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THE MACROPHAGE IN ACUTE NEURAL INJURY: CHANGES IN CELL NUMBERS OVER TIME AND LEVELS OF CYTOKINE PRODUCTION IN MAMMALIAN CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

ALENKA LESKOVAR[1,2], LOREN J. MORIARTY[1,2], JOHN J. TUREK[2], INGRID A. SCHOENLEIN[2] AND RICHARD B. BORGENS[1,2,*]

[1]*Center for Paralysis Research* and [2]*Department of Basic Medical Sciences, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907, USA*

*Author for correspondence (e-mail: rbb@vet.purdue.edu)

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Summary

We evaluated the timing and density of ED-1-positive macrophage accumulation (ED 1 is the primary antibody for the macrophage) and measured cytokine production by macrophages in standardized compression injuries to the spinal cord and sciatic nerves of individual rats 3, 5, 10 and 21 days post-injury. The actual site of mechanical damage to the nervous tissue, and a more distant site where Wallerian degeneration had occurred, were evaluated in both the peripheral nervous system (PNS) and the central nervous system (CNS) at these time points. The initial accumulation of activated macrophages was similar at both the central and peripheral sites of damage. Subsequently, macrophage densities at all locations studied were statistically significantly higher in the spinal cord than in the sciatic nerve at every time point but one. The peak concentrations of three cytokines, tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6), appeared earlier and were statistically significantly higher in injured spinal cord than in injured sciatic nerve. We discuss the meaning of these data relative to the known differences in the reparative responses of the PNS and CNS to injury.

Key words: spinal injury, macrophage, neurotrauma, cytokine, inflammation, central nervous system, peripheral nervous system, mammal.

Introduction

One of the unresolved riddles of neurobiology has centered on the question of the ease with which the mammalian peripheral nervous system (PNS) regenerates contrasted to the abortive regeneration of the central nervous system (CNS) (Ramon y Cajal, 1928). It is probable that the lack of regenerative ability of CNS axons and the overall poor functional recovery from injury are related to the CNS environment and are not intrinsic to central neurons (Berry, 1979; Kiernan, 1979; Reier and Houle, 1988; Schwab et al., 1993). It has long been thought that the non-neuronal cells of the CNS (astrocytes and oligodendrocytes) provide physical, physiological and molecular impediments to functional reconnection or recovery following injury to the CNS (Hatten et al., 1991; Liuzzi and Lasek, 1987; Reier et al., 1983; Schwab et al., 1993). Their homologues in the PNS, Schwann cells, are, however, seen as facilitators of functional regeneration (de Vellis, 1993). Here, we consider another type of cell that is dominant in numbers in the early phase following nervous system injury, phagocytes of myelomonocytic lineage, the macrophages.

Macrophages play a key role in the early inflammatory responses in all soft-tissue wounds (Clark, 1995; DiPietro, 1995; Kirsner and Eaglstein, 1993; Perry and Gordon, 1991). They may also play a pivotal role in nerve injury and contribute to control of the regenerative response, although the nature of their role is a matter of debate.

One view holds that an early and robust accumulation of macrophages in the PNS is key to the regeneration of peripheral axons (George and Griffin, 1994; Griffin and George, 1993; Perry and Brown, 1992; Perry and Gordon, 1988, 1991; Perry et al., 1987, 1993). Another view holds that an early and robust accumulation of macrophages in the injured brain or spinal cord causes more damage than is prevented by the inflammatory reaction (Blight, 1985, 1992, 1994; Giulian and Robertson, 1990; Giulian et al., 1989). These opinions have emerged in the absence of a systematic evaluation of the timing and density of macrophage accumulation following spinal cord, brain or peripheral nerve injury.

Macrophages not only produce and release many hydrolytic enzymes useful for debridement of dead and dying cells, they are also a particularly rich source of growth factors and cytokines useful for promoting intracellular signaling, mitosis and extracellular matrix production (Gordon, 1995; Lotan and Schwartz, 1994; Perry and Gordon, 1991). The responses of

the CNS and the PNS to injury are also mediated by the specific molecular products secreted by macrophages following their activation (David et al., 1990; Lazarov-Spiegler et al., 1996; Prewitt et al., 1997; Franzen et al., 1998). There is a belief that macrophages, activated at different regions of the nervous system, produce and secrete variable types and amounts of molecular products, thereby stimulating or preventing the regenerative ability of the nervous tissue (Lazarov-Spiegler et al., 1996). The identification of some of these molecular factors, in conjunction with an understanding of peak numbers of activated macrophages recruited to different injury sites, might lead to a deeper understanding of the role of these inflammatory cells in neurotrauma, and in this paper we set out to provide such data.

Materials and methods

Experimental animals

Laboratory rats (210–380 g; Sprague-Dawley) were used in these experiments. Following surgery, they were housed two animals per cage, fed *ad libitum*, and their health monitored daily. Animals were killed by an overdose of sodium pentobarbital (0.8 ml of 1 g ml^{-1}) at 3, 5, 10 and 21 days post-surgery (five animals per time point for morphometric studies; six animals per time point for cytokine assays). In the morphometry study, this overdose was immediately followed by perfusion/fixation with 6 % paraformaldehyde, 0.1 % glutaraldehyde in phosphate buffer. The spinal cords and sciatic nerves were dissected free and immersion-fixed for another 1–2 days. They were later stored in phosphate buffer prior to embedding for histology (see below). For cytokine assays, the tissues were dissected free and stored at 4 °C until further processing (see below).

Surgical procedures

Anesthesia was performed using an intraperitoneal injection of 0.15 ml 100 g^{-1} body mass of a standardized mixture of 10 ml of ketamine HCl (100 mg ml^{-1}) and 1.1 ml of xylazine (20 mg ml^{-1}). The spinal cord was exposed by a partial laminectomy of the caudal thoracic region (approximately T10–T12). A compression injury of the dorsal hemisphere of the exposed spinal cord was performed for 3 s at this site using blunted watchmaker's forceps (Moriarty et al., 1998). This standardized lesioning technique has been shown to produce lesions that are not statistically significantly different in unit area, volume or pathological features between animals (Moriarty et al., 1998). The wounds were closed in two layers with a 3-0 silk suture, and the skin was closed with wound clips. The sciatic nerve of the left hindlimb was subsequently exposed by a diagonal incision leading from the tuber coxae towards the knee. The biceps femoris muscle was retracted caudally, and the exposed sciatic nerve was crushed with watchmaker's forceps for 3 s for the morphometric study. For the cytokine assays, the sciatic nerve was crushed 5–7 times, over a length of approximately 1 cm, thus producing a stimulus sufficient to recruit large numbers of phagocytes which would produce enough cytokines for reliable assay and normalization per microgram of macrophage DNA. Next, the retracted biceps femoris muscle was sutured back to the gluteus superficialis muscle and the tensor fasciae latae muscle with 3-0 silk suture. The skin was closed with wound clips. Following surgery, each animal was subcutaneously injected with 3 ml of lactated Ringer's solution to prevent dehydration and placed under a heat lamp for approximately 24 h to reduce post-surgical mortality due to hypothermia.

