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Immunization With A91 Peptide or Copolymer-1 Reduces the Production of Nitric Oxide and Inducible Nitric Oxide Synthase Gene Expression After Spinal Cord Injury

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Immunization with neurally derived peptides (INDP) boosts the action of an autoreactive immune response that has been shown to induce neuroprotection in several neurodegenerative diseases, especially after spinal cord (SC) injury. This strategy provides an environment that promotes neuronal survival and tissue preservation. The mechanisms by which this autoreactive response exerts its protective effects is not totally understood at the moment. A recent study showed that INDP reduces lipid peroxidation. Lipid peroxidation is a neurodegenerative phenomenon caused by the increased production of reactive nitrogen species such as nitric oxide (NO). It is possible that INDP could be interfering with NO production. To test this hypothesis, we examined the effect of INDP on the amount of NO produced by glial cells when cocultured with autoreactive T cells. We also evaluated the amount of NO and the expression of the inducible form of nitric oxide synthase (iNOS) at the injury site of SC-injured animals. The neural-derived peptides A91 and Cop-1 were used to immunize mice and rats with SC injury. In vitro studies showed that INDP significantly reduces the production of NO by glial cells. This observation was substantiated by in vivo experiments demonstrating that INDP decreases the amount of NO and iNOS gene expression at the site of injury. The present study provides substantial evidence on the inhibitory effect of INDP on NO production, helping our understanding of the mechanisms through which protective autoimmunity promotes neuroprotection.

Key words: A91; Cop-1; neural antigens; protective autoimmunity; paraplegia

Immunization with neurally derived peptides (INDP) has become a promising strategy in promoting neuroprotection and neurorestoration after spinal cord (SC) injury (Ziv et al., 2006; Martiñon et al., 2007; Ibarra et al., 2010). It is essential to elucidate the possible mechanisms by which INDP is exerting its beneficial effects. Previous studies have demonstrated that INDP boosts the beneficial action of protective autoimmunity, which is an autoreactive physiological response to CNS trauma (Yoles et al., 2001). Protective autoimmunity is spontaneously evoked in strains that are resistant to the CNS autoimmune disease experimental autoimmune encephalomyelitis (EAE) than in susceptible strains; this suggests that a genetic background determining susceptibility to autoimmune CNS diseases resolves the final response of protective autoimmunity (Kipnis et al., 2001).

INDP has been shown to reduce the lipid peroxidative process observed after SC injury. This effect is dependent on protective autoimmunity, because the functional elimination of CNS-specific T cells avoids the

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beneficial effect on lipid peroxidation (Ibarra et al., 2010). Lipid peroxidation is caused mainly by the action of reactive oxygen species (ROS), which are abundant at the site of injury. Inflammatory cells are one of the main sources of ROS, and, because protective autoimmunity modulates the action of these cells (Shaked et al., 2005), it could also be neutralizing the release of ROS. Nitric oxide (NO) is one of the main ROS, contributing to lipid peroxidation. NO is a free radical that is produced at high concentrations after SC injury (Nakahara et al., 2002) and has been strongly associated with significant tissue damage after injury (Bao and Liu, 2003). NO combines with superoxide anion (another free radical released after SC injury) and gives rise to peroxynitrite ($ONOO^-$), a very well-known neurotoxic agent (Xiong and Hall, 2009). The formation of peroxynitrite is the most harmful mechanism by which NO causes tissue damage. Previous studies have suggested that some derivatives from the immune response (i.e., interferon-γ [IFNγ]) could reduce the amount of NO produced by glial cells (Shaked et al., 2005). The microenvironment induced by immunizing with neural-derived peptides such as A91 and Cop-1, which produce an antiinflammatory Th2-type response (Gaur et al., 1997; Arnon and Sela, 2003), could theoretically diminish the expression and activity of inducible NO synthase (iNOS; Bhat et al., 1998; Gordon, 2003). Previous work has shown that iNOS is the most important enzyme in NO synthesis after SC injury (Kwak et al., 2005; Pannu and Singh, 2006). To understand this phenomenon better, we performed both in vitro and in vivo studies to evaluate the effect of INDP on the production of NO.

