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Effect of anti–rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5′-triphosphatase and superoxide dismutase activation, and ultrastructural changes

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Object. The inflammatory cells that accumulate at the damaged site after spinal cord injury (SCI) may secrete interleukin-6 (IL-6), a mediator known to induce the expression of inducible nitric oxide synthase (iNOS). Any increased production of NO by iNOS activity would aggravate the primary neurological damage in SCI. If this mechanism does occur, the direct or indirect effects of IL-6 antagonists on iNOS activity should modulate this secondary injury. In this study, the authors produced spinal cord damage in rats and applied anti–rat IL-6 antibody to neutralize IL-6 bioactivity and to reduce iNOS. They determined the spinal cord tissue activities of Na^+-K^+/Mg^{++} adenosine-5′-triphosphatase (ATPase) and superoxide dismutase, evaluated iNOS immunoreactivity, and examined ultrastructural findings to assess the results of this treatment.

Methods. Seventy rats were randomly allocated to four groups. Group I (10 rats) were killed to provide normal spinal cord tissue for testing. In Group II 20 rats underwent six-level laminectomy for the effects of total laminectomy alone to be determined. In Group III 20 rats underwent six-level T2–7 laminectomy and SCI was produced by extradural compression of the exposed cord. The same procedures were performed in the 20 Group IV rats, but these rats also received one (2 μg) intraperitoneal injection of anti–rat IL-6 antibody immediately after the injury and a second dose 24 hours posttrauma. Half of the rats from each of Groups II through IV were killed at 2 hours and the other half at 48 hours posttrauma. The exposed cord segments were immediately removed and processed for analysis.

Conclusions. The results showed that neutralizing IL-6 bioactivity with anti–rat IL-6 antibody significantly attenuates iNOS activity and reduces secondary structural changes in damaged rat spinal cord tissue.

KEY WORDS • inducible nitric oxide synthase • adenosine triphosphatase • spinal cord injury • superoxide dismutase • rat

TRAUMA to the spinal cord produces tissue damage through direct and indirect mechanisms.[1,19,22] The ultimate results of this damage are influenced by the severity of the initial physical impact and by various secondary factors that occur near the site of primary injury.[3,44] The extent and severity of the secondary injuries depend on the magnitude of the initial insult, as well as on contributing factors that include substances released into the microenvironment, cell reactions in the spinal cord tissue, reduction in blood flow to the site, changes in the use of O_2 and glucose by the damaged tissue, reduction of extracellular CA^{++}, release of free radicals, lipid peroxidation, changes in levels of neuropeptides and monoamines, and the production of arachidonic acid metabolites.[17,23,24,33,52–55,58,64,69–76,78,79,89]

Nitric oxide is a tiny molecule that plays a pivotal role in maintaining cellular homeostasis.[46,48] It is synthesized from L-arginine and O_2 by the enzyme NOS,[42] which exists in three isoforms. Neuronal NOS mediates synaptic plasticity, neuronal signaling and, after ischemic damage, neurotoxi-

Abbreviations used in this paper: ATPase = adenosine-5′-triphosphatase; CNS = central nervous system; CSF = cerebrospinal fluid; IL = interleukin; iNOS = inducible nitric oxide synthase; ND_{50} = neutralization dose$_{50}$; SCI = spinal cord injury; SOD = superoxide dismutase.

city. Nitric oxide production via endothelial NOS protects brain tissue by maintaining regional cerebral blood flow.[63] The third isoform has been labeled iNOS because it is only expressed under pathological conditions, such as inflammation.[82,83] Once expressed, iNOS catalyzes the production of an excessive amount of NO (nanomolar quantities) for several hours, and this disrupts cellular homeostasis and leads to neurotoxicity.[6,20,32,51,63,66] In vitro induction of iNOS results in delayed neuronal cell death.[11,15]

Cytokines are known mediators of immune and inflammatory responses.[27] Inflammatory cells secrete these substances, some of which induce iNOS expression.[48] A significant increase in the level of IL-6 in CSF reflects severe inflammation of the CNS.[40,43] In experimental studies investigators have documented elevated levels of IL-6 in the CNS after traumatic brain injury in the rat.[80]

Based on current knowledge, it is conceivable that inflammatory cells may secrete IL-6 after SCI and, thus, induce iNOS expression. Excessive production of NO through iNOS induction would aggravate the primary injury, causing more serious damage to the spinal cord tissue. If this mechanism holds true, it follows that direct or indirect effects of IL-6 antagonists on iNOS activity would modulate the amount of secondary damage that occurs after SCI. We sought to assess the effects of neutralizing rat IL-6 bioactivity and reducing iNOS induction by administering anti–rat IL-6 antibody after experimental SCI in the rat. We evaluated the results of this treatment by determining the activity of Na^+-K^+/Mg^{++} ATPase and SOD, examining iNOS immunoreactivity, and evaluating ultrastructural findings.

