Journal of Neuroscience Research 68:315–322 (2002)

Cytokine Activity Contributes to Induction of Inflammatory Cytokine mRNAs in Spinal Cord Following Contusion

Jonathan Z. Pan,[1] Li Ni,[1] Ajeet Sodhi,[2] Ann Aguanno,[1] Wise Young,[3] and Ronald P. Hart[1,3]*

[1]Department of Biological Sciences, Rutgers University, Newark, New Jersey
[2]Program in Biology, New Jersey Institute of Technology, Newark, New Jersey
[3]The W.M. Keck Center for Collaborative Neurosciences, Rutgers University, Piscataway, New Jersey

Injury of the spinal cord leads to an inflammatory tissue response, probably mediated in part by cytokines. Because a common therapy for acute spinal cord injury is the use of an antiinflammatory synthetic glucocorticoid (methylprednisolone), we sought to determine mechanisms contributing to inflammation shortly after acute injury. Cytokine mRNAs [interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)-α, and IL-6] were increased during the first 2 hr following weight-drop compression injury by RNase protection assay, prior to the reported appearance of circulating lymphocytes. This immediate pattern of cytokine mRNA induction could be replicated in cultured, explanted spinal cord slices but not in whole blood of injured animals, which is consistent with a tissue source of cytokine mRNAs. Western blotting detected IL-1β-like immunoreactivity released into culture medium following explantation and pro-IL-1β-like immunoreactivity in freshly dissected spinal cord tissue. Pharmacologically blocking IL-1 and TNF-α receptors significantly reduced expression of IL-1α, IL-1β, and TNF-α mRNAs. Finally, mice lacking both IL-1 and TNF-α receptors exhibited diminished induction of TNF-α, IL-6, and IL-1ra mRNAs following injury. Therefore, we conclude that contusion injury induces an immediate release of cytokines, which then contributes to the induction of cytokine mRNAs.

Key words: spinal cord injury; cytokine; inflammation; gene knockout; methylprednisolone

Spinal cord injury (SCI) has been shown to induce the cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-α and their mRNAs within as little as 1 hr of injury (Wang et al., 1996, 1997; Bartholdi and Schwab, 1997; Hayashi et al., 1997, 2000; Streit et al., 1998; Le et al., 2000; Ghirnikar et al., 2000). Cytokines, a group of proteins that evoke remarkable responses in a variety of cell types, initiate a complex cascade leading to inflammatory responses, including increased vascular permeability, leukocyte infiltration, and induction of acute-phase response. These cytokines may be synthesized by tissue macrophages/microglia or activated, infiltrating lymphocytes (Bartholdi and Schwab, 1997).

Inflammation would be expected to contribute to secondary tissue damage after compression injury, primarily because of promotion of tissue edema. Inflammatory cytokines induce several molecules capable of allowing vascular permeability as well as inducing cellular fluid loss. These probably include components of the complement cascade (C3a and C5a, which in turn may cause release of histamine, prostaglandins, and leukotrienes from resident mast cells), proteases such as plasminogen activator, and bradykinin. All of these signals lead to enhanced vascular permeability. Indeed, studies show that direct injection of IL-1β into spinal cord leads to enhanced vascular permeability and lymphocyte recruitment (Schnell et al., 1999). Lack of cytokine action, through IL-1β or TNF receptor gene knockout, reduced remyelination (Mason et al., 2001) or diminished functional outcome (Kim et al., 2001). Systemic administration of the antiinflammatory cytokine IL-10 can reduce inflammation, limit neuronal damage, and promote functional recovery following spinal cord injury in rats (Bethea et al., 1999; Plunkett et al., 2001). Similarly, selective cyclooxygenase (COX)-2 inhibition reduces pathological and behavioral deficits following contusion SCI (Hains et al., 2001). Finally, in a transgenic mouse expressing myelin basic protein (MBP)-specific T cell receptor, increased expression of proinflammatory cytokines was shown to be associated with neurolog-

Contract grant sponsor: Christopher Reeve Paralysis Foundation; Contract grant sponsor: Charles and Johanna Busch Bequest Fund of Rutgers University; Contract grant sponsor: New Jersey Commission on Spinal Cord Research; Contract grant sponsor: Spinal Cord Injury Project of the W.M. Keck Center for Collaborative Neuroscience of Rutgers University.

*Correspondence to: Ronald P. Hart, The W.M. Keck Center for Collaborative Neuroscience, Rutgers University, Rm. D251, 604 Allison Rd., Piscataway, NJ 08854. Phone: (732) 445-1783; FAX (732) 445-2063; E-mail: rhart@andromeda.rutgers.edu

Received 12 December 2001; Revised 24 January 2002; Accepted 29 January 2002

ical functional impairment after SCI (Jones et al., 2002). These results demonstrate that inflammation-promoted cellular damage exacerbates lesion volumes and SCI functional outcomes.

However, other studies have found that inflammatory responses promote beneficial responses at the site of injury. Indeed, treatment of SCI with direct application of inflammatory cytokines has been shown to reduce tissue loss (Klusman and Schwab, 1997). Infusion of macrophages also promoted tissue repair following SCI (Franzen et al., 1998), and optic nerve injury was ameliorated following infusion of activated macrophages (Lazarov-Spiegler et al., 1998) or autoimmune T cells (Moalem et al., 1999). Other pathways stimulated by inflammatory cytokines seem likely to promote wound repair in SCI. Adhesion molecules (ICAM, VCAM, and P- and E-selectins) would be expected to be induced, and chemokines (Gro-α, LIX, MCP-1 and -5, IP-10, and others) are synthesized (McTigue et al., 1998; Le et al., 2000), promoting monocyte and neutrophil infiltration (Ghirnikar et al., 2000) and phagocytic macrophages and myelin clearance from mouse spinal cord (Ousman and David, 2001). Cytokines directly stimulate leukocytes to become phagocytic, removing cellular debris from the site of injury (Matsumoto and Kreutzberg, 1995). Antiinflammatory treatment, such as with methylprednisolone, would be expected to inhibit these beneficial effects of the inflammatory cascade.