MATERIALS AND METHODS

Experimental Design

Four experiments were performed as follows.

First experiment. The efficacy of immunization with A91 or Cop-1 for inducing a specific immune response was evaluated. For this purpose, 12 days after immunization, a group of SC-injured mice or rats (n = 4, selected at random) was studied to corroborate the response against the peptides. Statistical analysis was by Student's *t*-test.

Second experiment. The effect of anti-A91- or anti-Cop-1-specific T cells on the production of NO by glial cells was assessed. For this purpose, lymphocytes obtained from Balb/c mice, immunized with either A91 or Cop-1, were cocultured with lipopolysaccharide (LPS)-activated glial cells (LPS stimulates the production of NO by glial cells) in the presence of the corresponding antigen. Statistical analysis was by one-way ANOVA followed by Tukey's test.

Third experiment. The amount of NO at the site of injury was explored. For this purpose, Balb/c mice (n = 5 per group) or F344 rats (n = 6 per group) were immunized, within a 60-min time frame after injury, with A91, Cop-1, or phosphate-buffered saline (PBS). NO concentrations were determined 7 days later. Statistical analysis was by one-way ANOVA followed by Tukey's test.

Fourth experiment. This experiment explored the expression of the iNOS gene after immunization with A91, Cop-1, or PBS in rats (n = 4 per group) or mice (n = 4 per group) with SC injury. Statistical analysis was by Kruskal-Wallis followed by Mann-Whitney U test.

Animals

Adult Fischer 344 (F344; 13–14 weeks old, 200–220 g) rats and adult or newborn BALB/c mice were used for the experiments. All animals were supplied by the Animal Breeding Center of the Camina Research Center and were handled according to NIH guidelines for the management of laboratory animals. Animals were age-matched and housed in a light- and temperature-controlled room. Efforts were made to minimize the number of animals used and their suffering. All procedures were in accordance with the National Institutes of Health *Guide for the care and use of laboratory animals* and the Mexican Official Norm on Principles of Laboratory Animal Care.

Antigens

A nonencephalitogenic myelin basic protein (MBP) peptide, A91 (Gaur et al., 1997; Martiñón et al., 2007), was derived from an encephalitogenic peptide, amino acids 87–99 of MBP, by replacing the lysine residue 91 with alanine. The modified peptide was purchased from Invitrogen Life Technologies (San Diego, CA). Reverse-phase HPLC confirmed that the purity of the A91 peptide was higher than 95%. Cop-1 (Sigma St. Louis MO) is a synthetic random copolymer prepared by polymerization of the *N*-carboxyanhydrides of L-alanine, L-lysine, L-glutamic acid, and L-tyrosine followed by the removal of the protecting groups (Teitelbaum et al., 1974). The end product is a mixture of random polypeptides with amino acid composition of Ala (6.0 residues), Glu (1.9 residues), Lys (4.7 residues), and Tyr (1 residue). A91 and Cop-1 peptides were selected for this study because both have been demonstrated to induce neuroprotection in neurodegenerative diseases (Martiñón et al., 2007; Ibarra et al., 2007).

SC Injury

Animals were anesthetized by intramuscular injection of ketamine (77.5 mg/kg; Probiomed, Mexico City, Mexico) and xylazine (12.5 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA). The spinal cord was exposed by laminectomy of the T9 vertebrae. One hour after induction of anesthesia, the rats were subjected to a moderate contusive injury by dropping a 10-g rod onto the laminectomized cord from a height of 25 mm. A well-calibrated contusive injury was achieved using the NYU impactor (Basso et al., 1996). Mice were subjected to a compressive SC injury. Compressive injury was obtained using a calibrated aneurysm clip that delivered a closing force of 50 g during a 30-sec period. After injury, the aponeurotic plane and the skin were separately sutured with nylon thread.