Photography and image reproduction

Viewing of spinal cord sections was accomplished with an Olympus Van Ox Universal Microscope (see Figs 1, 2). Morphometry was performed on digitized images acquired with a JVC TK-1070U color video camera mounted on the microscope (see Fig. 2). The acquisition of microscopic images was accomplished with RasterOps MediaGrabber 3.2 software captured to a Macintosh Quadra 800 computer. Final color plates were made on a Tektronix Phaser 400 dye-sublimation printer.

Immunocytochemistry

Fixed segments of spinal cord and sciatic nerve (each containing the site of damage) were dehydrated in alcohol, embedded in Paraplast (paraffin) using conventional methods and sectioned (15 μm thick) in the longitudinal plane on a rotary microtome. As described by Moriarty et al. (1998), rehydrated sections affixed to slides were first incubated in a commercial enzyme and tissue non-specific antigen blocker (Endo/Blocker M69 and tissue blocker, Biomeda), then exposed to the primary antibody for the macrophage, ED 1 (MCA-341, Serotech/Harlan Bioproducts), for 30 min, and rinsed with buffer (Automation Buffer, Biomeda). ED 1 is a monoclonal antibody that binds specifically to a single-chain glycoprotein expressed predominantly on the lysosomal membranes of activated macrophages (Damoiseaux et al., 1994). Next, a biotinylated secondary antibody (rabbit antimouse; Lab/Probe, Biomeda) was administered for 15 min, and the sections were then rinsed in buffer prior to exposure to the streptavidin peroxidase (10 min). The sections were rinsed and exposed to a commercially prepared diaminobenzidine reagent (Biomeda) for 5 min, staining ED-1-labeled macrophages brown. Sections were counterstained with hematoxylin and covered with a warm glycerol gelatin (Sigma Chemical Co.) and a coverslip.

Determination of macrophage diameter

Sections were examined under a light microscope and images acquired using a JVC TK-10700 video camera. Individual macrophage cells (full diameter and isolated from other cells) were chosen from a minimum of ten fields of view (200× digitized images, sampled from all animals) of the central lesion in both the CNS and PNS at 3, 5, 10 and 21 days post-surgery. To convert pixels to μm^2, a hemocytometer was captured to the computer at 200×. Average macrophage diameters at each time point post-surgery were calculated from these images to allow

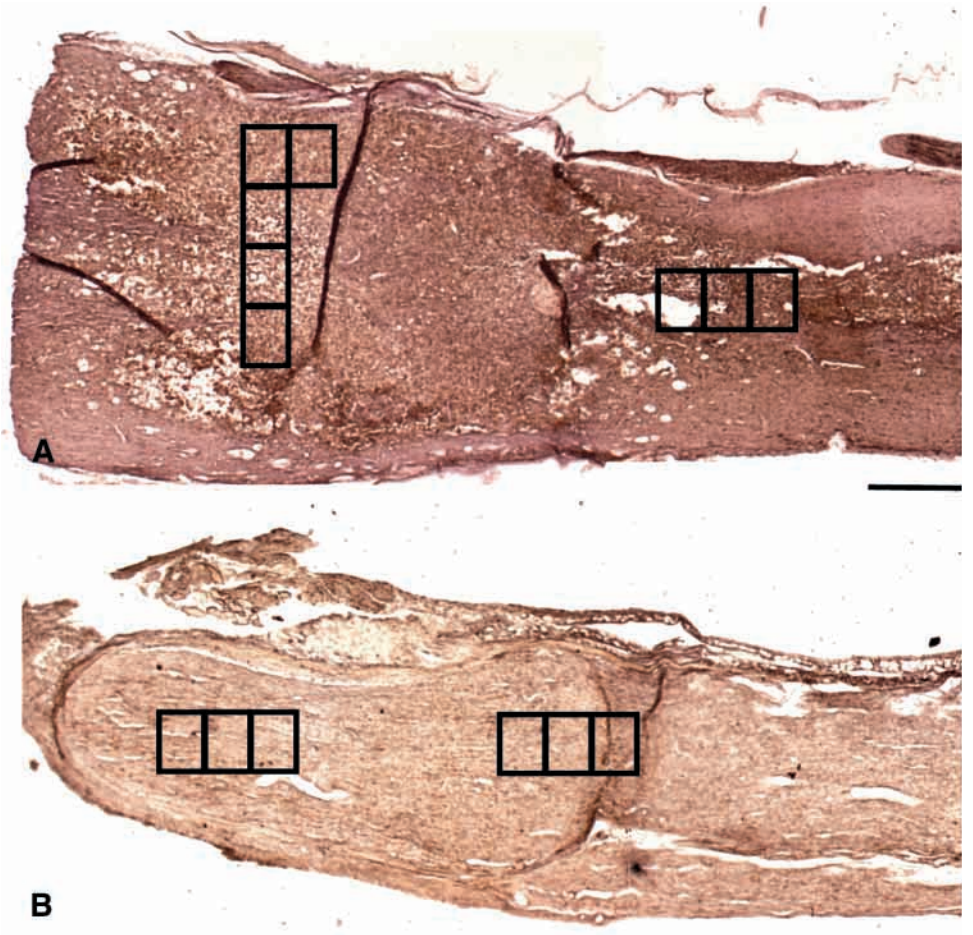

Fig. 1. Histological sections of spinal cord and sciatic nerve injuries. (A) A 15 μm thick, longitudinal horizontal section of rat spinal cord, 10 days post-injury. The site of direct compression of the cord is to the left (caudal), while an approximately 1 mm distant region of degenerating white matter is visible to the right (rostral). (B) A similar longitudinal horizontal section of the sciatic nerve of the same animal, injured at the same time as the spinal cord (see Materials and methods). The site of direct compression of the nerve is to the left (proximal), while approximately 1 mm distant is nerve trunk damage to the right (distal). Both these images are as they appear when captured to the computer at 20×. Scale bar, 500 μm (for A and B). The bold rectangles show the areas of injured nervous tissue where ED-1-labeled macrophages were counted for each sample at 200×. The choice and positioning of these regions are described in the experimental procedures.

conversion of the measured area into macrophage cell counts. A more detailed description of these methods and their verification can be found in Moriarty et al. (1998).

Determination of macrophage number

We chose to capture regions where the macrophage density was highest. In this way, peak macrophage numbers could be determined for each time point evaluated. In addition, we could estimate and compare peak amounts of cytokines produced by macrophages present in the injured nervous tissue at these different time points and locations.

In the CNS, this was achieved by capturing five (approximately 320 μm×240 μm) adjacent fields of view in the area of the highest macrophage density at a magnification of 200 diameters. A total area of approximately 0.4 mm^2 per histological section was thus captured to the computer at the site of compression of the spinal cord. At sites of Wallerian degeneration, beginning approximately 1.0 mm rostral to the center of the site of compression to the spinal cord, three (approximately 320 μm×240 μm) images were also captured, making a total area of 0.25 mm^2 (see Figs 1A, 2A,B).

A similar procedure was carried out in the sciatic nerve. Three 320 μm×240 μm adjacent fields of view were captured at the site of compression, where the macrophage density was greatest (see Figs 1B, 2C). The unit area captured was approximately 0.25 mm^2 per section. Three more 320 μm×240 μm fields of view were captured beginning approximately 1.0 mm distal to the center of the crush lesion (see Figs 1B, 2D).

