arterial infusion of heparin. The left femoral artery was exposed, and a Fogarty 2E balloon-tipped catheter (model CV 1035, American V. Mueller) was introduced for later advancement into the descending thoracic aorta. The left internal carotid artery was cannulated with a 20-gauge catheter and connected to a heated blood collection circuit (37.0–37.5 °C) that included a 54 cm vertical column of heparinized saline solution (1–4 U/mL of saline). When the aorta was occluded, proximal aortic blood was allowed to flow into the heparinized column, maintaining MPAP at 40 mm Hg.

Spinal cord ischemia

To induce spinal cord ischemia, the Fogarty 2F catheter in the left femoral artery was inserted retrogradely into the descending thoracic aorta 10.5 (10.8–11.4) cm from the femoral arteriotomy. The balloon was inflated with 0.05 mL of saline solution for the appropriate ischemic interval. In group 2, the balloon was not inflated. After ischemia, the balloon was deflated; in group 1, the blood from the carotid artery cannula was reinfused for 60 seconds. Finally, 0.4 mL of protamine sulfate (4 mg) was administered, catheters were removed, surgical wounds were closed, and the rats were returned to their cages for recovery. Animals were allowed to survive 1–7 days postischemia.

Motor function

Motor function was quantified by an assessment of ambulation, placing and stepping responses (Taira & Marsala, 1996). Ambulation (walking with lower extremities) was graded as follows: 0, normal (symmetrical and coordinated ambulation); 1, toes flat under body when walking but ataxia present; 2, knuckle-walking; 3, movement in lower extremities but unable to knuckle-walk; and 4, no movement, drags lower extremities. We assessed the placing/stepping reflex by dragging the dorsum of the hind paw over edge of a surface. This normally evokes a coordinating lifting and placing response, which was graded as follows: 0, normal; 1, weak; and 2, no stepping. A motor deficit index was calculated for each rat. The final index was the sum of the scores (walking with lower extremities and placing/stepping reflex). The maximum deficit was indicated by a score of 6.

Table 1. Motor index and microglial response to spinal cord ischemia of rat. [a]

Ischemia	Survival	Motor index contralat./ipsilat.	Microglial response L_3	L_4	L_5	L_6	S_{1-2}
8 min	1	5/6	+	+	+	+	+
8 min	1	5/6	+	+	+	+	+
8 min	2	1/5	+++	+++	+++	+++	+++
8 min	3	0/4	+++	+++	+++	+++	+++
10 min	2	6/6	++A	++A	++A	++A	++A
10 min	2	6/6	+++	+++A	+++A	+++A	+++A
10 min	3	0/5	+++A	+++A	+++A	+++A	++A
10 min	3	0/5	+++	+++	+++	+++	+++
10 min	5	0/0	+++	+++	+++	+++	+++
10 min	7	0/0	+	+	+	+	+
10 min	7	0/0	+	+	+	+	+
12 min	2	6/6	++A	++A	++A	++A	++A
12 min	2	6/6	++A	++A	++A	++A	++A
12 min	3	6/6	++	++	++	++	++

[a] Animals subjected to 8, 10 or 12 min ischemia with intra-ischemic control of MPAP at 40 mm Hg. Motor score: maximum deficit indicates score 6. Microglial activation response scores based on cell morphology and intensity of isolectin staining: baseline staining (−), mild response (+), moderate response (++), intense response (+++), A: amoeboid microglia/macrophages.

Histology/Lectin histochemistry

At the end of the postischemic reperfusion period the animals were reanesthetized with an overdose of pentobarbital (60 mg/kg i.v.) and transcardially perfused with saline followed by 4% paraformaldehyde in buffer (PBS). The spinal cords were stored *in situ* in a refrigerator for 24 hours and then they were removed and all postfixed in the same fixative. The L_3-S_2 segments were dissected out and then divided into the appropriate segments. Standard vibratome sections 50 μm thick were cut. Lectin staining was performed on sections using a free-floating procedure (Streit, 1990). Briefly, vibratome sections 50 μm thick were washed in 0.1 M sodium phosphate buffer (pH 6.8) containing $CaCl_2$, $MgCl_2$, $MnCl_2$ (all at 0.1 mM), and 0.1% Triton X-100 (microglia buffer). Subsequently, the sections were treated with 0.6% H_2O_2 in the microglia buffer, rinsed and incubated overnight at 4 °C with a horseradish peroxidase conjugate of *Griffonia simplicifolia* B_4-isolectin (GSA I-B_4-HRP, Sigma L-5391) diluted at 20 μg/mL in the microglia buffer. Lectin binding was developed using 3',3-diaminebenzidine tetrahydrochloride as a substrate.