Postoperative Care

Sterile beds and filtered water were replaced daily. Bladder expression was assisted by massage at least twice per day

TABLE I. PCR Primers Sequences*

Gene	Primer sequence	Product length (bp)	GenBank
iNOS mouse			
Forward	CAGCACAGGAAATGTTTCAGC	154	NM_010927.3
Reward	TAGCCAGCGTACCGGATGA		
iNOS rat			
Forward	AAGCTGGTGGCCGCCAAGCT	258	AY211532.1
Reward	ATGTGAGGGGTTTGGGGGGA		
HPRT mouse			
Forward	AAGCTTGCTGGTGAAAAGGA	184	NM_013556.2
Reward	GCGCTCATCTTAGGCTTTGT		
HPRT rat			
Forward	AA GCTTGCTGGT GAAAAGGA	192	NM_012583.2
Reward	CAAAGCCTAAAAGACAGCGG		

*iNOS, inducible nitric oxide synthase; HPRT, hypoxanthine guanine phosphoribosyl transferase

until normal function was regained. All animals were carefully monitored for evidence of urinary tract infection or any other signs of systemic disease. Animals received a 10-day postoperative course of enrofloxacine (Marvel, Mexico City, Mexico) in their drinking water (64 mg/kg/day) and an additional course if they developed hematuria after this period.

Active Immunization

Rats were injected subcutaneously at the base of the tail with 200 μg of A91, Cop-1, or 0.15 M PBS, pH 7.4. Mice were immunized subcutaneously in the back with 150 μg A91, Cop-1 or PBS. A subcutaneous injection was used because it has been demonstrated to boost protective autoimmunity without the risk of developing EAE (Hauben et al., 2001; Martiñón et al., 2007). All immunizations were emulsified in an equal volume of complete Freund's adjuvant (CFA) containing 0.5 mg/ml *Mycobacterium tuberculosis* and were administered within 60 min after injury.

Proliferation Assay

Lymph node cells, excised and pooled 12 days after SCI ($n = 4$), were cultured in quintuplicate in flat-bottomed microtiter wells in 0.2 ml RPMI-1640 medium (Gibco, Grand Island NY). The cells (3.0×10^5 cells per well) were cultured in antigen-free medium or together with A91 (10 μg/ml), Cop-1 (20 μg/ml), concanavalin A (Con A; 5 μg/ml; Sigma), or an irrelevant peptide (10 or 20 μg/ml SP10-27) corresponding to an 18-amino-acid sequence of a scorpion toxin. The proliferative response was determined by measuring the incorporation of [^{3}H]thymidine (1 μCi per well), which was added for the last 16 hr of a 96-hr culture. The stimulation index was calculated by dividing the mean value (in cpm) of experimental wells by the mean value (in cpm) of negative control wells (cells cultured without the antigen). The rank of proliferative response in negative controls was of 61–92 cpm.

Quantitative Polymerase Chain Reaction

iNOS gene expression was determined via Q-PCR 7 days after injury. The mRNA transcripts were isolated from 2.5 cm frozen injured spinal cord using the phenol-chloroform extraction method with Trizol (Life Technologies). cDNA synthesis was achieved using reverse transcription with oligo(dT) primers and Superscript II Reverse Transcriptase (Life Technologies) from 2 μg of total RNA. We analyzed cDNA levels using the iNOS and hypoxanthine guanine phosphoribosyl transferase (HPRT; as a control gene) gene-specific primer pairs listed in Table I. PCRs were carried out using 10 ng cDNA, 50 nM of each primer, 2 μl template DNA and LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche) in 10-μl reactions. Amplification was assessed by measuring SYBR green fluorescence collected during real-time PCR on a LightCycler 2.0 Instrument (Roche). For the initial denaturation step, both mice and rat samples were heated to 95°C for 10 min, followed by the first cycle consisting of a denaturation step (95°C, 10 sec), a primer annealing step (60 °C, 10 sec for mouse and 63°C, 8 sec for rat), an extension step (72°C, 4 sec), a melting curve (65°C, 1 min), and a cooling step (40°C, 30 sec). The reaction was carried out through 40 cycles for mouse and 37 cycles for rat. Each reaction was subjected to cross-point or cycle threshold analysis to confirm single amplified products. Aside from this, agarose gels were also used to confirm the correct size of the amplified product (see Supp Info. Fig.1).To analyze the cycles at take-off and the melting temperatures, we used the LightCycler software (build 4.1.1.21). Melting temperatures of genes were mouse HPRT, 82°C; mouse iNOS, 86°C; rat HPRT, 83°C; rat iNOS, 86°C. All experiments were performed in duplicate, and expression levels of individual genes were represented as arbitrary units after normalization with HPRT.