Materials and Methods

Seventy adult male Wistar rats, each weighing between 280 and 310 g, were used in the study. All animals received an anesthetic of ketamine (50 mg/kg) and xylazine (10 mg/kg). We used the clip compression model described by Rivlin and Tator[61] and produced SCI by extradural compression of a section of cord exposed via a six-level T2–7 laminectomy, in which the prominent spinous process of T-2 was used as a surgical guide. A six-level laminectomy was chosen to expedite timely harvest and to obtain enough spinal cord tissue for biochemical examination. With the aneurysm clip applicator oriented in the bilateral direction, an aneurysm clip with a closing force of 50 g was abruptly applied externally to the dura. The clip was then rapidly released from the clip applicator, which caused spinal cord compression. In the injured groups, the cord was compressed for 1 minute. The same clip was used in all the animals in which SCI was induced.

To assess for possible variation in lesion severity, we conducted a preliminary study and applied this method in a separate group of 20 rats. Ten weeks after clip compression injury, the rats' spinal cords were removed, fixed in buffered neutral formalin, embedded in paraffin, cut into 8-μm-thick serial sections, and stained by the Klüver–Barrera Luxol fast blue method. Five rats died during the 10-week postinjury period, and these animals were excluded. All specimens were assessed under light microscopy, according to the degree of cavitation over the entire surface of the cord and in the corticospinal tract. The results were all in the range of 75 to 85%, which indicated to us that there was no significant animal-to-animal difference in lesion severity when this method was used.

The 70 rats in the experiment were randomly allocated to a group of 10 uninjured controls (Group I), a group of 20 rats that underwent six-level laminotomy only (Group II, sham operated), and a set of 40 in which SCI was induced. Immediately after SCI was produced, each of these 40 rats was randomly assigned to either the trauma (Group III, 20 rats) or anti–rat IL-6 antibody–treated group (Group IV, 20 rats). All evaluations were performed in blinded fashion, and all surgical procedures were conducted with the aid of an operating microscope.

Group I control rats were used to determine normal Na^+-K^+/Mg^{++} ATPase and SOD activities, iNOS immunoreactivity, and spinal cord ultrastructure in the absence of surgery or medication. Group II (sham-operated) rats underwent six-level laminectomy and received an intraperitoneal injection of saline equal in volume to that received by anti–rat IL-6–treated rats in Group IV. The aim was to determine the impact of laminectomy alone on the biochemical factors measured, iNOS immunoreactivity, and cord ultrastructural findings. Group III (trauma-only) rats received an intraperitoneal saline injection—identical to that used in Group II rats—immediately after SCI was produced. Group IV (anti–rat IL-6 antibody–treated) rats received one intraperitoneal injection of anti–rat IL-6 antibody (2 μg) immediately post-SCI and received a second dose 24 hours later. The exact concentration of antibody required to neutralize rat IL-6 activity is dependent on the IL-6 concentration. The exact IL-6 concentration in rat spinal cord after clip-induced injury, however, is not known. To provide a guideline, the manufacturer of the antibody has determined the neutralization dose for this antibody under a specific set of conditions. The ND_{50} for this antibody is defined as the concentration of antibody required to yield one-half maximal inhibition of IL-6 activity on a responsive cell line. The ND_{50} of the anti–rat IL-6 antibody (immunoglobulin class: recombinant rat IL-6–specific goat immunoglobulin G) was determined to be 0.03 to 0.09 μg/ml in the presence of 0.6 ng/ml of recombinant rat IL-6. Accordingly, the ND_{50} of anti–rat IL-6 antibody in this study was determined to be approximately 2 μg based on the previous studies,[40,43,80] as well as the rat's body weight and blood and CSF volume.

Sample Collection

Immediately after Group I animals were killed, their spinal cord tissue was prepared for assays of Na^+-K^+/Mg^{++} ATPase and SOD, and iNOS immunohistochemical and ultrastructural analysis. In Groups II through IV, 10 animals from each group were killed 2 hours and 48 hours after surgery alone (Group II) or trauma. This created two subgroups per group, with Groups II-A, III-A, and IV-A killed at 2 hours and Groups II-B, III-B, and IV-B at 48 hours. Each rat was anesthetized and underwent transcardial perfusion, first with approximately 100 ml 0.1 M phosphate-buffered saline (pH 7.3) and then with 150 ml of 4% paraformaldehyde under constant pressure. Each animal's exposed spinal cord segment was removed immediately after perfusion. The sample obtained from the injury site was divided transversely into four blocks from cranial to caudal. The first and fourth blocks were stored in a –80˚C freezer for assays of Na^+-K^+/Mg^{++} ATPase and SOD, respectively. The second and third blocks were used for iNOS immunohistochemical and ultrastructural analysis, respectively.

Determination of Na^+-K^+/Mg^{++} ATPase Activity

Homogenates (10%) of the tissue were prepared in 0.3 M sucrose containing 1 mM Mg^{++} by homogenizing for 90 seconds with a Teflon pestle clearance of 0.25 to 0.38 mm at 1000 rpm. Adenosine triphosphatase activity was determined in the resulting supernatants by measuring the rate of liberation of inorganic phosphate from disodium ATP.[59] Incubation media were prepared as described previously.[60]

Adenosine-5′-triphosphates were as follows: Na^+-K^+ ATPase- 6 mM $MgCl_2$, 5 mM KCl, 100 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid, and 135 mM Tris-HCl buffer pH 7.4. After preincubation for 5 minutes at 37˚C, Na_2 ATP was added to each tube to reach a final concentration of 3 mM. The blank sample that contained no enzyme, the standard, and unknowns were incubated at 37˚C for 30 minutes. The reaction was stopped by placing the samples on ice. Inorganic phosphate was determined in 1-ml aliquots of the incubated mixtures by adding Lubrol–molybadate solution followed by centrifugation; the mixtures were then allowed to stand at ambient temperature for 10 minutes. Extinction at 240 nm was measured. All assays were conducted in triplicate and were run with enzyme and reaction blanks. Samples were compared for phosphate content with standards of KH_2PO_4. Specific activities were calculated as nanomoles of inorganic phosphate/hour/milligram of protein.