Fields of view from ten histological sections (every third section) were captured and evaluated in both the spinal cord and sciatic nerve of each animal (Fig. 1; see also Fig. 2).

Computer-assisted morphometry

Using software that transforms assigned color pixel values (Color Look-Up Tables) to a single color pixel value, the assigned colors (discriminating only labeled macrophages) were measured in pixels. Once the visual data had been digitized, color-transformed for counting and assigned a file name, the actual counting was performed using a custom-designed script (IPLab Spectrum) that did not require human interaction for the acquisition of these data (see Fig. 1 in Moriarty et al., 1998).

The use of this methodology for the counting of macrophages in spinal cord injuries and the verification of this method using two- and three-dimensional morphometric measurements and visual counts by a naïve investigator are described in detail by Moriarty et al. (1998).

Macrophage collection and culture

Isolated spinal cords and sciatic nerves were washed with sterile calcium- and magnesium-free Hanks' balanced salt

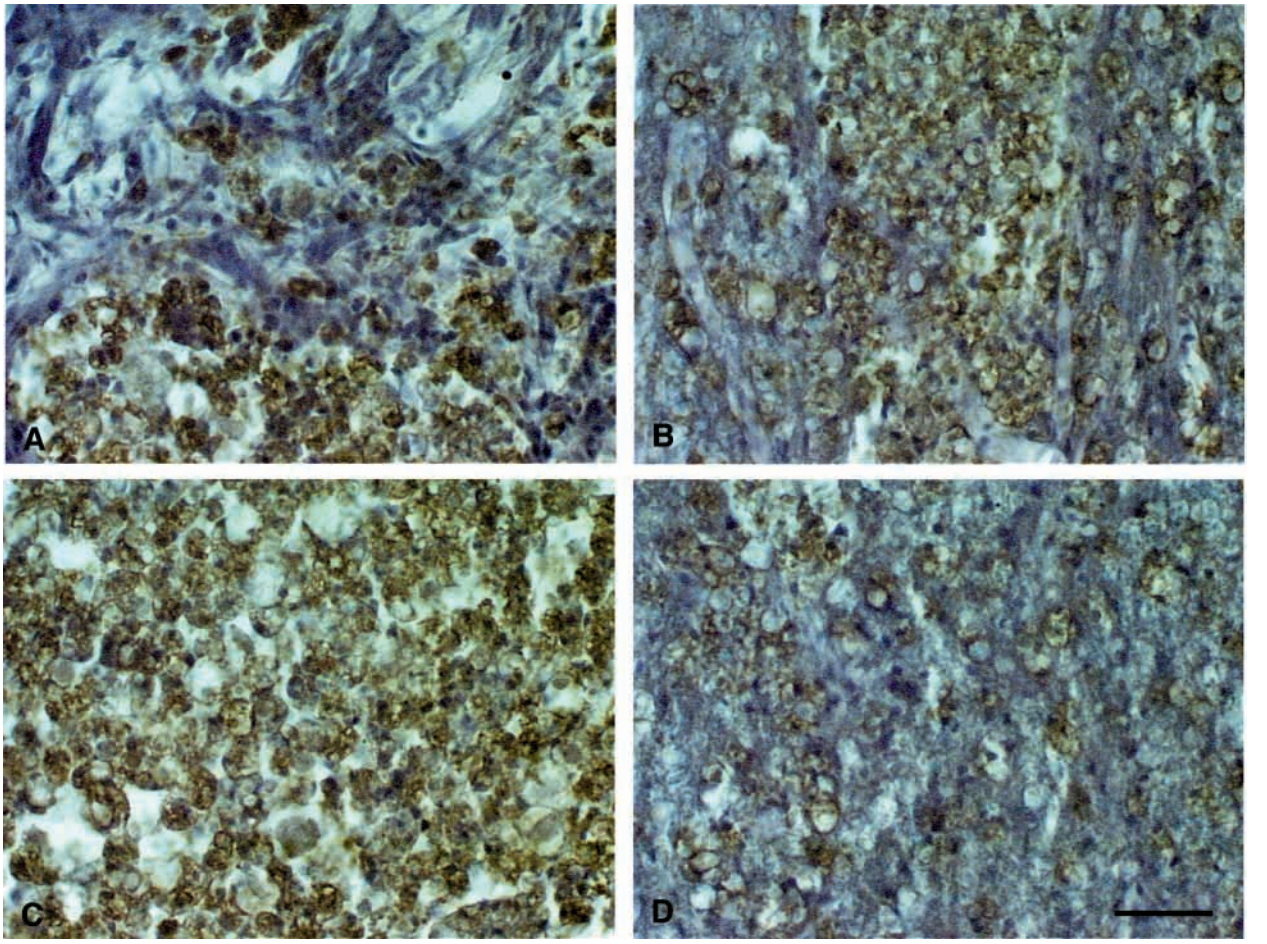

Fig. 2. Spinal cord and sciatic nerve compression injuries. These representative photomicrographs were taken 3 weeks post-injury. All images were captured to the computer at 200×. (A) A typical region of the center of the compression injury to the spinal cord. Fields of view captured to the computer for cell counting were chosen from such regions of high cell density (see Materials and methods). (B) A field of view 1 mm from the center of the compression injury (shown in A). (C) A typical sample region showing labeled cells at the central site of direct compression to the sciatic nerve; (D) reduced numbers of cells at more distal sampling sites. Scale bar, 50 μm (for all images).

solution (HBSS). Subsequently, the tissues, harvested in Corning 100 mm tissue culture dishes, were immersed in 3 ml of RPMI 1640 culture medium containing 5 mg ml^{-1} bovine serum albumin (BSA), 100 i.u. ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1 % L-glutamine. These tissues were mechanically macerated with a blunt probe. The suspensions were then transferred into 15 ml conical propylene-coated tubes (Falcon Blue Max Jr) and centrifuged for 30 s to separate the supernatant (containing macrophages) from tissue. Subsequently, supernatants were transferred into fresh tubes, and culture medium was added to 5 ml and kept cold on crushed ice. Equal amounts (approximately 2.5 ml) were then transferred from each tube into two Corning 35 mm polystyrene cell wells and cultured for 2 h in a humidified CO_2 incubator (5 % CO_2) at 37 °C. Thereafter, the supernatant was discarded, and adherent macrophages were incubated for another 24 h in 3 ml of freshly prepared culture medium containing 10 μg ml^{-1} lipopolysaccharide (LPS; 055: B5 *Escherichia coli*, Sigma, St Louis). Next, the cell-free supernatant fluid was divided into six 500 μl samples, and the adherent cells were digested with 1 mol l^{-1} ammonium hydroxide and 0.2 % Triton X-100 for total cellular DNA quantification (Downs and Wilfinger, 1983). These techniques specifically isolate macrophages from the separate regions of study (Edelson and Colin, 1976; Coligan et al.,1991), allowing cytokine production subsequent to stimulation by LPS to be determined in the microgram range. This concentration ensured that all cells would be stimulated identically and maximally (Turek et al., 1991). In addition, we further confirmed the identity of isolated cells by light and transmission electron microscopy. Anatomical details are to be published elsewhere.