Lymphocytes

A pure lymphocyte suspension was obtained from the cervical lymph nodes and the spleen of immunocompetent adult female BALB/c mice. Twelve days after immunization with 150 μg A91 or Cop-1, cervical lymph nodes and spleen were removed aseptically and immersed in cold RPMI culture medium. Tissues were gently homogenized in a dissociation cup. All suspensions were prepared in RPMI supplemented with L-glutamine (1 mM) and penicillin/streptomycin (100 IU/ml) and allowed to stand for 5 min at room temperature to allow detritus to sediment. The cellular suspension was then mixed with 3 ml Lymphoprep solution (Axis-Shield PoC

AS, Oslo, Norway), and the lymphocyte fraction was obtained according to the instructions provided by the manufacturer. Lymphocytes were then washed three times in DMEM and counted. Only lots with >93% viable cells were used.

Glial Cell Culture

Cells were prepared from 3-day-old BALB/c mice born to timed pregnant females, which were housed under pathogen-free conditions with 12-hr light-dark cycles and provided with food and drinking water ad libitum. The pups were anesthetized with CO_2 before decapitation. The cerebellum was quickly dissected under sterile conditions according to previously reported methods (Sass et al., 2001). The pooled cells were suspended at a density of 7.5×10^5 cells/ml in complete medium (MEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, and 10% fetal bovine serum), and 2 ml was plated on poly-D-lysine-coated six-well plates (2 ml/well) and incubated at 37°C, 5% CO_2. Glial cells were obtained at day 13 as described by (Gilad et al., 1990). This method yields a glial cell culture of astrocytes (40.19%), microglia (31.86%), and oligodendrocytes (28.38%). These ratios were consistent among wells.

Coculturing of Glia and Lymphocytes

After 13 days of culture, glial cells were harvested and seeded on 24-well plates and cocultured with lymphocytes from mice immunized with either A91 or Cop-1 (1:100; 5×10^3 lymphocytes and 4.9×10^5 glial cells). Cocultured cells were then incubated at 37°C, 5% CO_2 for 48 hr in the presence of A91 (20 μg/ml), Cop-1 (20 μg/ml), or an irrelevant synthetic peptide (20 μg/ml SP10-27) corresponding to an 18-amino-acid sequence of a scorpion toxin (Calderon-Aranda et al., 1999). To evaluate the effect of autoreactive lymphocytes on the production of NO^-, glial cells were activated with LPS (100 ng/ml, *E. coli*; Sigma-Aldrich).

Analysis of Nitric Oxide (NO^-) Concentrations

In vitro studies. NO concentrations were determined as nitrites, a stable oxidative product of NO, according to the Griess method (Green et al., 1982) after 48 hr of coculture. Briefly, equal volumes of Griess' reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) were incubated with supernatant samples (100 μl of medium in which glia had been cultured) for 10–20 min at room temperature. Absorbance was measured at 550 nm in an ELISA reader with a reference filter of 620 nm. The NO concentration was determined using KNO_2 as a standard; results were expressed as concentration of nitrites (nanomolar).

In vivo studies. Seven days after SC injury, NO concentrations were determined at the site of injury. After an HCl extraction of SC tissue, equal volumes of Griess' reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) were incubated with the soluble fraction of extract samples for 10–20 min at room temperature. The samples' absorbance was measured at a 550-nm wavelength; results were expressed in concentrations of nitrites per milligram tissue.