All reagents were of Analar grade unless otherwise stated. The Lubrol–molybadate solution was prepared according to the method described by Reading and Isbir.[59,60] The protein content was determined according to the method reported by Lowry, et al.,[41] and bovine serum albumin was used as a standard.

Determination of SOD Activity

We isolated SOD from the cord tissue in a preparation in the manner described by Yarbrough.[85] The pyrogallol method was used to measure SOD activity, with slight modification, as described by Roth and Gilbert.[62] Specific activities were calculated as units/gram protein.

Electron Microscopy

Each tissue block was immediately placed in 5% glutaraldehyde buffered at pH 7.4 with Milloning phosphate buffer[45] for 3 hours. The samples were then fixed in 1% osmic acid for 2 hours, after which they were dehydrated in graded ethanol baths, embedded in Araldite, and processed for transmission electron microscopy in which conventional methods were used.

Immunohistochemical Analysis of iNOS

On removal, the spinal cord blocks designated for immunohistochemical analysis were placed in the buffered neutral formalin and refrigerated overnight at 4°C. The next day, the tissue samples were dehydrated in graded ethanol baths, embedded in paraffin, and sliced in 4- to 6-μm-thick sections. The sections were then deparaffinized with 100% xylene and rehydrated in graded ethanol baths. Next, the sections were incubated in 3% H_2O_2 to block endogenous peroxidase activity and washed in phosphate-buffered saline. To block nonspecific binding, the sections were then incubated for 10 minutes in serum-blocking solution. Thereafter, they were incubated overnight at 4°C with anti-iNOS antibody. Finally, we used the streptavidin–biotin technique for visualization. For negative controls, some of the spinal cord sections were treated with a nonimmune serum instead of the same concentration of primer antibody. Aminoethylcarbazole was used as the chromogenic substrate.

The Mann–Whitney U-test was used for all statistical analyses.

Sources of Supplies and Equipment

The aneurysm clip (FE 691K) was purchased from Aesculap AG (Tuttlingen, Germany). The anti–rat IL-6 antibody was obtained from R & D Systems (Minneapolis, MN). Boehringer-Mannheim (Mannheim, Germany) produced the Na^{++} ATPase, and Sigma Chemical Co. (St. Louis, MO) manufactured the Lubrol-type Px reagent. We acquired the anti-iNOS antibody (SA-200) from Biomol (Hamburg, Germany). For processing the immunohistochemical sections, we used the Zymed kit obtained from Zymed Lab (San Francisco, CA).

Results

Activity of Na^+-K^+/Mg^{++} ATPase

Figure 1 shows the effect of injury with and without the intraperitoneal administration of anti–rat IL-6 antibody on Na^+-K^+/Mg^{++} ATPase activity in spinal cord homogenate measured 2 hours and 48 hours after trauma. Injury alone resulted in significantly decreased Na^+-K^+/Mg^{++} ATPase activity in the damaged segments compared with both control and sham-operated rats at both time points posttrauma ($p < 0.05$). The decreased activity noted in the sham-operated subgroups did not differ statistically from the values determined in the control group ($p > 0.05$). Intraperitoneal administration of anti–rat IL-6 antibody (Groups IV-A and IV-B) attenuated the decrease in Na^+-K^+/Mg^{++} ATPase activity noted in the trauma-only group, and this was observed both 2 hours and 48 hours after treatment ($p < 0.05$).

TABLE 1

*Inducible NOS immunoreactivity in spinal cord tissue**

Group	Staining Score
I	+
II-A	+
III-A	+++
IV-A	+++
II-B	+
III-B	+++
IV-B	++

* + = mononuclear and microglial cells; ++ = mononuclear cells, microglial cells, and some capillary walls; +++ = mononuclear cells, microglial cells, capillary walls, axons, and dendrites.

Activity of SOD

Figure 2 shows the effect of injury with and without anti–rat IL-6 antibody treatment on SOD activity in spinal cord homogenates 2 hours and 48 hours posttrauma. Superoxide dismutase activity was higher in the sham-operated rats than in control rats, but the difference was statistically insignificant ($p > 0.05$). The activity was significantly higher in the traumatized rats than in those in the sham-operated and control groups ($p < 0.05$), and anti–rat IL-6 antibody treatment significantly decreased SOD activity at both time points posttrauma compared with the values observed in the trauma-only groups ($p < 0.05$).