Cytokine bioassay

Spectrophotometric cell growth/cell kill analysis

To determine cytokine concentration, we used the principle of cell growth/cell kill originally described by Mosmann (1983) and further developed by Hansen et al. (1989). Using this method, the colored conversion product formazan, formed from the tetrazolium salt (MTT) mainly in intact mitochondria,

can be measured spectrophotometrically. Depending on the concentration of the evaluated cytokine in the sample, the number of cells in culture changes, as can be seen by the intensity of formazan formation. Comparisons of the optical density signal with standard curves of cytokine levels yields the cytokine concentration of the sample. This concentration was then normalized per microgram of DNA of the plated macrophages. The individual TNFα, IL-1 and IL-6 bioassay methodologies are described briefly below and in detail by Turek et al. (1996, 1998).

TNFα bioassay

Macrophage culture supernatants were assayed in octuplicate for TNFα activity as previously detailed (Turek et al., 1996). Dilutions of the supernatants (1:10 to 1:100) were placed in wells containing L929 murine fibroblasts with a growth medium containing actinomycin D. Following a 20 h incubation, the fluid in the wells was discarded, and tetrazolium salt in HBSS was added to each well. After 3 h, 100 µl of a solution of sodium dodecyl sulfate (SDS) in *N*,*N*-dimethylformamide (DMF, pH 4.7) was added to each well and incubated overnight. The absorbance at 600 nm was read in a microplate reader. Standard curves using recombinate human TNFα (Genzyme, Boston, MA, USA) were used to determine the amount of TNFα activity.

IL-1 bioassay

A murine plasmacytoma cell line (T 1165.17) that proliferates in response to IL-1 was used as the bioassay. The IL-1 receptor on the T 1165.17 cell line was blocked with a monoclonal antibody (LA 15.6) to the IL-1 receptor (Karavodin et al., 1989). The cells were grown in a Minimal Essential Medium supplemented with 10 % fetal calf serum (FCS), 0.05 mol l^{-1} 2-mercaptoethanol (2-ME) and 200 pg l^{-1} IL-1β. Cells were seeded in 96-well plates and samples assayed in quadruplicate. Thirty minutes before the addition of the samples, four wells were blocked with antibody (1 µg ml^{-1}) and four were left unblocked. Macrophage culture supernatants were diluted (1:10 to 1:200) with growth medium, and 200 µl was added per well in quadruplicate. After incubation for 18 h, MTT and SDS–DMF were added, and the absorbance was measured at 550 nm after an overnight incubation. The difference in absorbance between blocked and unblocked wells, adjusted for control levels, was a measure of the amount of IL-1 activity in the macrophage culture medium. Standard curves were constructed using recombinant murine IL-1β (Genzyme).

IL-6 bioassay

IL-6 bioassays were performed using B 9 cells (Aarden et al., 1987). The cells were grown in Iscove's modified Dulbecco's medium supplemented with 100 % FCS, 4 pg per 100 ml of mouse recombinant IL-6 and 0.05 mol l^{-1} 2-ME. Macrophage culture supernatants were diluted (1:100 or 1:200) with growth medium. Next, 200 µl was added per well in octuplicate to a 96-well culture plate containing B 9 cells. After 72 h of incubation, 20 µl of 7.24×10^{-7} mol l^{-1} MTT in HBSS was added to each well, followed by 100 µl of SDS–DMF after 6 h. The absorbance was measured as described above. Standard curves using recombinant murine IL-6 (Genzyme) were used to determine IL-6 bioactivity.

Statistical analyses

Statistical analyses were performed using Microsoft Excel 5.0 and InStat 2.00 software. Kruskal–Wallis non-parametric analysis of variance (ANOVA) tests were performed, and Mann–Whitney non-parametric *post-hoc* two-tailed tests were used to determine the significance of data, which are presented as means ± standard error of the mean (S.E.M.).

Results

Fifty-five rats were used: 28 in the morphometry study and 27 in the cytokine study. Eight rats died prematurely, two cords were lost to the study because of poor perfusion/fixation and one autophagic animal had to be killed. These animals and tissues were replaced to give a total of five animals evaluated per time point (3, 5, 10, and 21 days post-injury) for the morphometry study and six for the cytokine assay study.

Morphometry

Macrophages in subacute nerve injury

By 3 days post-injury, identifiable macrophages were present in the injury to both spinal cord and sciatic nerve. These cells were large, relative to other blood-borne elements, modestly vacuolated and ED-1-positive. In the spinal cord, these cells were present not only at the site of damage (usually at the periphery of the lesion) but had also accumulated in white matter tracts. At this time, a considerable amount of degenerating myelin and tissue cavitation was evident in both the injured spinal cord and sciatic nerve.

Five days post-injury, the accumulations of macrophages within the hemorrhagic lesion of the spinal cord were not confined to the periphery of the lesion, but could also be found in degenerating parenchyma and cysts. Cystic cavitation of the cord extended in a rostral/caudal direction from the site of mechanical damage, compromising white matter. Such significant enlargement of the wound was characteristic of the spinal cord, but not of the injury to the sciatic nerve of the same animal.

At 10 days and 3 weeks post-injury, contusion of the spinal cord had produced an increasingly dense cicatrix at the site of damage (Reier et al., 1983). The borders of large cavities and cysts were better defined, there was no evidence of recent bleeding, and the lesion site contained cells of many different types. In particular, macrophages in this region and at more remote sites of Wallerian degeneration were intensely labeled, larger in diameter (see below) and more vacuolated than observed at earlier time points. The physical appearance of activated macrophages did not differ between spinal cord and peripheral nerve injuries in the same animal (Fig. 2).

Macrophage size in CNS and PNS injury

The diameters of individual macrophages (N=50) were measured independently at 3, 5 and 10 days post-injury by two investigators (A.L. and L.J.M.). The mean macrophage diameter within the central lesion of the spinal cord was remarkably, and statistically, similar during this early phase of the injury (13.5±0.2 μm, $P \geqslant 0.5$ for each comparison; Mann–Whitney, two-tailed tests). However, macrophage diameters increased to approximately 15 μm by 3 weeks post-injury (14.9±0.3 μm), a result confirmed by additional independent measurements (N=195). These data were compared with the individual data from each successive time point and with the pooled data of all measurements taken at 3, 5 and 10 days post-injury. In every comparison, except one, the 3 week data indicated a statistically significant ($P \leqslant 0.03$; Mann–Whitney, two-tailed test) increase in macrophage size. We performed a similar statistical evaluation of macrophage size in the sciatic nerve. There was no statistical difference between the diameters of macrophages sampled at any time point post-injury using an identical comparison (mean diameter 14.3±0.2 mm; $P \geqslant 0.23$; Mann–Whitney, two-tailed tests).

Time-dependent changes in the density of macrophages

Tables 1 and 2 provide a summary of macrophage density data.

Comparison of CNS and PNS sites of injury. Macrophage numbers and densities were generally greater (and significantly different) at the actual site of spinal cord injury and a remote region of injury compared with corresponding loci of the sciatic nerve injury ($P \leqslant 0.003$; Mann–Whitney, two-tailed tests). The 10 day time point (distant site comparison) showed a trend towards significance (P=0.06; Mann–Whitney, two-tailed test), and only one time point (3 day; site of compression injury comparison) was not significantly different (P=0.46; Mann–Whitney, two-tailed test).