TABLE II. Lymph Node Cell Proliferative Response After Peptide Immunization

	Stimulation index after immunization with	
Animals	A91	Cop-1
Mice	2.8 ± 0.39[a],⋆	2.70 ± 0.40[a],⋆
	Con A control: 15.19 ± 2.90[b]; IP: 0.61 ± 0.10[c]	
Rats	3.01 ± 0.18[a],⋆	2.93 ± 0.24[a],⋆
	Con A control: 52.69 ± 8.92[b]; IP: 0.73 ± 0.21[c]	

[a]Mean ± SD of four mice or rats Con A, concanavalin A; IP, irrelevant peptide.
[b]Con A proliferation as positive control values.
[c]Proliferative response to an irrelevant peptide.
⋆Statistically different from IP ($P < 0.05$, Student's *t*-test).

RESULTS

A91 or Cop-1 Immunizations Induced an Immune Response

After immunization, peptides were capable of inducing an immune response in both mice and rats (Table II).

T Cells Activated Against Neurally Derived Peptides Are Capable of Counteracting the Production of NO by Glial Cells

A91- and Cop-1-specific T lymphocytes were capable of counteracting NO production (Fig. 1A and B, respectively). The amount of nitrites, as an indirect determination of NO concentration, was significantly diminished when glial cells were cocultured with specific T cells activated in vitro with neurally derived peptides: A91 = 5.4 ± 0.9 and Cop-1 = 2.6 ± 0.3 (mean ± SD; $P < 0.001$ vs. glia + LPS = 9.8 ± 1.9 and 7.4 ± 0.3). In contrast, when lymphocytes from either A91- or Cop-1-immunized mice were cocultured with LPS-activated glial cells in the presence of an irrelevant peptide, there was no effect on the production of NO: A91 = 10.24 ± 1.5 and Cop-1 = 7.8 ± 0.1.

INDP Reduces the Amount of NO at the Injury Site

Immunization with A91 or Cop-1 significantly reduced the amount of NO in both mice and rats. Figure 2A shows that PBS-immunized mice had 523.7 ± 20 nM/g tissue (mean ± SD), whereas in animals immunized with A91 and Cop-1 the concentrations were 374.3 ± 6 and 319.2 ± 80.1, respectively ($P = 0.05$). A similar effect was observed in SC-injured rats; A91 or Cop-1 immunizations induced a significant reduction in the amount of NO: PBS = 1,437.5 ± 239 nM/g tissue, A91 = 372.8 ± 80 and Cop-1 = 304.3 ± 121 ($P < 0.05$; Fig. 2B).

Expression of the Gene Encoding for iNOS Is Decreased in Animals Treated With INDP

Immunization with either A91 or Cop-1 peptides reduced mRNA iNOS expression in both mice and rats.