Immunoreactivity of iNOS

The spinal cord tissue obtained in rats from Groups I, II-A, and II-B showed weak immunoreactivity with anti-iNOS antibody. The mononuclear cells of the pia-arachnoid membranes and microglial cells in the white matter of the cords stained positive. In sections obtained in the trauma-only rats (Groups III-A and III-B), in addition to staining in the mononuclear cells and microglial cells, we noted a positive reaction in the axons, dendrites, and capillary vessel walls (Fig. 3 *upper*). Inducible NOS immunoreactivity in the cord tissue obtained in Group IV-A rats was similar to that observed in Group III-A rats (Fig. 3 *center*). Group IV-B showed weak immunoreactivity to anti-iNOS antibody (Fig. 3 *lower*). Table 1 summarizes the qualitative scoring conducted according to the level of iNOS-positive staining observed in the control, sham-operated, trauma-only, and anti–rat IL-6 antibody–treated groups.

Ultrastructural Findings

Group I. Spinal cord tissue samples obtained in control rats exhibited gray and white matter, with intact nerve cells, glial cells, and nerve fibers. The nerve cells contained a vesicular nucleus, and the cytoplasm of many of the cytoplasmic organelles was mildly stained. The healthy nerve fibers were composed of myelinated and unmyelinated axons. The capillary walls were also normal, as were the pericapillary glial cell extensions.

Group II-A. In the sham-operated group, the spinal cord ultrastructure was similar to that demonstrated in the normal control rats; however, some of the myelinated nerve fibers showed focal degeneration of the myelin sheath.

Group III-A. In this trauma-only group, in addition to

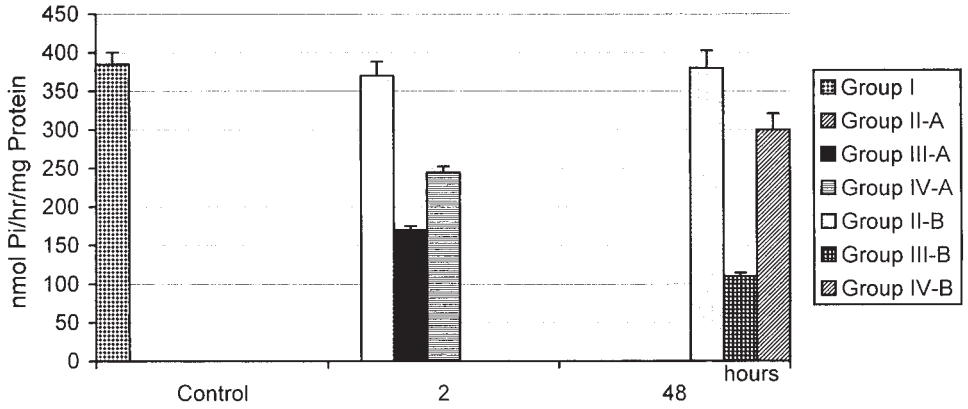

FIG. 1. Graph depicting the effect of injury with and without anti–rat IL-6 antibody treatment on Na^+K^+/Mg^{++} ATPase activity of in the spinal cord. Values are expressed as the means; vertical bars indicate standard errors of the means. Pi = inorganic phosphate.

extensive hemorrhagic areas, structural degeneration was more prominent in both the gray and white matter. The gray matter nerve cells showed changes in the nuclear chromatin, vacuolation of cytoplasmic organelles, and disintegration of mitochondrial membranes. We also observed vacuolation of the perineuronal cell feet and mild-to-moderate ultrastructural changes in the glial cells as well. In the white matter, the oligodendrocytes showed cytoplasmic vacuolation. The myelinated nerve fibers exhibited degeneration of the axons and myelin sheaths. Separation and disruption of the myelin lamellae, as well as invaginations of the myelin sheath to the level of the axon, were also common findings. Some of the myelinated nerve fibers showed axonal changes (Fig. 4 *upper left*). The capillary endothelial cells exhibited abnormal nuclear chromatin and degeneration of cytoplasmic organelles, as well as irregular thickening of the basal lamina. Additionally, the pericapillary astrocytic processes were enlarged.

Group IV-A. In this treated group, we noted intercellular and pericapillary edema. There were structural changes in the neurons and glial cells in the gray matter. The appearance of the myelinated nerve fibers was similar to that observed in Group III-A (Fig. 4 *upper right*). The capillary wall showed changes in the endothelium and the basal lamina.

Group II-B. The ultrastructural organization in this sham-operated group was similar to that in the control group.

Group III-B. The edematous areas seen in the cord tissue obtained in rats in this trauma-only group were similar to those seen in Group III-A, but the hemorrhage was less extensive. The nerve cells in the gray matter exhibited changes in the nucleus and cytoplasmic organelles. The ultrastructural irregularities were even more prominent in the white matter, where the myelinated nerve fibers showed myelin sheath disruptions and axonal organelle changes (Fig. 4 *lower left*). The capillary endothelial cells exhibited mild-to-moderate organelle changes as well. Pericapillary edema was also noted.