Therefore, to identify the source of inflammatory signaling following SCI, we studied the induction of inflammatory cytokine mRNAs following contusion injury. We found that cytokine mRNAs are detected as soon as 15 min after injury. Our results are consistent with a local source of inflammatory cytokine. IL-1β-like immunoreactivity (IL-1β-LI) is found released into culture medium upon explantation, and a propeptide form of IL-1β is found in freshly dissected spinal cord tissue. Furthermore, a lack of IL-1 and TNF-α signaling reduces the induction of IL-1β mRNA, suggesting that it is the activity of these cytokines that contributes to their own mRNA regulation.

MATERIALS AND METHODS

Long-Evans hooded rats (half male and half female) were contused using the MASCIS standard weight-drop impactor (formerly known as the NYU impactor) with a 10 g weight dropped 25.0 mm onto T9–T10 spinal cord exposed by laminectomy. At various times following impact, 5 mm tissue sections were collected and stored frozen at −80°C.

Wild-type (C57Bl/6 × 129J progeny) mice or mice lacking IL-1RI and TNFRp55 receptor genes (Jackson Labs Strain JR.3244) were contused using a modified MASCIS impactor (the impact head was replaced with a smaller diameter head). Three groups of mice (two per group) were anesthetized, injured by contusion with a 10.0 g weight dropped 6.25 mm, sutured, and allowed to recover. Uninjured mice (0 hr) or injured mice (4 hr) were euthanized, and a 5 mm segment of spinal cord from the site of injury was collected for RNA preparation.

Total cellular RNA was prepared by homogenization of tissue in Ultraspec (Biotecx, Houston, TX) or Trizol (Invitrogen, La Jolla, CA), followed by a final precipitation from 2 M ammonium acetate with 2.5 volumes of ethanol. RNA was washed, dried, and resuspended in sterile water, then stored at −80°C.

Cytokine mRNAs were assayed by RNase protection assay (RPA) using the Pharmingen Riboquant kit for rat cytokine mRNAs (BD Biosciences). This kit allows simultaneous detection of up to 13 mRNAs, including 11 cytokine mRNAs and 2 control mRNAs. Generally, 2–4 μg of total cellular RNA was hybridized with 8×10^5 cpm of probe in a 10 μl reaction. After hybridization, RNase treatment, and proteinase K treatment (performed according to the kit instructions), protected fragments were run on a 4% acrylamide-8 M urea sequencing gel. The gel was dried and exposed to a phosphorimaging screen, then read on a Molecular Dynamics (Sunnyvale, CA) phosphorimager and analyzed with ImageQuant software. GAPDH mRNA levels were higher in more distal segments of spinal cord and lower in more proximal segments, consistently with the relative proportion of cell-rich gray matter.

In some experiments, cultured spinal cord slices were used as a model of SCI. Uninjured adult rat spinal cords were quickly isolated, and then 1 mm slices were placed in wells of a 24-well tissue culture plate. Four slices were typically placed in a single well with 0.5 ml of Opti-MEM medium with 1% N2 supplement (Invitrogen) and antibiotics (penicillin 25 U/ml, streptomycin 25 μg/ml; Sigma, St. Louis, MO).The cultures were incubated at 37°C in 5% CO_2 for up to 6 hr, then frozen for RNA analysis.

Spinal cord slices were placed in serum-free culture medium; then, tissue and medium were prepared for Western blotting in lysis buffer (New England Biolabs, Beverly, MA). Crude proteins were separated by SDS-PAGE, then detected by immunoblotting with 1:500 diluted Armenian hamster monoclonal anti-mouse IL-1β (Biogenesis) and visualized with secondary alkaline phosphatase-conjugated goat anti-Armenian hamster IgG. In competitive Western blotting, the monoclonal anti-IL-1β primary antibody was first mixed with recombinant rat IL-1β (rrIL-1β; Liu et al., 1995) at various concentrations overnight, then incubated with individual strip of blot, which had equal amounts of protein loaded as control strips without rrIL-1β pretreatment. We then followed the procedure described above.

Because so little RNA was available in the mouse contusion studies, we assayed selected cytokine mRNAs by quantitative reverse transcription-PCR (Q-RT-PCR) using selected gene-specific primer pairs (Table I). RNA was purified from a 4 mm segment of cord centered at the contusion site using the Trizol protocol and reverse transcribed with SuperScript II and random primers as suggested by the manufacturer (Invitrogen). The PCRs were carried out using the equivalent of cDNA from 10 ng of mRNA, 50 nM of each primer, and SYBR green master mix (Applied Biosystems, Foster City, CA) in 10 μl reactions. Levels of Q-RT-PCR product were measured using SYBR green fluorescence (Ririe et al., 1997; Wittwer et al., 1997) collected during real-time PCR on an Applied Biosystems 7900HT system. A control cDNA dilution series was created for

TABLE I. Oligonucleotide Primers Used in Q-RT-PCR Assays

GenBank accession number	Gene name	Sequence
NM_00836	IL-1β	5′-CCAAAAGATGAAGGGCTGCT-3′
		5′-TCATCAGGACAGCCCAGGTC-3′
NM_031167	IL-1ra	5′-CGCTTTACCTTCATCCGCTC-3′
		5′-TGACTCAAAGCTGGTGGTGG-3′
NM_013693	TNF-α	5′-ATGCTGGGACAGTGACCTGG-3′
		5′-CCTTGATGGTGGTGCATGAG-3′
NM_031168	IL-6	5′-TTCCATCCAGTTGCCTTCTTG-3′
		5′-GAAGGCCGTGGTTGTCACC-3′

each primer pair to establish a standard curve. Each reaction was subjected to melting-point analysis to confirm the presence of a single amplified product.