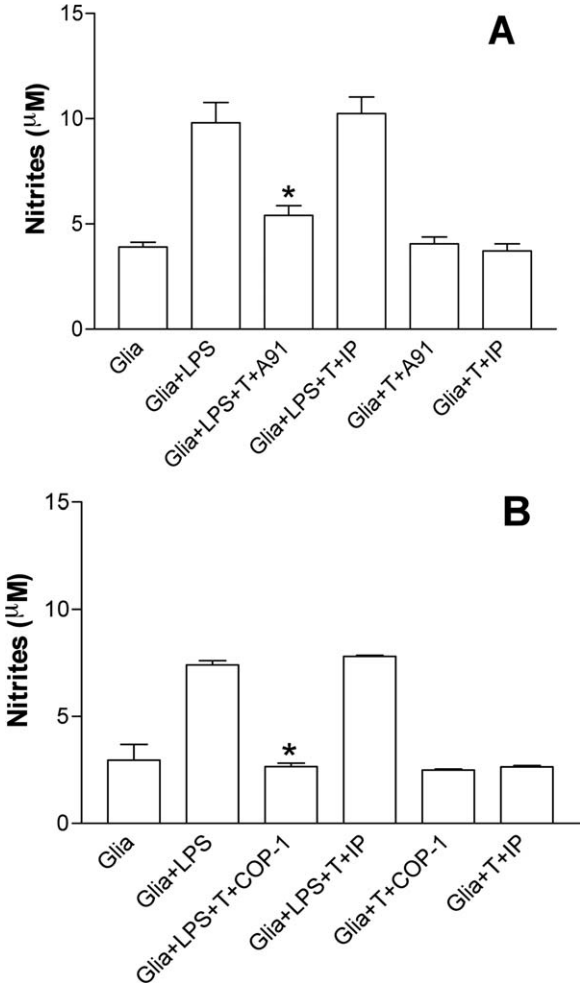

Fig. 1. Production of nitric oxide by glial cells cocultured with lymphocytes obtained from mice immunized with A91 (**A**) or Cop-1 (**B**) *Different from glia + LPS and glia + LPS + T + IP groups ($P < 0.001$; one-way ANOVA followed by Tukey's test). Bars represent mean ± SD of four observations. Each experiment is one of three in which we observed the same effect. LPS, lipopolysaccharide; T, lymphocytes; IP, irrelevant peptide.

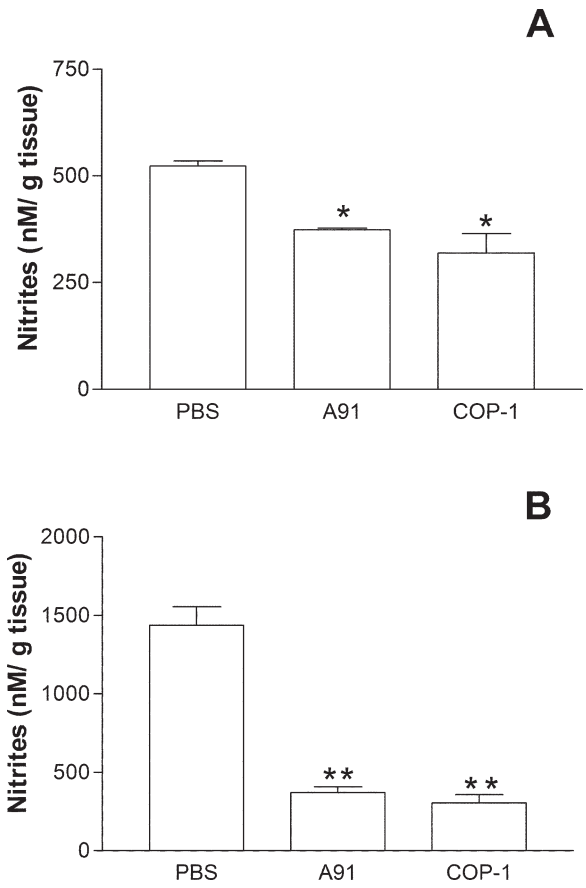

Fig. 2. Effect of immunization with neurally derived peptides (A91 or Cop-1) on the amount of nitric oxide 7 days after SC injury. The production of nitric oxide was analyzed in mice (**A**) and rats (**B**) that had been immunized immediately after injury. Bars represent the mean ± SD of three different experiments (four animals per group for each experiment). *Different from PBS-immunized mice ($P = 0.05$, Kruskal Wallis followed by Mann Whitney U test). **Different from PBS-immunized rats ($P = 0.004$, Kruskal Wallis followed by Mann Whitney U test).

Figure 3A shows that PBS-treated mice presented a significantly higher mRNA iNOS expression compared with A91- or Cop-1-immunized mice: PBS = 9.1 ± 1.2, mean ± SD; A91 = 2.9 ± 1.7; Cop-1 = 1.19 ± 0.7 ($P = 0.01$ and 0.001, A91 and Cop-1 vs. PBS, respectively). Similarly, when rats were immunized with A91 or Cop-1, there was a significant reduction in mRNA iNOS expression compared with PBS-immunized rats: A91 = 2.7 ± 0.2, Cop-1 = 0.5 ± 0.4, PBS = 5.4 ± 1.4 ($P = 0.05$ and 0.01, A91 and Cop-1 vs. PBS, respectively; Fig. 3B).