Comparison of the compression site and remote site of Wallerian degeneration. With only one exception, the actual site of the compression injury was strikingly inundated with macrophages relative to the region of axonal degeneration approximately 1–2 mm distant. These differences between the actual site of mechanical damage and a more distant site were independent of whether the spinal cord or sciatic nerve was evaluated and independent of the time point at which the comparison was made (P<0.0001; Mann–Whitney, two-tailed tests). This dominance of macrophage numbers at the site of damage can be appreciated in Fig. 3. The only exception was observed at 10 days post-injury in the sciatic nerve, where no difference in the numbers of macrophages accumulating at the site of the crush and a more remote site was detected (P=0.7; Mann–Whitney, two-tailed test).

Comparison of macrophage densities at the same location at different time points. At the site of the compression injury to the spinal cord, macrophage density increased dramatically between 3 and 5 days post-injury. Macrophage numbers peaked at 5 and 10 days post-injury (a significant difference in numbers at these time points was not detected; P=0.37; Mann–Whitney, two-tailed test, Fig. 3). Macrophage numbers declined by 3 weeks post-injury, although not to the densities observed at 3 days post-injury (approximately 1800 cells mm^{-2} at 3 weeks post-injury compared with approximately 1100 cells mm^{-2} at 3 days post-injury). The densities at the distant site of injury similarly increased to a maximum, followed by a 50 % decline between 10 days and 3 weeks post-injury (Fig. 3).

At the site of compression injury to the sciatic nerve, macrophage densities were initially equivalent to those observed in the spinal cord. However, the macrophage densities at this location in the PNS increased only slightly to a maximum of approximately 1500 cells mm^{-2} at 10 days. The density of macrophages at later time points was approximately

Table 1. *A summary of macrophage densities after spinal cord injury*

	Macrophage density (cells mm^{-2})			
Time post-injury	Mean	S.E.M.	Minimum	Maximum
3 day central	1128.1	75.6	119.8	2302.0
3 day distant	540.9	48.5	37.4	1287.9
5 day central	2524.6	173.4	60.9	4568.6
5 day distant	1433.0	166.6	1.4	3663.7
10 day central	2758.3	197.8	31.5	4977.9
10 day distant	1456.0	253.7	0.8	4983.3
3 week central	1769.7	101.1	488.8	3952.1
3 week distant	740.0	57.8	180.5	1738.9

The mean, standard error of the mean (S.E.M.) (N=5) and range of data for all time points evaluated post-injury are given in cell densities (macrophage mm^{-2}).

Central means that morphometry was performed at the lesion epicenter; distant means that morphometry was performed approximately 1–2 mm from the lesion epicenter.

Table 2. *A summary of macrophage densities after sciatic nerve injury*

	Macrophage density (cells mm^{-2})			
Time post-injury	Mean	S.E.M.	Minimum	Maximum
3 day central	1293.0	100.6	546.7	2815.7
3 day distant	311.9	51.5	6.7	1415.1
5 day central	1161.5	135.1	44.8	3570.9
5 day distant	495.1	89.5	1.5	2184.5
10 day central	1529.0	125.0	55.8	3520.1
10 day distant	1451.1	101.1	25.6	3184.8
3 week central	1014.8	89.1	188.4	2781.8
3 week distant	494.4	54.4	83.2	1890.2

The mean, standard error of the mean (S.E.M.) (N=5) and range of data for all time points evaluated are given in cell densities (macrophages mm^{-2}).

Central means that morphometry was performed at the lesion epicenter; distant means that morphometry was performed approximately 1–2 mm from the lesion epicenter.

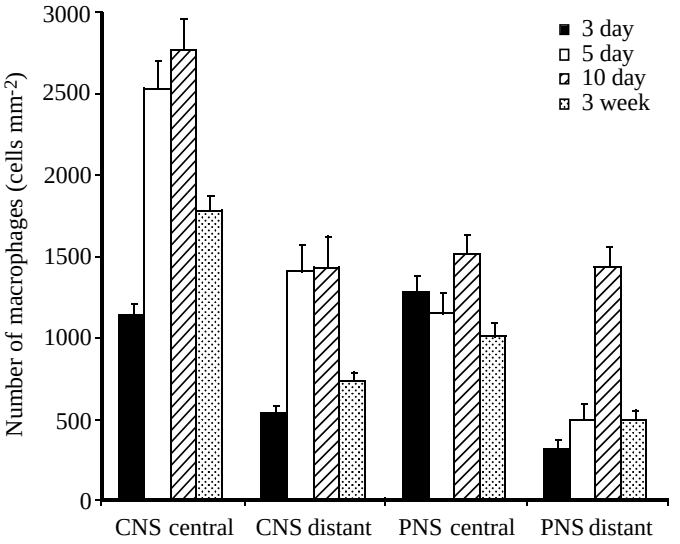

Fig. 3. Pooled densities of macrophages in central and peripheral nerve injury. Peak macrophage densities and their standard errors (N=5) are shown for all animals at all time points sampled. In all data sets shown, 'central' refers to the region of peak macrophage density at the site of compression, while 'distant' refers to regions sampled 1 mm or more from this site (see Materials and methods). Note that at both central and distant spinal cord injury sites, peak densities of macrophages were reached approximately 1 week post-injury, with densities declining by over one-third by 3 weeks post-injury. Also, densities of macrophages at the site of direct compression of the spinal cord were approximately double those counted in regions of Wallerian degeneration at all time points studied. These differences were statistically significant. In the sciatic nerve (PNS), macrophage accumulations peaked at 10 days at only the distant site of injury. CNS, central nervous system; PNS, peripheral nervous system.

half that at the corresponding location in the injured spinal cord. At the distant site of Wallerian degeneration, the trend in macrophage accumulations was similar to that at both sites evaluated in the spinal cord. Cell densities gradually increased with time from 3 days to a statistically significant maximum at 10 days, and then fell precipitously to approximately 30 % of the peak level by 3 weeks post-injury (Fig. 3).

Normalized LPS-inducible cytokine production per microgram of DNA

Cytokine concentrations (pg) were determined and normalized per microgram of DNA obtained from the same plated macrophages that produced the cytokines.

TNFα bioactivity

In the spinal cord, we observed the highest TNFα bioactivity at 3 and 5 days post-injury (1276 pg μg^{-1} DNA at 3 days and 650 pg μg^{-1} DNA at 5 days), while significantly lower bioactivity was seen at 10 days (101pg μg^{-1} DNA) and 3 weeks (17 pg μg^{-1} DNA) post-injury (Table 3). These differences were statistically significant ($P \leqslant 0.04$; Mann–Whitney, two-tailed tests) and demonstrated a distinct rise and fall in TNFα bioactivity (Fig. 4A).