Group IV-B. The spinal cord tissue obtained in the anti–rat IL-6–treated rats exhibited interstitial edema in some regions, but this was less extensive than that seen in the corresponding trauma-only rats. The nerve cells in the gray matter showed mild-to-moderate organelle changes, including vacuolation of membranous organelles and higher numbers of lipofuscin granules. Glial cell structure was generally normal. In the white matter, the myelinated

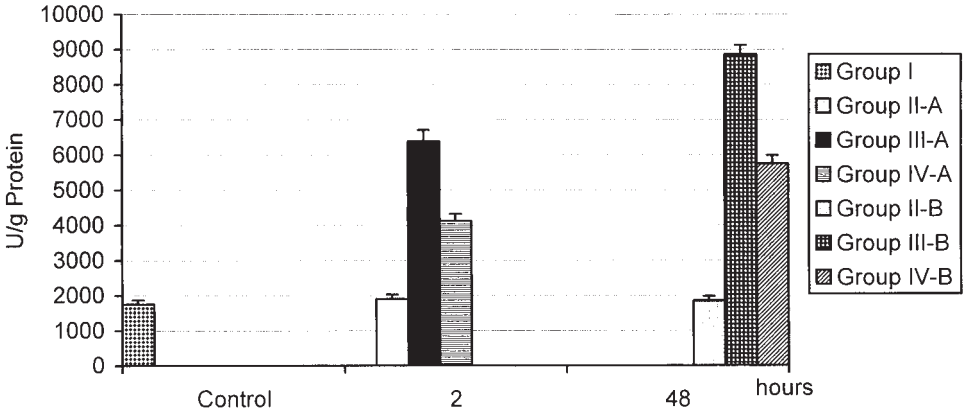

FIG. 2. Graph showing the effect of injury with and without anti–rat IL-6 antibody treatment on the SOD activity in the spinal cord. Values are expressed as means; vertical bars indicate standard errors of the means.

nerve fibers showed mild abnormalities of the myelin sheaths, such as separation and partial disruption of the lamellae. The ultrastructure of the axolemma and axonal organelles was normal (Fig. 4 *lower right*), and the capillary endothelial cells and basal lamina were also intact.

Discussion

The involvement of iNOS expression has been demonstrated in such pathological CNS conditions as cerebral ischemia,[35] experimental allergic encephalitis,[88] and multiple sclerosis.[2] The cytotoxic action of NO, as driven by iNOS induction, appears to play a critical role in each of these conditions. Initiation of iNOS is most striking in immunocompetent cells that have been exposed to cytokines. Microglia are the immunocompetent cells in the CNS, and they are known to express iNOS when subjected to adequate stimulation.[11] In addition to microglial cells, iNOS expression increases under pathological conditions when cells such as macrophages, neurons, astroglia, vascular smooth muscle cells, and endothelial cells are exposed to cytokines.[47,49]

The mechanism responsible for iNOS upregulation after focal SCI is not clear. Alterations in the microenvironment at the damaged site and the release of several neurochemicals after injury appear to be important elements.[44,53,75,76] Neurochemicals may trigger the opening of cation-permeable channels, resulting in greater accumulation of intracellular Ca^{++} than that which occurs under normal conditions.[3,25,79] Interleukin-6 is one of the major inflammation-associated cytokines. Researchers have demonstrated local production of IL-6 and elevated CSF levels of this mediator in noninfectious CNS inflammatory disease.[29] It is believed that applying antiserum raised against a particular hormone or chemical compound can neutralize the function of the compound itself in vivo,[75] and this was the premise for our use of the anti-IL-6 antibody. One group of authors reported that systemically administered antibodies to IL-6 penetrate the CSF in animals with noninfectious CNS inflammatory disease, hampering the development of the normally induced disease process.[28] The mechanism behind iNOS upregulation may be that release of IL-6 from inflammatory cells triggers increased production of one or several intracellular neurochemicals, which, in turn, initiate iNOS activity.

After trauma, eicosanoids, leukotrienes, and superoxide anions accumulate in the intra- and extracellular spaces. These substances exert a toxic effect on the cells that respire. It is well known that iNOS induction can lead to the production of free radicals and/or lipid peroxidation.[4,5] Lipid peroxidation activates the arachidonic acid cascade and the protein kinase C system.[56,68] When NO is overproduced, it contributes to cytotoxic effects through a number of mechanisms, including inhibition of mitochondrial respiration via inhibition of the citric acid cycle. Additionally, the cytotoxic action of NO can be augmented by the generation of the highly reactive free-radical species peroxynitrite, which can contributes to cellular dysfunction through lipid peroxidation.[4,7,12,37,38,50,57] These effects prompted us to evaluate Na^{+}-K^{+}/Mg^{++} ATPase activity, SOD level, iNOS immunoreactivity, and ultrastructural findings as indicators of the value of anti–rat IL-6 antibody in the treatment of SCI.