Glial scoring

The assessment of microglial activation response was performed on spinal cord sections stained with lectin for five or more sections from each segmental level (L_3-S_2). The minimum distance between sections was 200 μm. The score was based on the following scale: baseline staining (·), mild response (+), moderate response (++), intense response (+++) (Colburn et al., 1999). The microglial activation response scoring was based on the observed cell morphology, local cell density and intensity of lectin labeling. Microglial cells were judged throughout the entire spinal cord gray matter with respect to its laminar distribution (Rexed, 1954; Molander, 1984).

Results

Neurological outcome

The neurological outcome for individual animals is shown in Table 1. The motor index of animals differed for the ipsilateral compared with the contralateral side. The reason for the higher ipsilateral motor index could be the short time allowed for recovery of rats following experimental surgery on the left limb. In the majority of animals with intra-ischemic control of MPAP at 40 mm Hg, spastic paraplegia developed during the first 24 hours postischemia. The recovery of the neurological status in rats subjected to 8 min ischemia started on day 2 and on day 3 postischemia in rats with 10 min ischemia. In the group with 12 min of aortic occlusion, an acute and persistent spastic paraplegia remained unchanged for 3 days following occlusion.

Lectin histochemistry

Lectin staining revealed the presence of microglial cells in all lumbosacral segments of the spinal cord affected by ischemia/reperfusion. Although differing intermediate forms of resident microglia

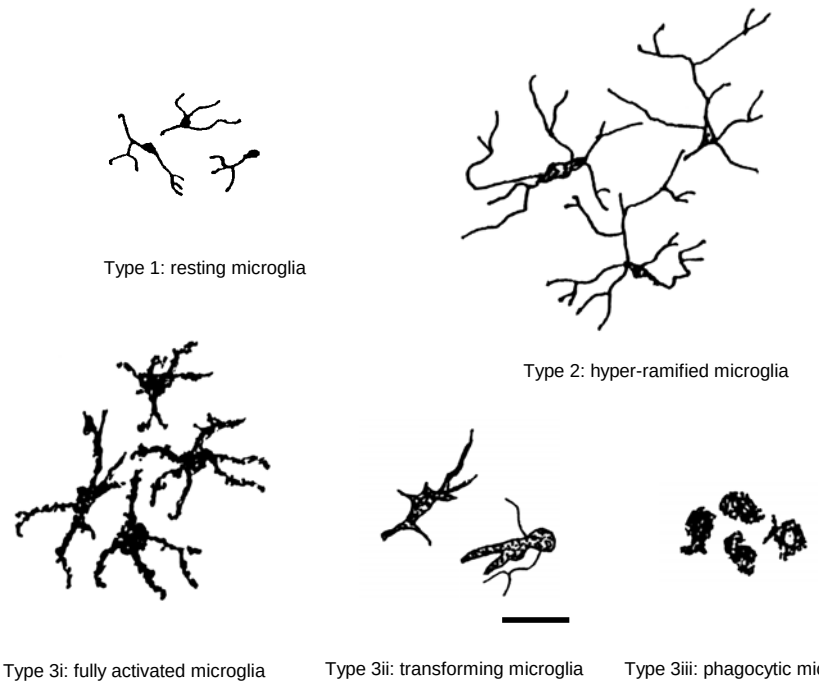

Fig. 1. Camera lucida drawing of the different stages in cellular transformation of resident microglial cells as visualized by lectin staining. Type 1: Basic staining. Weak lectin positive, solitary microglial cells with typical small cell bodies bearing a few ramified thin processes occur in the control spinal cord tissue. Type 2: Mild response. Microglial response characterized by cellular hyper-ramification and intensive lectin labeling induced by the mildest ischemic insult. Type 3: Strong response. (3i) Intensively labeled hypertrophic microglia with still long but less arborised processes can transform into (3ii) reactive microglia with enlarged cell bodies and short stout processes. (3iii) Microglial transformation can continue by the appearance of ameboid cells characterized by rounded cell bodies and the loss of processes. Bar = 25 μm.

were found in the ischemic spinal cord sections, three main types could be distinguished by means of their lectin labeling (Fig. 1): (1) basic staining type, characterized by weak perinuclear lectin staining of quiescent microglia; (2) mildly responding type, with proportional lectin labeling of hyper-ramified activated microglia similar in their morphology to fully-developed resting microglia; and (3) strongly responding type, characterized by intense lectin labeling of: (i) fully activated, hypertrophic microglia with still long but less arborised processes; (ii) transformed microglia with an enlarged cell body and short stout processes; and (iii) phagocytic, ameboid microglia characterized by rounded cells and the loss of processes.