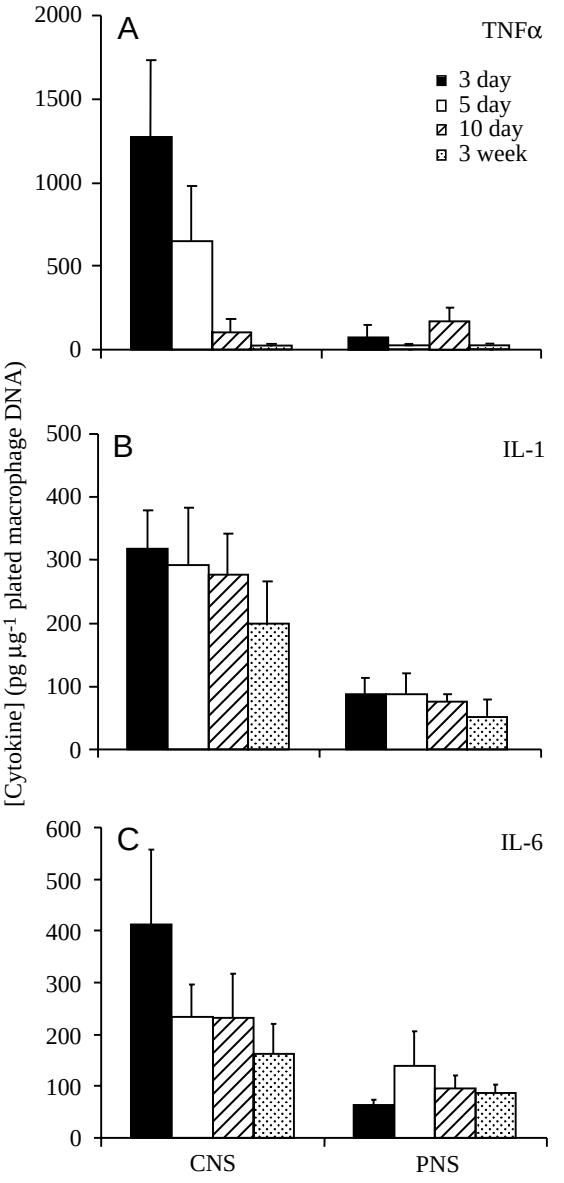

Fig. 4. Macrophage cytokine levels in the central nervous system (CNS) and peripheral nervous system (PNS) at four time points post-injury. (A–C) Mean values of lipopolysaccharide (LPS)-inducible cytokine concentrations and their standard errors (N=6) are presented in pg μg^{-1} plated macrophage DNA. Note that normalized cytokine concentrations from CNS macrophages were initially several-fold higher than those produced by PNS macrophages. TNFα, tumor necrosis factor α; IL-1, interleukin-1; IL-6, interleukin-6.

In the sciatic nerve, TNFα bioactivity reached a modest peak at 10 days post-injury (Table 4), but only when compared with the 5 day and 3 week time points ($P \leqslant 0.027$ compared with the 5 day level and $P \leqslant 0.009$ compared with 3 week level; Mann–Whitney, two-tailed tests). Three day and 10 day levels were not significantly different from each other.

TNFα bioactivity was significantly higher in the CNS at 3 and 5 days post-injury compared with the PNS for all eight

Table 3. *A summary of lipopolysaccharide-inducible cytokine levels in cultured macrophages derived from injured spinal cord*

	Cytokine level (pg μg^{-1} DNA)			
Time post-injury	Mean	S.E.M.	Minimum	Maximum
3 day TNFα	1276	459	114	3057
5 day TNFα	650	331	67	2059
10 day TNFα	101	77	0	471
3 week TNFα	17	13	0	76
3 day IL-1	319	60	153	584
5 day IL-1	293	91	74	576
10 day IL-1	278	65	138	511
3 week IL-1	200	66	25	403
3 day IL-6	412	145	136	909
5 day IL-6	234	63	64	434
10 day IL-6	233	84	75	570
3 week IL-6	162	59	63	414

The mean, standard error of the mean (S.E.M.) (N=6) and range of cytokine data for all time points evaluated post-injury are given in pg μg^{-1} DNA.

TNFα, tumor necrosis factor α; IL-1, interleukin-1; IL-6, interleukin-6.

Table 4. *A summary of lipopolysaccharide-inducible cytokine levels in cultured macrophages derived from injured sciatic nerve*

	Cytokine level (pg μg^{-1} DNA)			
Time post-injury	Mean	S.E.M.	Minimum	Maximum
3 day TNFα	70	66	0	397
5 day TNFα	9	4	0	25
10 day TNFα	157	74	9	479
3 week TNFα	4	4	0	26
3 day IL-1	87	27	28	171
5 day IL-1	87	33	8	209
10 day IL-1	76	11	48	127
3 week IL-1	52	27	0	170
3 day IL-6	63	10	37	99
5 day IL-6	139	66	39	449
10 day IL-6	96	25	18	176
3 week IL-6	86	16	58	150

The mean, standard error of the mean (S.E.M.) (N=6) and range of cytokine data for all time points evaluated post-injury are given in pg μg^{-1} DNA.

TNFα, tumor necrosis factor α; IL-1, interleukin-1; IL-6, interleukin-6.

possible comparisons except one ($P \leqslant 0.026$, Mann–Whitney, two-tailed tests, see Fig. 4A). The exception was the 5 day CNS to 10 day PNS comparison (P=0.18).

In contrast to early time points, TNFα bioactivity fell precipitously to values similar to, and even lower than, those measured in the sciatic nerve.

IL-1 bioactivity

In spinal cord, there was no evidence of a statistically significant peak in IL-1 bioactivity between 3 days and 3 weeks following injury: there was a graded decline in levels. Similarly, there was no peak in bioactivity observed in the injured sciatic nerve (Fig. 4B).

In general, IL-1 bioactivity was higher in the CNS than in the PNS (Fig. 4B). The measurements of IL-1 bioactivity obtained from CNS macrophages at 3 and 10 days post-injury were statistically significantly higher than comparable values from PNS macrophages at any time point evaluated ($P \leqslant 0.04$; Mann–Whitney, two-tailed tests).

IL-6 bioactivity

In spinal cord, an apparent peak in IL-6 bioactivity was observed at earlier time points, declining thereafter (Fig. 4C). However, the means at these times post-injury were not significantly different given the large range of values measured and the low number of samples. Similarly, the slight peak in IL-6 bioactivity at 5 days post-injury in the sciatic nerve was not statistically significant.

IL-6 bioactivity in the CNS macrophages at 3 days post-injury was statistically significantly higher than all values obtained from the sciatic nerve ($P \leqslant 0.04$; Mann–Whitney, two-tailed tests, Fig. 4C).

Normalized LPS-inducible cytokine production per cell counts

Cytokine levels were adjusted to the normalized macrophage densities encountered at the sites of study within the spinal cord and the sciatic nerve at all time points and locations studied. Cytokine levels can be given as:

$$CL(c_i, t_i, l_i) = [MD(t_i, l_i) \times CP(c_i, t_i, l_i)/(MD \times CP)_{max}] \times 100,$$

where CL is cytokine level (%), c_i is TNFα, IL-1 or IL-6, t_i is 3, 5, 10 or 21 days post-injury, l_i is CNS central, CNS distant, PNS central or PNS distant, MD is macrophage density (mm^{-2}) and CP is cytokine level (pg μg^{-1} macrophage DNA). These concentrations are presented as percentages of the peak cytokine concentrations encountered for any of the three pro-inflammatory cytokines evaluated.