RESULTS

To detect the inflammatory response to SCI quantitatively, we assayed 11 cytokine mRNAs simultaneously by RPA. It is important to consider several inflammatory cytokines at once, because they have many overlapping functions and because one inflammatory cytokine is likely to induce the expression of others. Figure 1 depicts the time course for the first 2 hr following injury. During this period, IL-1α, IL-1β, TNF-α, and IL-6 are significantly increased over sham surgery control by 15 min (ANOVA; 95% confidence levels). IL-1α and IL-1β mRNAs continue to rise, reaching peak levels at 6 hr, then falling by 12–24 hr (Hart et al., 1999). Previous studies found a peak of IL-1β mRNA at 1 hr and IL-1β protein at 8 hr (Wang et al., 1997). TNF-α mRNA peaks quickly by about 60 min, then falls slightly by 120 min. IL-6 mRNA increases more slowly, reaching peak levels by 6–12 hr, then falling off by 24 hr (Hart et al., 1999). Levels of these mRNAs are nearly undetectable in sham-injured animals. Because both IL-1 mRNAs and TNF-α mRNA are induced by IL-1 and TNF-α action (Dinarello et al., 1986; Witsell and Schook, 1993; Tada et al., 1994; Imaizumi et al., 2000), we conclude that the induction of these mRNAs may indicate an immediate release of stored inflammatory cytokines following SCI. The induction of IL-1 and TNF-α mRNAs, then, is likely to be the response to this initial release, with a replenishment of newly accumulated cytokine mRNA. Both immunocytochemistry and in situ hybridization have detected intracellular cytokines following SCI (Wang et al., 1996; Bartholdi and Schwab, 1997; Streit et al., 1998). Most authors conclude that expression is probably limited to the microglia within the first few hours after injury.

Interestingly, several cytokine mRNAs are *not* detected during this period. Notably, IL-2 and interferon (IFN)-γ mRNAs would be expected to be found if large numbers of T cells infiltrated the injury during this period. Both IL-2 and IFN-γ were clearly detected in positive-control RNA, demonstrating that our assay could detect these species.

Because one concern is that cytokine mRNAs may be due to the presence of pooled blood cells at the site of injury, we assayed peripheral blood from injured animals (Fig. 1A). The only cytokine mRNA detected was IL-1β. Therefore, the presence of other cytokine mRNAs in injured spinal cord could not have been due to the presence of blood cells.

To develop a more controllable model of this tissue inflammation following SCI, we tested cytokine mRNA levels in cultured spinal cord slices. In this culture system, the slicing of the spinal cord prior to culture is intended to be the model of spinal cord injury. Therefore, time in culture should correlate with time following injury. RPA at several time points shows a pattern of cytokine gene expression similar to that following weight-drop compression injury in vivo (Fig. 2). The induction of these cytokine mRNAs in excised tissue slices implies that no infiltrating blood-borne cells were required for cytokine mRNA accumulation, because the sliced tissue was completely removed from the circulating blood system. Furthermore, any blood cells included in the slice explant had the capability of diffusing away from the slices during culturing. The progression from initial stage (IL-1α, IL-1β, and TNF-α beginning at 30 min) to later stage (IL-6 at 120 min) is similar to that in in vivo injury. In conclusion, the slice cultures at least replicate the initial pattern of cytokine mRNA accumulation following SCI.

We next examined the presence of IL-1β protein in injured and uninjured spinal cord. Western blotting shows that rrIL-1β was detected at the expected mobility for bacterially produced protein and that two bands (35 and 18 kDa) can be detected in homogenates of cultured spinal cord tissue (Fig. 3A). After preincubating the monoclonal anti-IL-1β primary antibody with rrIL-1β, we found that both rrIL-1β and 35 kDa bands were completely blocked and disappeared in a concentration-dependent manner. The intensity of the 18 kDa band also decreased at least 50% after blocking with higher concentrations of rrIL-1β. These results confirmed that both propeptide (35 kDa) and mature (18 kDa) IL-1β-LI could be identified in our model system. Furthermore, this IL-1β-LI can be detected in various explanted culture conditions (Fig. 3B). The mature form of IL-1β-LI is detectable in medium by 2 hr following explantation, suggesting that inflammatory cytokine is released very quickly upon tissue damage. Moreover, the presence of the precursor peptide band in freshly dissected spinal cord slices argues that cells within spinal cord contain pools of inactive procytokine and that they are, therefore, armed for an injury-induced processing and release.

If spinal cord injury leads to inflammatory signaling, bioactive cytokines must be released. Enzymes required for cytokine synthesis and receptor activity would have to be present in spinal cord prior to injury for an immediate release of mature cytokines to be possible. In the example of IL-1β, the mRNA encodes pro-IL-1β peptide, which must be cleaved by a cysteinyl aspartate-specific protease called *ICE* (also known as *caspase-1*) to produce bioactive

IL-1β. We determined that ICE mRNA is present in uninjured spinal cord by RT-PCR (not shown). The mRNA encoding signal-transducing IL-1 receptor, IL-1RI, is also present (not shown), suggesting that cells may be responsive to inflammatory signaling prior to injury and throughout this experiment.

If cytokine mRNAs are postulated to be regulated by cytokine bioactivity released by injury, then a blockade of early proinflammatory cytokine receptors should diminish the induction of cytokine mRNA by injury. To test this hypothesis, we resorted to both genetic and pharmacological approaches. We reasoned that a genetic knockout of only the IL-1RI gene would be "rescued" by the use of a similarly acting TNF-α-signaling pathway. Therefore, double-knockout mice, lacking both IL-1RI and TNFRp55, were used to test our hypothesis. Mice were injured with a modified MASCIS impactor (the impact head was replaced with a smaller diameter head), and mouse spinal cord RNA was assayed by Q-RT-PCR because of the small quantities of tissues available. First, wild-type mice (of genetic background similar to that of knockouts, C57bl/6 × 129J F1) were tested for cytokine mRNA induction following injury. By 4 hr after contusion injury, significantly increased levels of IL-1ra and IL-6 mRNAs were clearly detected by Q-RT-PCR (Fig. 4; $P < 0.05$, Student's t-test). Because IL-1ra is often induced following IL-1 action at its receptor (Martel-Pelletier et al., 1993; Ilyin and Plata-Salamán, 1996), its regulation is consistent with the presence of IL-1 bioactivity following spinal cord injury. In these experiments, IL-1β and TNF-α mRNAs were not significantly regulated, probably because of variability in their control mRNA levels observed in our experiments. RPAs were able to demonstrate induced IL-1β mRNA levels (not shown). Mice lacking both IL-1RI and TNFRp55 exhibited significantly reduced IL-1ra, IL-6, and TNF-α mRNAs in the injured IL-1RI$^{-/-}$/TNFRp55$^{-/-}$ mice compared with injured wild-type (Fig. 4; $^{**}P < 0.05$, Student's t-test). These results demonstrate that IL-1 and TNF-α receptor signaling contributes to the induction of cytokine mRNAs following injury.