Ischemia/Microglial response

The severity of ischemia was defined on the basis of an examination of neurological status.

Mild ischemia: 8 min aorta cross-clamping. As early as 24 h following intraluminal occlusion of the thoracic aorta increased lectin staining and morphological transformation of microglial cells were observed in the examined lumbosacral segments in comparison with resting microglia (Fig. 2A). Hyper-ramified, lectin stained microglia of type 2 were widespread in the spinal cord gray matter at this earliest examined time point (Fig. 2B). Morphological changes proceeding together with prolonged postischemic interval revealed activated, strongly lectin stained microglia with various morphological features, mostly type 2 and 3i, distributed throughout the spinal cord gray matter at day 2 (Fig. 2C, D) and day 3 postischemia. Morphological response was accompanied with an increase in the number of microglial cells distributed in the preserved spinal cord gray matter.

Moderate ischemia: 10 min aorta cross-clamping. Different degrees of microglial morphological transformation were observed throughout the spinal cord gray matter following 10 min ischemic

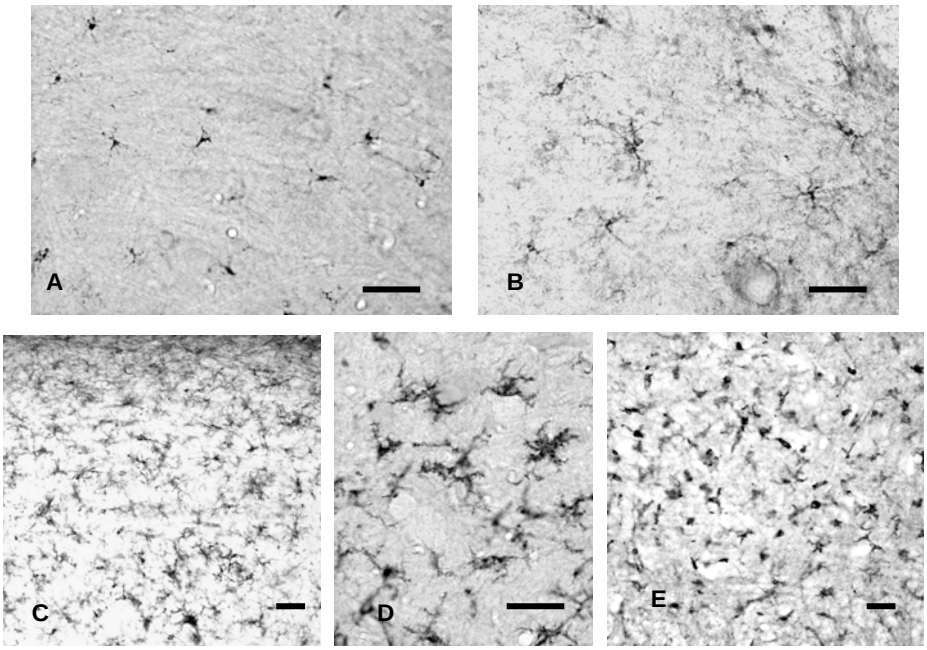

Fig. 2. Visualization of the different stages of microglial cell transformation in the ventral lumbar spinal cord gray matter of L_5 segment. (**A**) Type 1: basic staining; control rats. Solitary microglial cells widespread in the ventral horns. Microglia with typical small cell bodies bearing a few ramified thin processes. (**B**) Type 2: mild response; 8 min ischemia, 1 day reperfusion. Hyper-ramified microglia resembling fully developed resident microglia. (**C**, **D**) Type 2 and 3 of microglial transformation develops in the ventral horn with prolonged ischemia and/or postischemic reperfusion (8 min ischemia, 2 days reperfusion). (**C**) Microglial response of type 2 and 3i; (**D**) detail of type 3i microglia. (**E**) Strong response; 12 min ischemia, 2 days reperfusion. Loss of microglial processes develops in types 3ii-3iii cells. Bars = 50 μm.

insult. At day 2 postischemia, a large number of intensively lectin stained microglial cells of type 3i and 3ii were widespread in the preserved spinal cord gray matter (Fig. 3A, B). Irregular lesion areas delineated by ameboid microglia (type 3iii) and hematogenous macrophages emerged in the intermediate zone and ventral horn at day 3 postischemia (Fig. 3A, C). Reduction of microglial hypertrophy was apparent at day 5 following ischemia (Fig. 3A, D) and the number of activated microglial cells declined at day 7 postischemia.