Fig. 5A demonstrates that high levels of TNFα are produced by macrophages in the damaged spinal cord at 3 and 5 days post-injury, when the production of this cytokine by macrophages is barely detectable in the injured sciatic nerve. However, at the 10 day time point, TNFα production by macrophages is similar in both the injured CNS and PNS. At 3 weeks post-injury, TNFα levels in both the CNS and the PNS decline precipitously.

IL-1 levels in the injured spinal cord rise and then fall, reaching a peak at 5 and 10 days post-injury. IL-1 production by macrophages of the injured sciatic nerve is much lower and appeared to reach a peak only in the area of Wallerian degeneration (PNS) at 10 days (Fig. 5B).

Similarly, IL-6 levels in the injured spinal cord were much higher than in the injured sciatic nerve (Fig. 5C). However, in

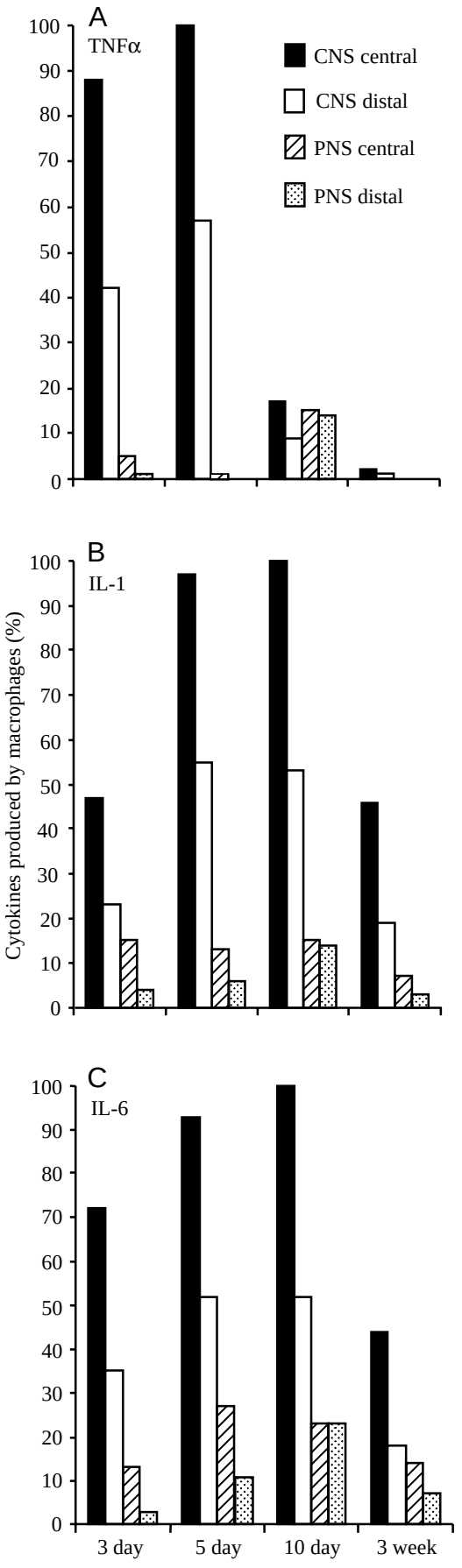

Fig. 5. Cytokine levels normalized for macrophage densities. (A–C) The percentage of peak lipopolysaccharide (LPS)-inducible cytokine levels as a function of time post-injury. Note that the levels of pro-inflammatory cytokines present in the injured spinal cord are strikingly higher than those present in the injured sciatic nerve at early time points. Values are means (N=6). 'Central' refers to the region of peak macrophage density at the site of compression, while 'distant' refers to regions sampled 1 mm or more from this site (see Materials and methods). TNFα, tumor necrosis factor α; IL-1, interleukin-1; IL-6, interleukin-6; CNS, central nervous system; PNS, peripheral nervous system.

both the CNS and the PNS, IL-6 concentrations peak at 5 and 10 days post-injury.

Discussion

Over the last decade, there has been an increasing interest in the role of the inflammatory reaction in the process of secondary injury to the central nervous system. Moreover, this interest has focused on the dominant cell type observed during inflammation, the macrophage. More recently, it has been suggested that the site of activation and origin of these phagocytes may play an important role in the outcome after injury, and even in the difference in response to injury between the CNS and PNS (Lawson et al., 1994; Lazarov-Spiegler et al., 1996; Perry and Gordon, 1991; Zeev-Brann et al., 1998). Macrophages arise from two different sources, from the resident population present in tissues at all times, and from the non-resident population of circulating blood monocytes recruited into tissues and transformed into macrophages. In the CNS, ramified microglia represent the resident macrophage population. Activated macrophages arising from both sources are specifically labeled by the monoclonal antibody ED 1 (Damoiseaux et al., 1994). ED 1 does not label untransformed CNS resident microglia or resident macrophages of peripheral connective tissue (Damoiseaux et al., 1994) and is, therefore, the best label for fully activated macrophagic phagocytes (for a review, see Moriarty et al., 1998). Interestingly, microglia are also of myelomonocytic origin, reaching the CNS during early development (Ling et al., 1980; Miyake et al., 1984). In a damaged CNS in which the blood–brain barrier is broken, and at distant sites of Wallerian degeneration in damaged axons in the PNS, activated macrophages are derived mainly from blood-borne recruited cells (Giulian et al., 1989; Lunn et al., 1989). However, at the site of Wallerian degeneration in the CNS, Ling (1978) and Lawson et al. (1994) have shown using carbon labeling, [^{3}H]thymidine labeling and X-irradiation that activated macrophages can also be derived from resident microglia. In this paper, we are interested in those activated macrophages, irrespective of origin, that accumulate in injured nervous tissue and that may alter the local microenvironment through the production and liberation of their cell-specific products such as cytokines.

The following observations were made. (i) Similar densities of macrophages (approximately 1200 cells mm^{-2}) occurred at

the site of actual damage in acutely injured spinal cords and in peripheral nerves. (ii) At 5, 10 and 21 days post-injury, significantly higher densities of macrophages were found at the site of actual damage and at a more remote site in the CNS than in the PNS. This was not true for the distant-site comparison at 10 days post-surgery. (iii) Macrophage accumulations were more impressive at the actual site of damage than at a more distant site of Wallerian degeneration at most time points in response to CNS and PNS injuries. (iv) Macrophage densities rose to a peak level and then declined in both spinal cord sample sites and also in the distant site of the injured peripheral nerve. (v) Macrophages harvested from the injured spinal cord had a much greater ability to produce LPS-inducible cytokines when stimulated than those harvested from the injured sciatic nerve. TNFα production at 10 days was the only exception. (vi) In the spinal cord, cytokine levels were maximal between 5 and 10 days for IL-1 and IL-6, whereas TNFα levels peaked between 3 and 5 days. (vii) In the sciatic nerve, TNFα levels showed a distinct peak at 10 days post-injury, whereas both IL-1 and IL-6 levels at the same time point rose only at the site of Wallerian degeneration.