To confirm further our hypothesis using a pharmacological approach, rat spinal cord slice cultures were

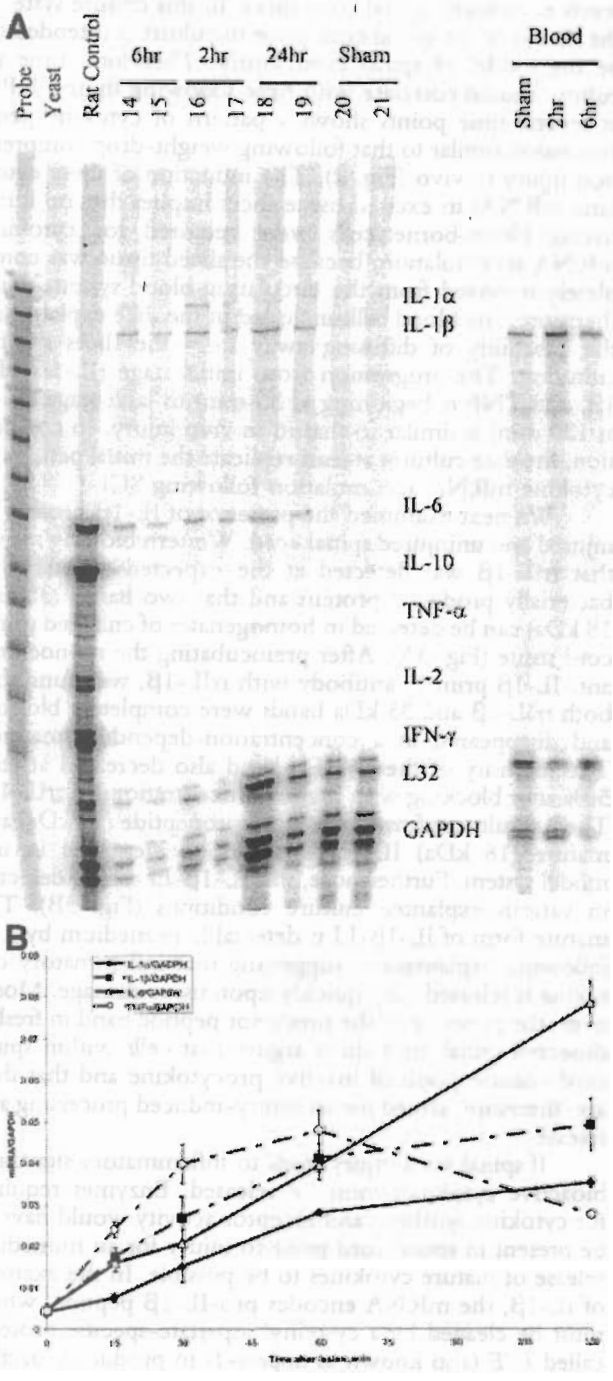


Fig. 1. Example multiple-probe RNase protection assay (RPA) of contused rat spinal cords. **A:** Sample RPA. Individual rats were injured by weight-drop compression with a 10.0 g weight dropped 25.0 mm onto exposed T9–T10 spinal cord. Muscle and skin were temporarily sutured. At the times indicated, rats were euthanized, and a 5 mm section containing the site of contusion was excised for analysis. Total cellular RNA prepared from a single cord segment was used in an RPA with the BD Pharmingen Riboquant rat cytokine probe set (Probe). After RNase treatment, protected fragments were loaded onto a sequencing gel, electrophoresed, dried, and imaged using a phosphorimager. Full-length probes were loaded as markers. One assay contained an equivalent amount of yeast RNA as control (Yeast). Rat Control, a positive control RNA provided by Pharmingen. The tag numbers of individual rats are shown above each lane along with the time postinjury. On the right is an RPA showing cytokine mRNA levels detected in blood from sham-injured animals (Sham) or animals contusion injured for 2 or 6 hr. **B:** Time course. Results from several RPA analyses, including the one shown in A, were quantified and assembled into a time course. For each mRNA sample, the total radioactivity (as volume) was expressed as a ratio to GAPDH mRNA internal standard volumes (mRNA/GAPDH). Measured volumes were corrected for size of the protected band to correspond with molar ratios of different mRNAs. Each point is the mean ± SEM for n = 4, except at 0 (n = 3) and 120 (n = 2) min.

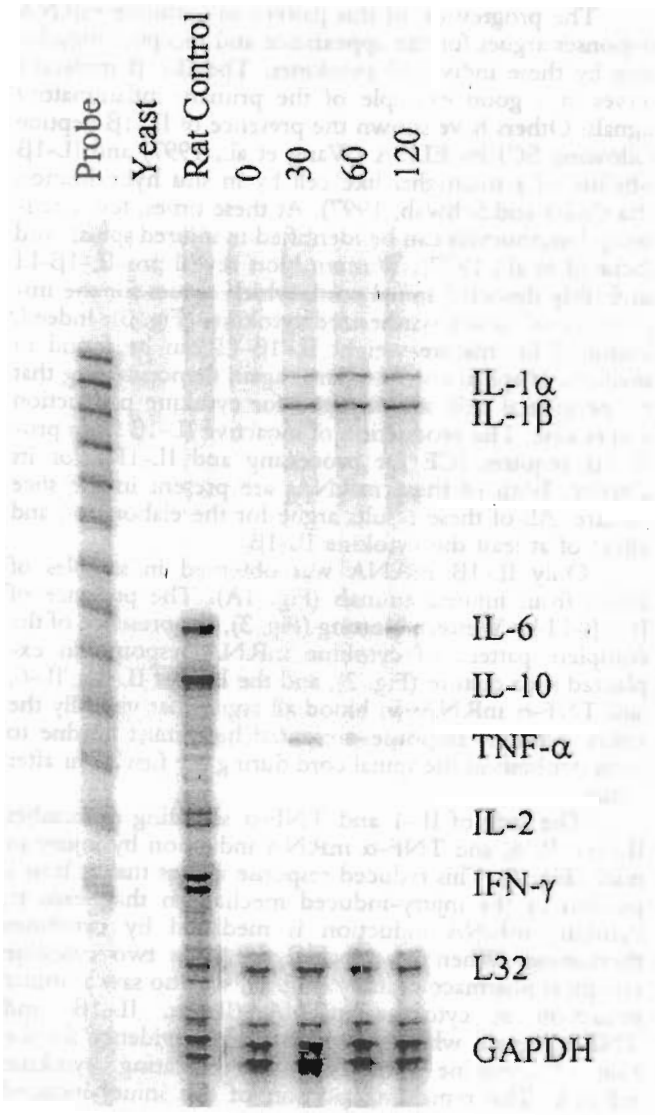


Fig. 2. Cytokine mRNA expression in spinal cord slice cultures. Adult rat spinal cords were dissected and sliced into 1 mm segments. Four slices were added to 0.5 ml of culture medium in a 24-well culture plate. At the time indicated, slices were harvested and used to prepare total cellular RNA. The RNase protection assay used the BD Pharmingen Multiprobe rat cytokine probe.