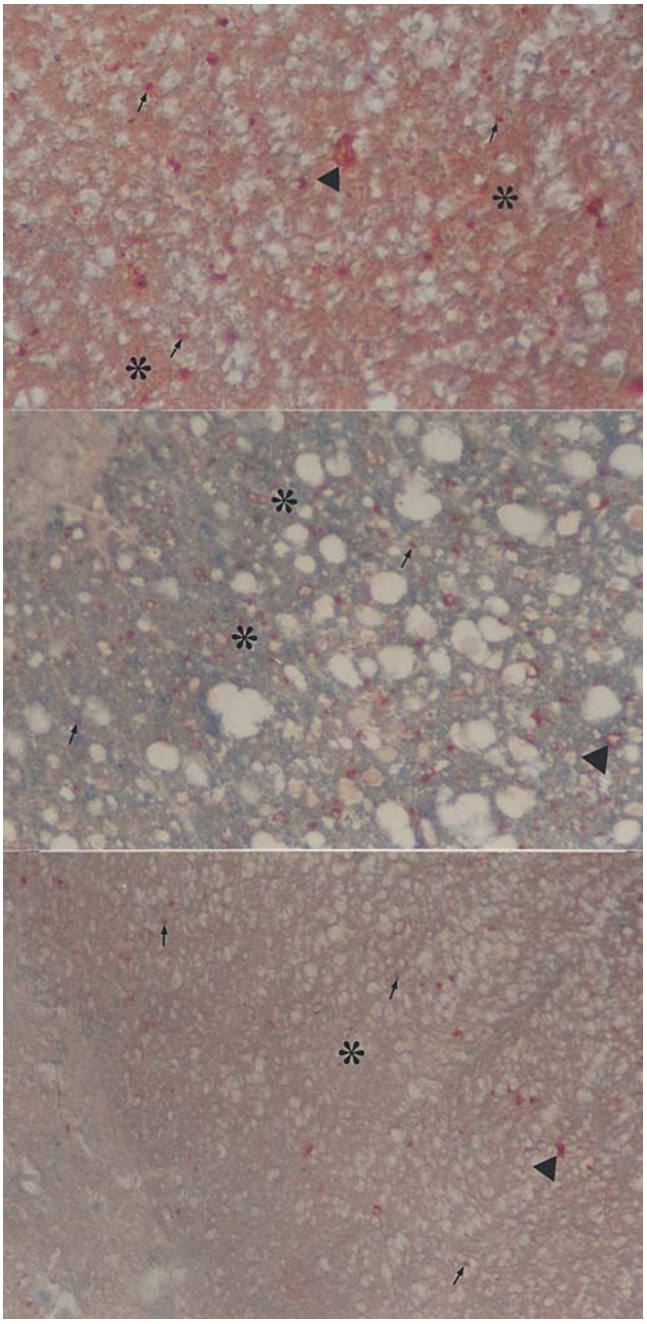

FIG. 3. Photomicrographs. *Upper:* Group IV-A. The white matter stained strongly positive for iNOS. *Center:* Group III-B. The white matter shows extensive iNOS immunoreactivity. *Lower:* Group IV-B. There was significantly less iNOS immunoreactivity in the spinal cord compared with that seen in Group III-B. Microglia *(arrows)*, axons and dendrites *(asterisk)*, and capillary *(arrowhead)* are shown. Aminoethylcarbazole and hematoxylin, original magnifications × 200 (*upper* and *lower*) and × 250 *(center)*.

Because SOD is a specific free-radical scavenger,[14] its activity in the rats' spinal cords reflected the impact that neutralizing rat IL-6 bioactivity had on free-radical production. The SOD activity in the anti–rat IL-6–treated rats was significantly lower than that in the trauma-only group. This is also good evidence that anti–rat IL-6 an-