The usual precautions should be taken when extrapolating these *in vitro* data to the *in vivo* injury, although it is probable that our measurements show actual functional differences between CNS and PNS macrophages. The data reflect functional differences in the capacity of PNS and CNS macrophages to express different levels of cytokines at different times post-injury *in vivo*. Determination of cytokine levels in the injured tissues would be less informative, since these data would reflect the products of many cell types inhabiting the injury, including fibroblasts, Schwann cells and epithelial cells (Rotshenker et al., 1991), and would not provide a specific understanding of the possible role of the macrophage in neural injury.

CNS and PNS: differences in macrophage numbers

These data do not support the idea that there is a significantly earlier, or more robust, accumulation of macrophages in the PNS nor the corollary that a failure in regeneration of CNS neurons might be due to a delayed and weak macrophage response to injury in this tissue (Avellino et al., 1995; George and Griffin, 1994; Griffin and George, 1993; Lawson et al., 1994; Perry and Brown, 1992; Perry and Gordon, 1988, 1991; Perry et al., 1987, 1993). Furthermore, we show that macrophages in injured spinal cord are activated to secrete high levels of pro-inflammatory cytokines, particularly at early time points. Macrophages in injured PNS do not display this early and elevated cytokine production (Fig. 5).

Some investigators have viewed the macrophage response to injury in the CNS as extreme, with the large build-up of these cells even causing ‘bystander’ damage to undamaged parenchyma. They further suggest that this exaggerated macrophage response to CNS injury may be one factor responsible for poor functional outcome (Blight, 1985, 1992, 1994; Carlson et al., 1998; Fujita et al., 1998; Giulian and Robertson, 1990; Giulian et al., 1989). In support of this view, Blight (1994) and Giulian and Robertson (1990) have observed a better anatomical and functional outcome in animals with CNS injuries when macrophage numbers are reduced experimentally. In addition, the effects of macrophage-produced pro-inflammatory cytokines appear to be damaging, as shown in several studies (Barone et al., 1997; Nawashiro et al., 1997; Jacobs et al., 1991; Rothwell and Relton, 1993; Relton et al., 1996; Hagberg et al., 1996).

Another view holds that delayed accumulation of relatively few macrophages occurs in CNS injury, particularly in degeneration of distal axonal segments, and this may be associated with the lack of regenerative ability in this tissue. In support, several studies transplanting macrophages/microglia into an injured CNS showed increases in axonal regeneration (David et al., 1990; Lazarov-Spiegler et al., 1996; Prewitt et al., 1997; Franzen et al., 1998). However, this interpretation suffers from a dearth of quantitative, comparative cell counts conducted in the same animal (Avellino et al., 1995; George and Griffin, 1994; Lawson et al., 1994; Lotan et al., 1994; Perry et al., 1987). Many studies have compared damaged optic nerve (as representative of CNS tissue) with injury to the sciatic nerve, but we do not believe the optic nerve is a good model for CNS lesions. Both brain and spinal cord undergo a marked hemorrhagic and ischemic necrosis of gray and white matter and progressive ‘secondary’ destruction of remaining parenchyma near the site of damage (Tator and Fehlings, 1991; Young et al., 1995). There is usually extensive cavitation, cyst formation and progressive destruction of CNS parenchyma, producing stronger signals for myelomonocytic cell recruitment and activation than the more restricted injury to optic nerve axons. In general, the supraspinal inflammatory reaction to injury is weaker than that following damage to the spinal cord (Schnell et al., 1999).

Furthermore, there are only a few reports with which we can compare the numerical data presented here (Blight, 1985; Carlson et al., 1998; Fujita et al., 1998; Lotan et al., 1994). Summarizing what can be gleaned from these reports, it is probable that 1000–3000 macrophages mm^{-2} of tissue are observed accumulating within a central lesion within 72 h of the injury. The data of Carlson et al. (1998) and those from the present study are mutually corroborative in another way. Approximately 500 cells mm^{-2} were observed in both studies at a site of Wallerian degeneration 1–2 mm from the site of actual damage to the spinal cord.

There are even fewer studies with which to compare macrophage accumulations at the site of mechanical damage to peripheral nerve. Our data reveal cell densities between 1000 and 1500 cells mm^{-2} at this site in the sciatic nerve. Avellino et al. (1995) reported approximately one-third of this number, but did not sample cell numbers at the crush site. Their sample site was approximately 0.42 mm from it. At more distant sites of Wallerian degeneration, the data from Avellino et al. (1995) are consistent with those reported here. However, since a 10 day time point was not evaluated in their study, the actual peak density was probably not observed by this group.

Cytokine production in injured nerves

We found early and high pro-inflammatory cytokine levels in the spinal cord relative to the injured sciatic nerve. Others have noted a very early (minutes to hours) and transient increase in pro-inflammatory cytokine production (and/or mRNA content) in the injured CNS and PNS (Minami et al., 1992; Taupin et al., 1993; Shohami et al., 1994; Fan et al., 1995; Klusman and Schwab, 1997; Rotshenker et al., 1991; Reichert et al., 1996). Days after injury to the CNS, however, pro-inflammatory cytokine mRNAs were again expressed at high concentrations and could also be identified in mononuclear cells surrounding the lesion site (Holmin et al., 1997). This is consistent with our data showing that macrophages harvested from injured spinal cord produce higher levels of pro-inflammatory cytokines than their counterparts in damaged sciatic nerve. Overall, our data support the idea that the microenvironment in which macrophages are activated directly impacts on the character of their production and liberation of cell-specific products such as cytokines, which may secondarily alter the reparative or regenerative response to injury (Lazarov-Spiegler et al., 1996; Rapalino et al., 1998; Schwartz et al., 1999).

The pro-inflammatory cytokine TNFα, rapidly present at high levels at the injured CNS, plays a role in fibroglial scar formation, in neovascularization, in 'secondary injury' and in demyelination (Barone et al., 1997; Nawashiro et al., 1997).

The pro-inflammatory cytokine IL-1, also present at higher levels in the injured CNS than PNS, similarly enhances the formation of a fibroglial scar, worsening the effects of injury. Several studies administering recombinant IL-1 to an injured CNS report an exacerbation of damage, while studies administering IL-1 antagonists or inhibitors report a reduction in CNS damage (Hagberg et al., 1996; Jacobs et al., 1991; Relton et al., 1996; Yamasaki et al., 1995).

In contrast, there have been reports of beneficial effects of the cytokine IL-6 to CNS injury (Schindler et al., 1990; Lotan and Schwartz, 1994; Kim, 1996; Holmin et al., 1997; Toulmond et al., 1992). Klusman and Schwab (1997) infused a mixture of TNFα, IL-1 and IL-6 onto rat spinal cords at 4 days post-injury and showed a reduction in the pathology of the injury. We suggest this may have been due largely to IL-6, which overcame the deleterious effects of the other cytokines. It is probable that reparative mechanisms in the spinal cord (and perhaps in the brain) may be enhanced by altering the timing and concentrations of these factors, combined with a strategy of overall reduction in macrophage recruitment to the site(s) of damage.

In summary, our studies show that high numbers of macrophages are recruited early to spinal cord injury and that these cells have a greater capacity for cytokine production than the macrophages present in PNS injury. This greater secretory capacity may contribute to the inability of the CNS to recover from injury.

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