treated with both IL-1 and TNF-α receptor blockers (IL-1ra and sTNFR:Fc) during the 4 hr incubation (Fig. 5). Not surprisingly, after a 4 hr culture period, cytokine mRNA induction (IL-1α, IL-1β, and TNF-α) was significantly reduced in the drug-treated group compared with the untreated group (Fig. 5; $P < 0.05$, Student's *t*-test). Therefore, we conclude that cytokines signaling through their own receptors contribute to increased levels of inflammatory cytokine mRNA.

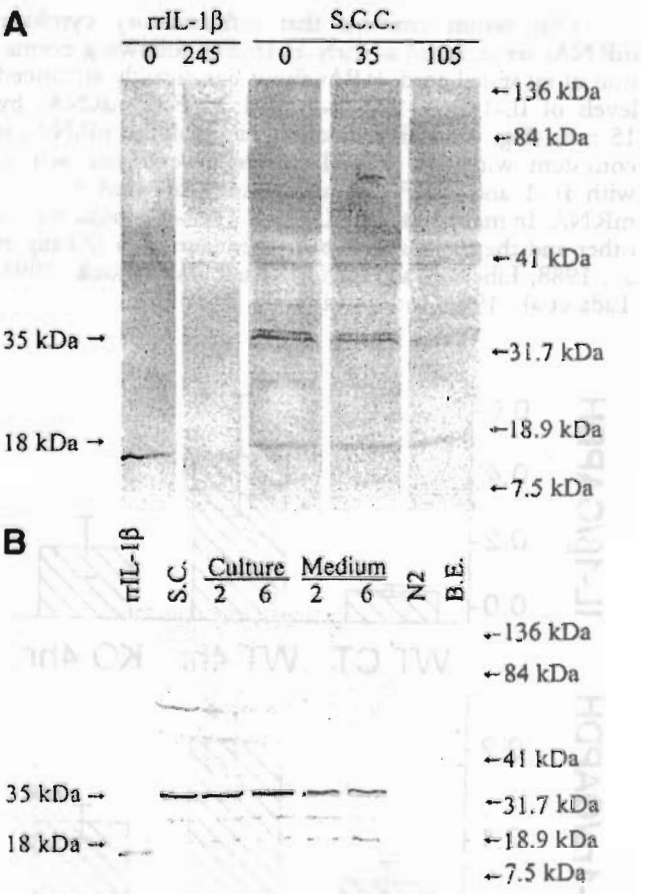
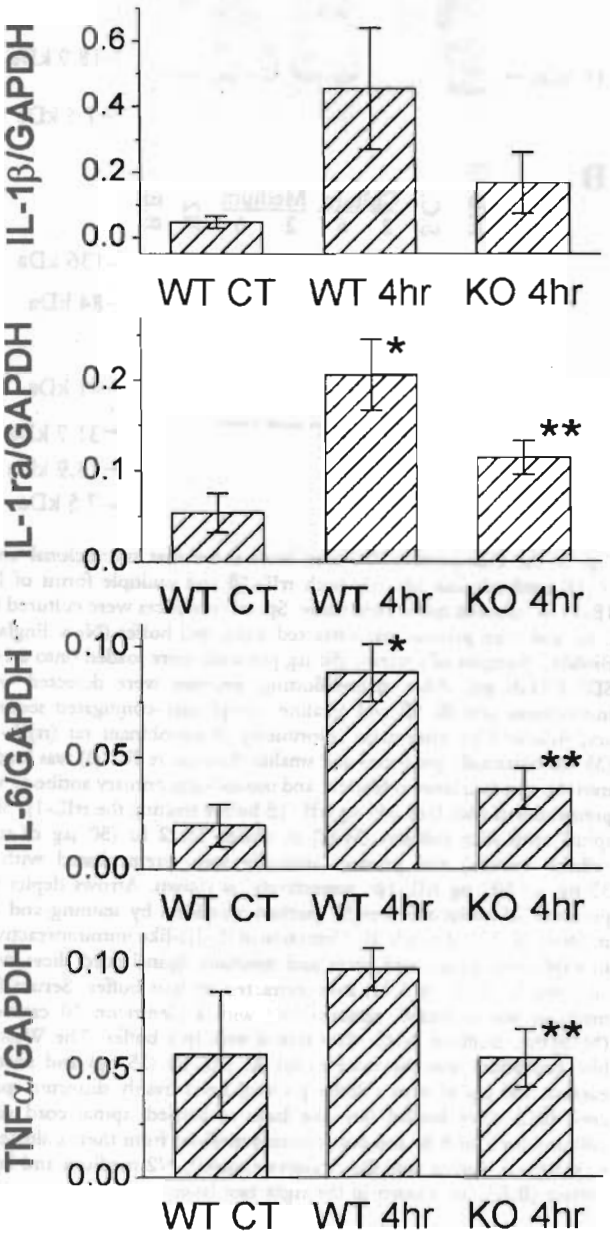