Severe ischemia: 12 min aorta cross-clamping. Severe ischemic insult leading to significant neuronal damage adversely influenced the identification of lectin labeled glial cells. Lectin positive cells of type 3ii and 3iii (Fig. 2D) prevailed in the spinal cord gray matter at day 2 and 3 postischemia.

Discussion

In our study, a model of transient spinal cord ischemia giving rise to neurological impairment of varying intensity depending on the duration and completeness of the insult (Degirolami & Zivin, 1982; Coston et al., 1983) was used for the examination of morphological microglial transformation. The ischemic episode may present itself initially as spastic paraparesis or paralysis, suggesting preferential injury to small inhibitory interneurons in the intermediate zone, followed at longer intervals by flaccid paralysis as a result of lower motor neuron lesion. Our results are consistent with those revealing that the reduction of intra-ischemic MPAP evidently has a deteriorating effect on the neurological outcome, varying after 8 and 10 min ischemia and producing permanent motor deficit in animals subjected to 12 min aorta cross-clamping (Taira & Marsala, 1996).

The present findings show that microglial cells respond to transient ischemic injury of differing intensity with increased lectin-binding and morphological alterations such as enlargement of the cell body and retraction and swelling of microglial processes. Cellular hypertrophy continued in specific ischemic conditions with the loss of pro-