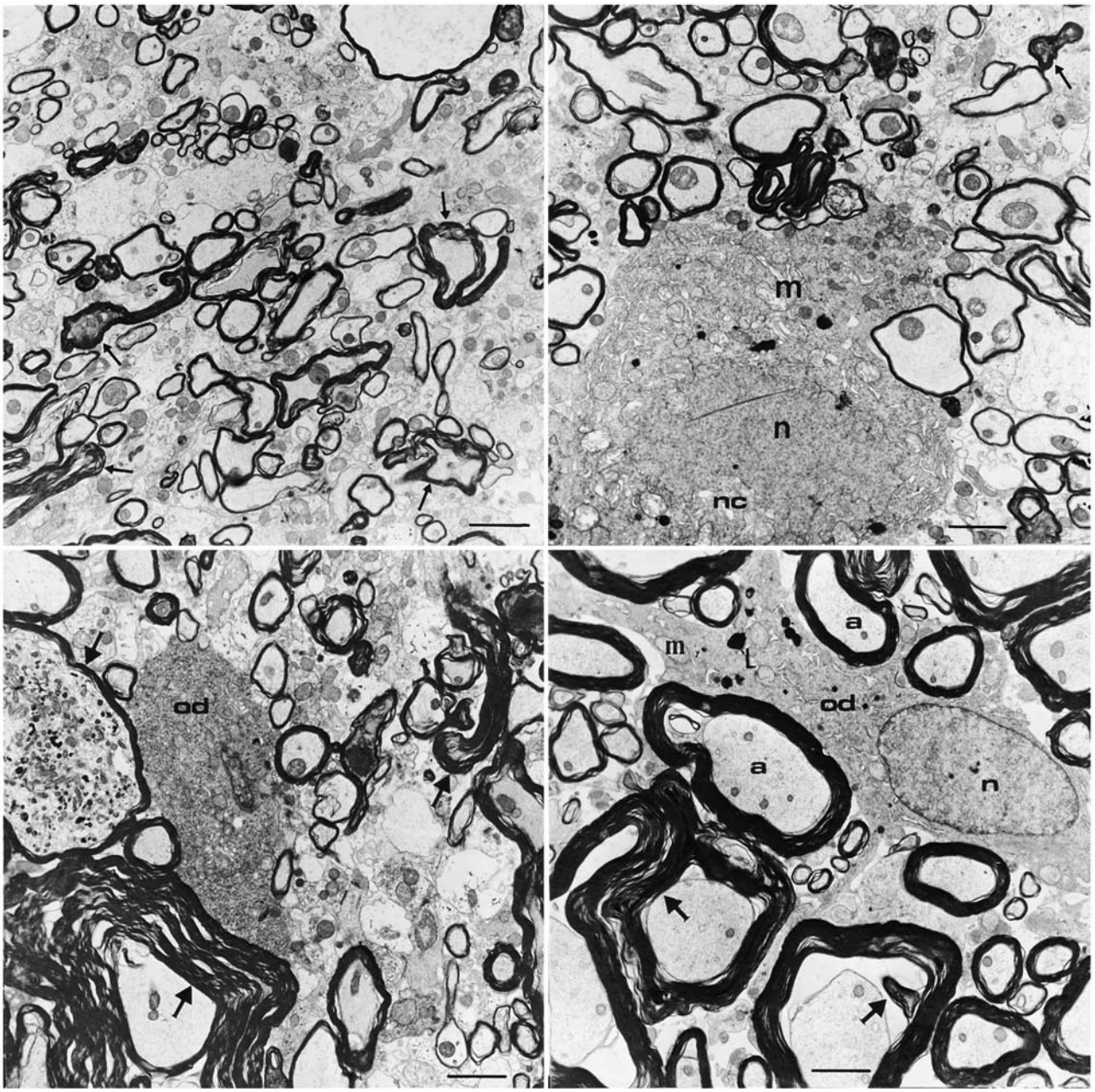

FIG. 4. Electron microscopy studies. *Upper Left:* Group III-A. Most myelinated nerve fibers in the white matter show axon and myelin sheath abnormalities *(arrows)*. Bar = 2.5 μm. *Upper Right:* Group IV-A. The nerve cell (nc) exhibits enlarged mitochondria (m) and disintegration of mitochondrial cristae. The myelinated nerve fibers show changes in the axons and myelin sheaths *(arrows)*. Nucleus (n). Bar = 1.7 μm. *Lower Left:* Group III-B. The myelinated nerve fibers show axon and myelin sheath degeneration *(arrows)*. Oligodendrocyte (od). Bar = 1.7 μm. *Lower Right:* Group IV-B. The oligodendrocyte exhibits increased numbers of lysosomes (L) and mild mitochondrial abnormalities. The myelinated nerve fibers show focal myelin sheath degeneration *(arrows)*. Axons (a). Bar = 1.7 μm.

tibody neutralizes IL-6 and attenuates iNOS induction, which, in turn, decreases the level of free-radical production in the spinal cord tissue. Another possible mechanism for our findings might be that the inflammatory response is attenuated via neutralizing rat IL-6 bioactivity, which decreases the entry of inflammatory cells, such as polymorphs, to the CNS because IL-6 is one of the mediators of inflammatory response.[27] Thus, it might also decrease free-radical generation. The end result is reduced secondary neurological damage.

Schettini, et al.,[67] studied the fluctuations in SOD that occur over time after ischemia–reperfusion injury in the dog. They reported that SOD activity increases fivefold 60 minutes postinjury, drops to twice normal at 150 minutes, and rises again to reach three times the normal rate at 24 hours. When we determined the SOD levels in all groups,

the mean values in Groups III-B and IV-B (rats killed 48 hours posttrauma) were higher than those in Groups III-A and IV-A (those killed 2 hours posttrauma). It is logical that the iNOS induced by cytokines during secondary injury causes free-radical production and that, over time, SOD would rise in conjunction with the increasing levels of free radicals.

Adenosine-5′-triphosphatases are enzymes that execute important cellular functions in ionic and osmotic balance and in active transport. Because ATPases are very susceptible to free-radical exposure and lipid peroxidation, their activity is reduced in damaged tissue.[10,39] In several recent reports the authors have focused on the process of free radical–induced lipid peroxidation and its negative impact on lipid-dependent enzymes such as Na^+-K^+–activated ATPase and Na^+-K^+/Mg^{++} ATPase, after SCI.[8,13,18,23,31,65] Analysis of the results indicates that the activity of lipid-dependent enzymes is a useful parameter for evaluating cellular disturbance caused by SCI. We used ATPase activity levels to gauge the therapeutic action of anti–rat IL-6 antibody as effected through indirect inhibition of iNOS induction during secondary SCI. The levels of the Na^+-K^+/Mg^{++} ATPase activity in the trauma-only rats were markedly reduced at 2 hours and 48 hours after injury compared with that in the sham-operated rats at both time points. This lower activity was also associated with increased SOD levels, and this finding fits with the aforementioned interpretation for SOD. In contrast, the Na^+-K^+/Mg^{++} ATPase activity in the antibody-treated rats was significantly higher than that in the trauma-only group 2 hours and 48 hours postinjury. These biochemical findings also correlated well with milder ultrastructural changes observed in the spinal cords removed from the antibody-treated group. Our findings show that anti–rat IL-6 antibody significantly inhibits the decrease in Na^+-K^+/Mg^{++} ATPase activity that typically occurs after SCI. This effect reflects attenuation of iNOS induction and in vivo lipid peroxidation during the process of secondary injury.