Fig. 3. **A:** Competitive Western blots show that monoclonal anti-IL-1β antibody can identify both rrIL-1β and multiple forms of IL-1β-LI in cultured spinal cord tissue. Spinal cord slices were cultured for 2 hr, and then protein was extracted using lysis buffer (New England Biolabs). Samples of proteins (50 μg per well) were loaded onto a 14% SDS-PAGE gel. After electroblotting, proteins were detected with monoclonal anti-IL-1β and alkaline phosphatase-conjugated secondary, followed by enzymatic colorimetry. Recombinant rat (rr)IL-1β (35 ng; bacterially produced and smaller than native IL-1β) was loaded into the first two lanes as labeled, and monoclonal primary antibody was preincubated with 0 or 245 ng rrIL-1β before treating the rrIL-1β blot; spinal cord slice cultures (SCC) incubated for 2 hr (50 μg of total cellular protein) and primary antibody were preincubated with 0, 35 ng, or 105 ng rrIL-1β, respectively, as shown. Arrows depict the positions of molecular weight markers visualized by staining and the multiple IL-1β-LI bands. **B:** Detection of IL-1β-like immunoreactivity in explanted spinal cord slices and medium. Spinal cord slices were cultured for 0, 2, or 6 hr, then extracted by lysis buffer. Serum-free medium was collected, concentrated with a Centricon 10 cartridge (Millipore, Bedford, MA), then mixed with lysis buffer. The Western blot procedure was the same as for A. rrIL-1β (35 ng) and protein extracts (50 μg of total cellular protein) from freshly dissected spinal cord (SC) were loaded into the lanes as labeled; spinal cord slices cultured for 2 or 6 hr and concentrated medium from these cultures (2 or 6 hr) are shown, and the negative controls, N2 medium and brain extract (B.E.), are shown in the right two lanes.

DISCUSSION

Our results confirm that inflammatory cytokine mRNAs are induced as early as 15 min following contusion of rat spinal cord. RPAs show significantly enhanced levels of IL-1α, IL-1β, IL-6, and TNF-α mRNAs by 15 min (Fig. 1). This induction of cytokine mRNAs is consistent with the general pattern of cytokine action, with IL-1 and TNF-α peaking first, followed by IL-6 mRNA. In many systems, IL-1 and TNF-α stimulate each other and themselves, and both stimulate IL-6 (Zhang et al., 1988; Libert et al., 1990; Witsell and Schook, 1993; Tada et al., 1994; Imaizumi et al., 2000).



The progression of this pattern of cytokine mRNA responses argues for the appearance and receptor stimulation by these individual cytokines. The IL-1β molecule serves as a good example of the primary inflammatory signals. Others have shown the presence of IL-1β peptide following SCI by ELISA (Wang et al., 1997) and IL-1β labeling of a microglial-like cell by in situ hybridization (Bartholdi and Schwab, 1997). At these times, few circulating lymphocytes can be identified in injured spinal cord (Schnell et al., 1997). Western blots reveal pro-IL-1β-LI in freshly dissected spinal cord, which argues for the importance of locally synthesized cytokines (Fig. 3). Indeed, within 2 hr, mature-weight IL-1β-LI can be found in medium of spinal cord explants, again demonstrating that no peripheral cells are required for cytokine production and release. The production of bioactive IL-1β from pro-IL-1β requires ICE for processing and IL-1RI for its activity. Both of these mRNAs are present in the slice culture. All of these results argue for the elaboration and effect of at least the cytokine IL-1β.

Only IL-1β mRNA was observed in samples of blood from injured animals (Fig. 1A). The presence of IL-1β-LI by Western blotting (Fig. 3), the presence of the complete pattern of cytokine mRNA response in explanted slice culture (Fig. 2), and the lack of IL-1α, IL-6, and TNF-α mRNAs in blood all argue that virtually the entire cytokine response presented here must be due to local synthesis in the spinal cord during the first 24 hr after injury.

The lack of IL-1 and TNF-α signaling diminishes IL-1ra, IL-6, and TNF-α mRNA induction by injury in mice (Fig. 4). This reduced response argues that at least a portion of the injury-induced mechanism that leads to cytokine mRNA induction is mediated by cytokines themselves. When we blocked the same two cytokine receptors pharmacologically (Fig. 5), we also saw a similar reduction in cytokine mRNA (IL-1α, IL-1β, and TNF-α) levels, which provided further evidence for the role of cytokine bioactivity in regulating cytokine mRNA. The remaining portion of the injury-induced

Fig. 4. Reduced cytokine mRNA expression in IL-1RI$^{-/-}$/TNFRp55$^{-/-}$ mice by Q-RT-PCR. Wild-type and IL-1RI$^{-/-}$/TNFRp55$^{-/-}$ mice were contused using a modified MASCIS impactor with a 10.0 g weight dropped 6.25 mm onto exposed midthoracic spinal cord. RNA was purified from a 4 mm segment of cord centered at the contusion site and reverse transcribed. The PCRs were carried out using the equivalent of 10 ng of cDNA, 50 nM of each primer, and SYBR green master mix in 10 μl reactions. Levels of Q-RT-PCR product were measured using SYBR green fluorescence detected by an Applied Biosystems 7900HT system. Each reaction was subjected to melting-point analysis to confirm the presence of a single amplified product (not shown). IL-ra and IL-6 were significantly induced after 4 hr injury in wild-type mice ($P < 0.05$, Student's t-test; n = 3). IL-1ra, IL-6, and TNF-α expression were significantly reduced after injury in knockout mice compared with wild type ($P < 0.05$, Student's t-test; n = 3). *Significant difference from wild-type, sham-injured mice; **Significant difference from wild-type, injured mice.