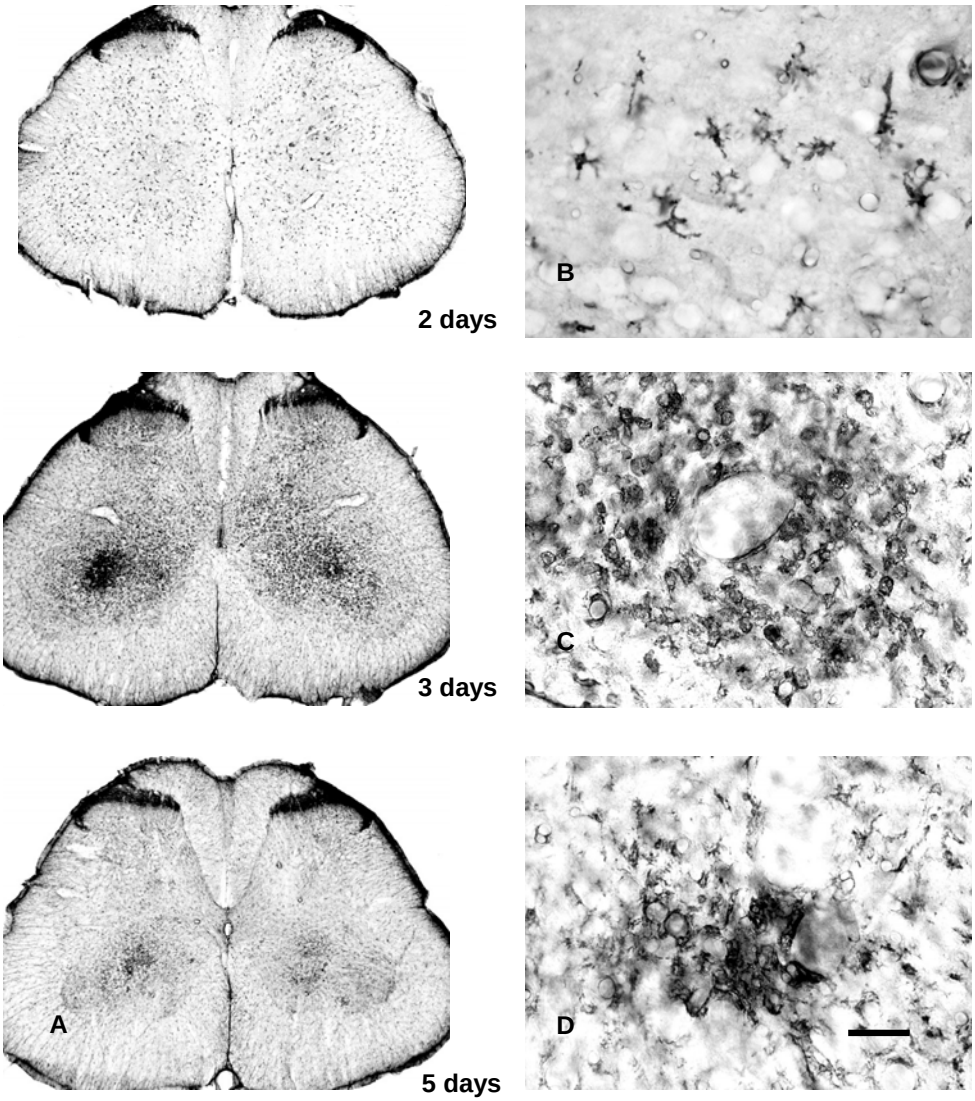

Fig. 3. Microglial response to 10 min aorta cross-clamping. (**A**) Representative sections of L_4 spinal cord segments; day 2, 3 and 5 after ischemic insult. At day 2, activated resident microglia almost homogenously distributed in the entire spinal cord gray matter, massive microglial proliferation together with infiltration of macrophages appeared with prolonged postischemic interval (day 3 and 5). (**B**) Resident microglia activated to type 3i-3ii cells. (**C**) Rounded phagocytic cells laden with lipid droplets distributed in the intermediate zone and center of ventral horn at day 3 postischemia. (**D**) Reduction in the number of the phagocytic microglia/macrophages in the center of the ventral horn at day 5 postischemia. Bars = 500 μm (**A**), 50 μm (**B**–**D**).

cesses and transformation of microglia into ameboid cells. We discovered that lectin stains microglial cells at any stage of activation, as well as in their ameboid form**.** Graded response of microglial cells is a consistent feature in almost all forms of CNS pathology (RAIVICH et al., 1999; STREIT et al., 1999). *In vitro* analyses support this pattern of microglial activation and it has been demonstrated that microglia progress to ameboid macrophages through a stereotyped sequence of steps (BEYER et al., 2000; STENCE et al., 2001). The initial response of microglia to ischemia and ischemia-like conditions begins with morphological activation, but without the expression of macrophage-

like antigens that appear with neuronal damage (KATO & WALZ, 2000). Although the function of the microglial cells activated at this stage is not fully understood, apparently these cells do not harm neurons but, on the contrary, possibly help them to survive (KATO et al., 1995).

In the group of animals with moderate ischemic insult, the generalized response of resident microglia was followed by massive infiltration of microglia/macrophages into the central portions of the spinal cord gray matter, which are the regions where asymmetric liquefactive necroses appear following severe ischemic insults over a period of time (DEGIROLAMI & ZIVIN, 1982). The effects of accumulation of phagocytic cells in the ischemic regions are not fully understood. Detrimental effects of mononuclear phagocytes in the ischemic spinal cord have been reported (GUILIAN & ROBERTSON, 1990), and the possibility has been raised that dysfunction and death of glial cells may also underlie the exacerbation of neurodegeneration (KITAMURA et al., 1999). Considering the fact that microglial cells produce neurotrophic factors, modify the response to tissue injury and assist in the structural repair, it is possible to contemplate upon the ameliorative role of microglia accumulated at ischemic lesion sites as well.

Divergent spatial and temporal patterns of microglial and hematogenous macrophage activation may have an important role in reparative or secondary degenerative sequel after spinal cord ischemia. Comparative *in vitro* studies reveal that the magnitude and composition of cytokines and neurotoxic molecules released by microglia and peripheral macrophages can differ (BANATI et al., 1993; MOSLEY & CUZNER, 1996). Visualization of microglia by antibodies (OX-42, ED1) or lectin, as used in our study, reveals morphological transformation of microglia/macrophages (LING et al., 2001), however, all activated microglia and recruited blood monocytes/macrophages are labeled by these methods. In a recent study with chimeric animals it was shown, that the onset and plateau of macrophage activation is dominated initially by microglial-derived macrophages and is then supplanted by hematogenous cells (POPOVICH & HICKEY, 2001). The origin of the microglial/macrophages population is thus of some importance, since unique functions have been attributed to macrophages derived from resident microglia compared with those originating from infiltrating monocytes. Consequently, differences in the magnitude, timing, and spatial distribution of activated microglia relative to recruited macrophages could produce conflicting effects on neurons and glia in and around the site of injury (POPOVICH et al., 2003).

The present study shows that resident microglia in the spinal cord, just like microglia in the brain, rapidly undergo activation in response to transient ischemia. The induction of different degrees of ischemic injury revealed variations in the lectin labeled microglia that accompanied their transient morphological changes. Our observation of microglial response in ischemic spinal cord tissue corresponds well with other descriptions of the role of activated microglia in respect to neuronal injury. Further investigation of the factors governing the microglial activation and the consequences of different aspects of activation in the ischemic spinal cord is needed.

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