Electron microscopy showed marked ultrastructural changes in the gray and white matter of the spinal cords obtained in the trauma-only rats. In contrast, the spinal cords obtained in the anti–rat IL-6–treated group exhibited milder subcellular damage and less edema. These findings demonstrate that the application of anti–rat IL-6 antibody partially blocks the progression of edematous and structural changes in the rat spinal cord after clip compression injury. On this basis, it appears that there may be therapeutic value in administering anti–rat IL-6 antibody to reduce secondary spinal cord damage.

A single 2-μg dose of anti–rat IL-6 antibody had no effect on clip injury–induced ultrastructural changes or iNOS immunoreactivity in the spinal cord when testing was administered 2 hours after injury. Administering two doses of anti–rat IL-6 antibody resulted in milder ultrastructural changes and decreased iNOS immunoreactivity compared with that demonstrated in the trauma-only group. It seems that the single dose of antibody was either ineffective or required more time to neutralize IL-6 and attenuate the initiation of iNOS activity in the spinal cord. This observation is in agreement with previous findings: various NOS inhibitors act in a dose-dependent manner to reduce or aggravate neurotoxicity in both in vitro and in vivo settings.[16,84] To our knowledge, our immunohistochemical findings are the first to show that the application of anti–rat IL-6 antibody neutralizes IL-6 and thus attenuates iNOS induction in vivo.

We found that iNOS activity is upregulated in conjunction with prominent subcellular damage after SCI, and this finding supports the theory that abnormal production of NO via iNOS is harmful.[16,36,81] Specifically, we observed that the gray and white matter in nontreated animals showed marked ultrastructural changes and extensive iNOS immunoreactivity, and application of anti–rat IL-6 antibody reduced iNOS immunoreactivity and curbed some of the typical structural changes seen in the spinal cord after injury. Other investigators have noted similar observations in rats. It has been documented that intrathecal or topical application of antibodies to dynorphin A or 5-HT reduces the severity of neurological damage and diminishes gross expansion of the spinal cord and cell changes following focal trauma to the spinal cord.[21,71,75]

Normal spinal cord neurons contain NOS,[9,26] but iNOS is not detected in healthy tissue.[49] Research has demonstrated iNOS expression in cells that are positive for the monocyte/macrophage lineage marker in multiple sclerosis,[2] as well as in blood vessel walls and anterior horn cells in experimental allergic encephalitis.[88] In our study, control rats exhibited weak iNOS immunoreactivity in the mononuclear cells of the pia-arachnoid membranes and in microglial cells in the white matter of the spinal cord. These tissues and cells have not been shown to stain positive in previous immunohistochemical investigations,[2,35,49,88] and there is no definitive explanation for this discrepancy. The higher sensitivity of our testing method may be one reason for the difference. In addition, our results indicated there was marked upregulation of iNOS in the mononuclear cells, microglial cells, capillary wall, axons, and dendrites after clip compression SCI. This indicates that our antibody and immunohistochemistry protocol was sensitive enough to detect very small changes in iNOS immunoreactivity.

In some reports the authors have stated that iNOS is not involved in the early cell changes that follow SCI because upregulation requires relatively long survival (2–3 days) after ischemic injury.[36,87] In contrast to this contention in experiments on rat brain Iadecola, et al.,[35] found that iNOS protein and catalytic activity were detectable 12 hours after cerebral ischemia, that they peaked at 48 hours, and returned to baseline after 7 days. Hu, et al.,[34] reported that iNOS immunoreactivity and enzymatic activity increased at 4 hours and persisted for 24 to 48 hours after intrathecal injection of dynorphin A. Increased NOS immunoreactivity in neurons in which this does not normally occur has been described in cell bodies of brainstem nuclei several weeks after axotomy.[30,86] Other authors have noted several NOS-positive neurons in the spinal cord gray matter 5 hours postinjury.[77] We found iNOS expression in mononuclear cells of the pia-arachnoid membranes, white matter microglia, capillary wall, and axons and dendrites of the spinal cord at only 2 hours postinjury.

The most significant finding in our study is that clip compression SCI induces rapid and widespread expression of iNOS immunoreactivity in axons and dendrites that normally do not express this enzyme activity. Also interesting is the fact that we observed strong correlations among the levels of iNOS immunoreactivity in mononuclear cells,

microglia, capillary endothelium, axons, and dendrites. These levels of iNOS expression correlated well with the degree of edematous involvement and ultrastructural damage observed in the spinal cord tissue. Together, these findings indicate that iNOS plays a key role in secondary injury after spinal cord trauma.

Conclusions

To the best of our knowledge, this is the first study to demonstrate that anti–rat IL-6 antibody treatment for SCI simultaneously attenuates iNOS upregulation, reduces ultrastructural damage, decreases SOD level, and increases Na^+-K^+/Mg^{++} ATPase activity. These findings prove that anti–rat IL-6 antibody effectively neutralizes IL-6 bioactivity and that the resultant decrease in iNOS activity is key to inhibiting secondary spinal cord damage.

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