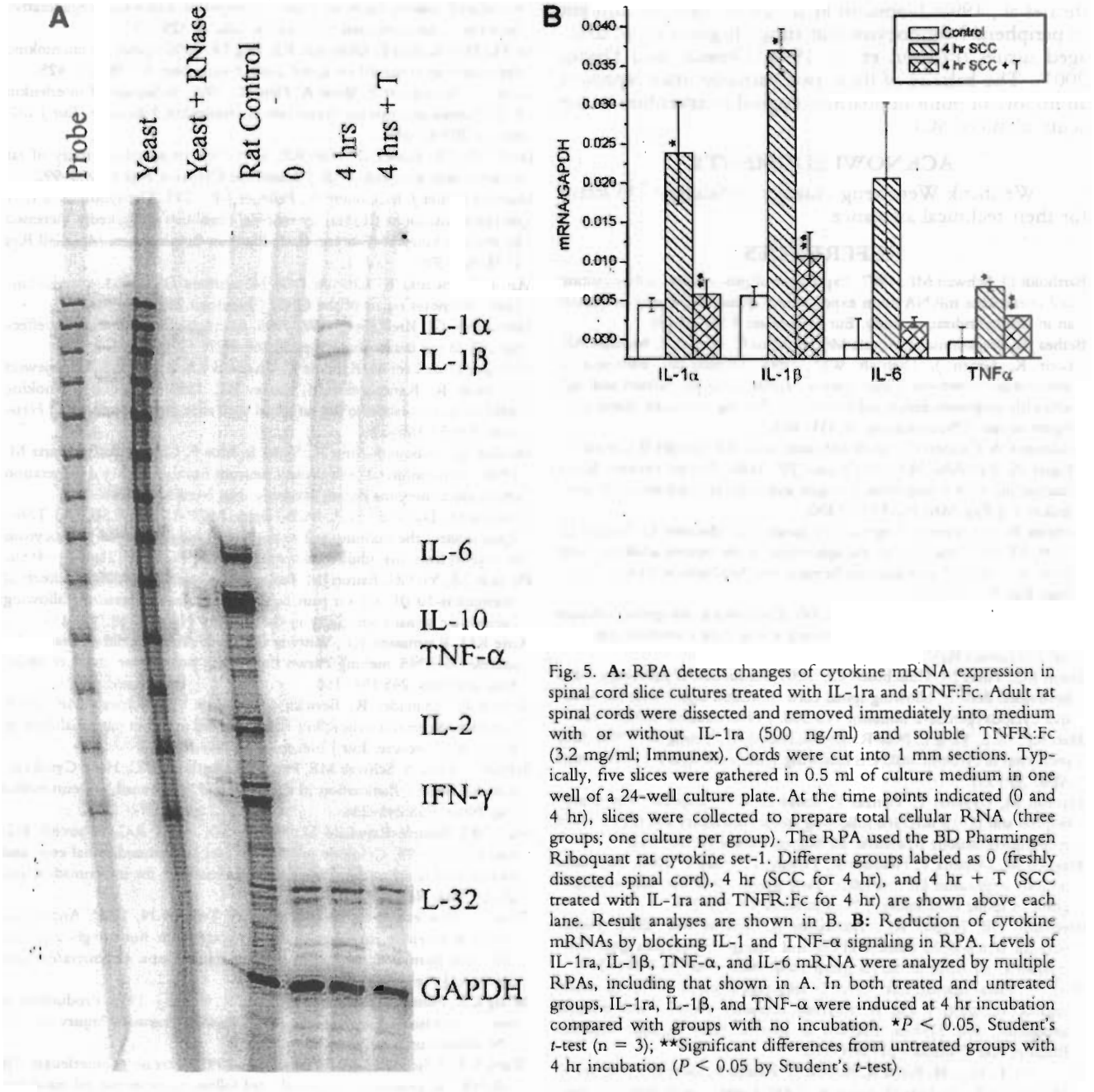


Fig. 5. **A:** RPA detects changes of cytokine mRNA expression in spinal cord slice cultures treated with IL-1ra and sTNF:Fc. Adult rat spinal cords were dissected and removed immediately into medium with or without IL-1ra (500 ng/ml) and soluble TNFR:Fc (3.2 mg/ml; Immunex). Cords were cut into 1 mm sections. Typically, five slices were gathered in 0.5 ml of culture medium in one well of a 24-well culture plate. At the time points indicated (0 and 4 hr), slices were collected to prepare total cellular RNA (three groups; one culture per group). The RPA used the BD Pharmingen Riboquant rat cytokine set-1. Different groups labeled as 0 (freshly dissected spinal cord), 4 hr (SCC for 4 hr), and 4 hr + T (SCC treated with IL-1ra and TNFR:Fc for 4 hr) are shown above each lane. Result analyses are shown in B. **B:** Reduction of cytokine mRNAs by blocking IL-1 and TNF-α signaling in RPA. Levels of IL-1ra, IL-1β, TNF-α, and IL-6 mRNA were analyzed by multiple RPAs, including that shown in A. In both treated and untreated groups, IL-1ra, IL-1β, and TNF-α were induced at 4 hr incubation compared with groups with no incubation. $*P < 0.05$, Student's *t*-test (n = 3); **Significant differences from untreated groups with 4 hr incubation ($P < 0.05$ by Student's *t*-test).

stimulation of cytokine mRNAs is likely due to several sources, including chemokines, prostaglandins, leukotrienes, reactive oxygen species, and lipid oxidation products. Therefore, the diminished cytokine mRNA levels following injury in the absence of IL-1 and TNF-α receptor activity in our experimental models confirm our hypothesis that cytokines at least contribute to cytokine mRNA induction following spinal cord injury.

The appearance of increased levels of cytokine mRNAs beginning as early as 15 min argues for a prior stimulation by cytokines or other inflammatory signals. Because cytokine mRNAs are not themselves bioactive and they are commonly produced in response to active cytokines, we conclude that injury stimulates the nearly immediate release of stored cytokines, and the induction of cytokine mRNAs demonstrates the effect of cytokines in the spinal cord. Regulation of cytokine mRNAs represents one of the earliest responses to SCI. This amplification of inflammatory signaling probably leads to both edema, producing ischemia and secondary cell death (Be-

thea et al., 1999; Hermann et al., 2001), and recruitment of peripheral lymphocytes, initiating phagocytosis of damaged tissues (Franzen et al., 1998; Ousman and David, 2001). The balance of these two pathways may represent an important point of pharmacological intervention in the acute phase of SCI.

ACKNOWLEDGMENTS

We thank Wencheng Huang and Suzanne D'Orazio for their technical assistance.

REFERENCES

Bartholdi D, Schwab ME. 1997. Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an in situ hybridization study. Eur J Neurosci 9:1422–1438.

Bethea JR, Nagashima H, Acosta MC, Briceno C, Gomez F, Marcillo AE, Loor K, Green J, Dietrich WD. 1999. Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats. J Neurotrauma 16:851–863.

Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beutler B, Cerami A, Figari IS, Palladino MAJ, O'Connor JV. 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J Exp Med 163:1433–1450.

Franzen R, Schoenen J, Leprince P, Joosten E, Moonen G, Martin D. 1998. Effects of macrophage transplantation in the injured adult rat spinal cord: a combined immunocytochemical and biochemical study. J Neurosci Res 51:316–327.

Ghirnikar RS, Lee YL, Eng LF. 2000. Chemokine antagonist infusion attenuates cellular infiltration following spinal cord contusion injury in rat. J Neurosci Res 59:63–73.

Hains BC, Yucra JA, Hulsebosch CE. 2001. Reduction of pathological and behavioral deficits following spinal cord contusion injury with the selective cyclooxygenase-2 inhibitor NS-398. J Neurotrauma 18:409–423.

Hart RP, Ni L, Yang L, Brus-Ramer M, Huang W, Young W. 1999. Early induction of cytokine mRNAs following spinal cord injury. Soc Neurosci Abstr 25:1331.

Hayashi M, Ueyama T, Tamaki T, Senba E. 1997. Expression of neurotrophin and IL-1β mRNAs following spinal cord injury and the effects of methylprednisolone treatment. Kaibogaku Zasshi 72:209–213.

Hayashi M, Ueyama T, Nemoto K, Tamaki T, Senba E. 2000. Sequential mRNA expression for immediate early genes, cytokines, and neurotrophins in spinal cord injury. J Neurotrauma 17:203–218.

Hermann GE, Rogers RC, Bresnahan JC, Beattie MS. 2001. Tumor necrosis factor-alpha induces cFOS and strongly potentiates glutamate-mediated cell death in the rat spinal cord. Neurobiol Dis 8:590–599.

Ilyin SE, Plata-Salamán CR. 1996. In vivo regulation of the IL-1β system (ligand, receptors I and II, receptor accessory protein, and receptor antagonist) and TNF-α mRNAs in specific brain regions. Biochem Biophys Res Commun 227:861–867.

Imaizumi T, Itaya H, Fujita K, Kudoh D, Kudoh S, Mori K, Fujimoto K, Matsumiya T, Yoshida H, Satoh K. 2000. Expression of tumor necrosis factor-alpha in cultured human endothelial cells stimulated with lipopolysaccharide or interleukin-1alpha. Arterioscler Thromb Vasc Biol 20:410–415.

Kim GM, Xu J, Xu J, Song SK, Yan P, Ku G, Xu XM, Hsu CY. 2001. Tumor necrosis factor receptor deletion reduces nuclear factor-kappaB activation, cellular inhibitor of apoptosis protein 2 expression, and functional recovery after traumatic spinal cord injury. J Neurosci 21:6617–6625.

Klusman I, Schwab ME. 1997. Effects of pro-inflammatory cytokines in experimental spinal cord injury. Brain Res 762:173–184.

Lazarov-Spiegler O, Solomon AS, Schwartz M. 1998. Peripheral nerve-stimulated macrophages simulate a peripheral nerve-like regenerative response in rat transected optic nerve. Glia 24:329–337.

Le YL, Shih K, Bao P, Ghirnikar RS, Eng LF. 2000. Cytokine chemokine expression in contused rat spinal cord. Neurochem Int 36:417–425.

Libert C, Brouckaert P, Shaw A, Fiers W. 1990. Induction of interleukin 6 by human and murine recombinant interleukin 1 in mice. Eur J Immunol 20:691–694.

Liu C, Bai Y, Ganea D, Hart RP. 1995. Species-specific activity of rat recombinant interleukin-1β. J Interferon Cytokine Res 15:985–992.

Martel-Pelletier J, McCollum R, Pelletier J-P. 1993. The synthesis of IL-1 receptor antagonist (IL-1ra) by synovial fibroblasts is markedly increased by the cytokines TNF-α and IL-1. Biochim Biophys Acta Mol Cell Res 1175:302–305.

Mason JL, Suzuki K, Chaplin DD, Matsushima GK. 2001. Interleukin-1beta promotes repair of the CNS. J Neurosci 21:7046–7052.

Matsumoto GJ, Kreutzberg GW. 1995. Microglia: intrinsic immunoeffector cell of the brain. Brain Res 20:269–287.

McTigue DM, Tani M, Krivacic K, Chernosky A, Kelner GS, Maciejewski D, Maki R, Ransohoff RM, Stokes BT. 1998. Selective chemokine mRNA accumulation in the rat spinal cord after contusion injury. J Neurosci Res 53:368–376.

Moalem G, Leibowitz-Amit R, Yoles E, Mor F, Cohen IR, Schwartz M. 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. Nat Med 5:49–55.

Ousman SS, David S. 2001. MIP-1alpha, MCP-1, GM-CSF, and TNF-alpha control the immune cell response that mediates rapid phagocytosis of myelin from the adult mouse spinal cord. J Neurosci 21:4649–4656.

Plunkett JA, Yu CG, Easton JM, Bethea JR, Yezierski RP. 2001. Effects of interleukin-10 (IL-10) on pain behavior and gene expression following excitotoxic spinal cord injury in the rat. Exp Neurol 168:144–154.

Ririe KM, Rasmussen RP, Wittwer CT. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245:154–160.

Schnell L, Schneider R, Berman MA, Perry VH, Schwab ME. 1997. Lymphocyte recruitment following spinal cord injury in mice is altered by prior viral exposure. Eur J Neurosci 9:1000–1007.

Schnell L, Fearn S, Schwab ME, Perry VH, Anthony DC. 1999. Cytokine-induced acute inflammation in the brain and spinal cord. J Neuropathol Exp Neurol 58:245–254.

Streit WJ, Semple-Rowland SL, Hurley SD, Miller RC, Popovich PG, Stokes BT. 1998. Cytokine mRNA profiles in contused spinal cord and axotomized facial nucleus suggest a beneficial role for inflammation and gliosis. Exp Neurol 152:74–87.

Tada M, Diserens A-C, Desbaillets I, De Tribolet N. 1994. Analysis of cytokine receptor messenger RNA expression in human glioblastoma cells and normal astrocytes by reverse-transcription polymerase chain reaction. J Neurosurg 80:1063–1073.

Wang CX, Nuttin B, Heremans H, Dom R, Gybels J. 1996. Production of tumor necrosis factor in spinal cord following traumatic injury in rats. J Neuroimmunol 69:151–156.

Wang CX, Olschowka JA, Wrathall JR. 1997. Increase of interleukin-1β mRNA and protein in the spinal cord following experimental traumatic injury in the rat. Brain Res 759:190–196.

Witsell AL, Schook LB. 1993. Tumor necrosis factor alpha is an autocrine growth regulator during macrophage differentiation. Proc Natl Acad Sci USA 90:4763.

Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22:130–138.

Zhang YH, Lin JX, Yip YK, Vilcek J. 1988. Enhancement of cAMP levels and of protein kinase activity by tumor necrosis factor and interleukin 1 in human fibroblasts: role in the induction of interleukin 6. Proc Natl Acad Sci USA 85:6802–6805.