DISCUSSION

Earlier studies demonstrated that INDP significantly reduces the lipid peroxidative process after SC injury (Ibarra et al., 2010). The present work attempted to elucidate one of the possible mechanisms by which INDP is contributing to this phenomenon. In line with this, we found that lipid peroxidation could be reduced, at least in part, as a consequence of a decreased production of NO. NO is a well-known free radical that promotes the formation of peroxynitrite and that has been involved in lipid peroxidation and neural tissue damage after injury (Liu et al., 2000; Kwak et al., 2005).

INDP could be reducing NO production through the effect of protective autoimmunity on the expression of iNOS. This study showed that INDP is capable of reducing the expression of the gene encoding for iNOS. Previous work has determined that iNOS is the main source of NO after SC injury, so a decrease in its expression could result in less synthesis of NO.

The exact mechanism by which INDP diminishes the expression of iNOS gene should be explored further; however, we expect that such an effect is caused by the

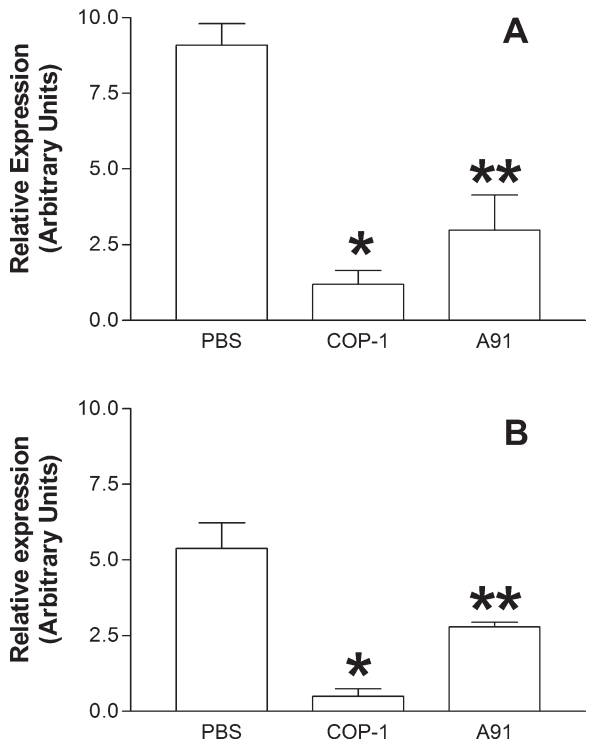

Fig. 3. Relative expression of iNOS enzyme gene at the site of SC injury. Mice (**A**) and rats (**B**) were immunized with A91 or Cop-1 immediately after spinal cord injury. Expression of the gene encoding for iNOS enzyme was significantly decreased in mice (different from PBS-treated animals $\star P = 0.001$, $\star\star P = 0.01$, Kruskal Wallis followed by Mann Whitney U test) and rats (different from PBS-treated animals, $\star P = 0.01$, $\star\star P = 0.05$), that had been immunized with A91 or Cop-1. Bars represent mean ± SD of four or five animals.

action of some molecules, such as interleukin-10 (IL-10), that are being produced at the site of injury (Shechter et al., 2009). A91- and Cop-1-specific T cells belong to the Th2 phenotype and predominantly release IL-10 (Gaur et al., 1997; Arnon and Sela, 2003). Another important source of this cytokine could be the $CD4^+$ $CD25^+$ regulatory T cells (T reg cells), which after CNS injury play an active role in repairing mechanisms by controlling the tolerance to self. T-reg cells maintain the balance between the need for autoimmunity and its attendant risks (Kipnis and Schwartz, 2005).

IL-10 could be playing a pivotal role in the down-regulated expression of the iNOS gene. When IL-10 interacts with its receptor, the signaling cascade activates the suppressor of cytokine signaling 3 (SOCS-3; McNamee et al., 2010). SOCS-3 interferes with the activity of the nuclear factor-κ light-chain-enhancer of activated B cells (NF-κB; Bruun et al., 2009), the main transcription factor responsible for the expression of the iNOS gene (Kakita et al., 2009). Therefore, it is plausible that an IL-10-predominant microenvironment will result in decreased iNOS gene expression. Accordingly, some studies have shown that the overproduction of IL-10 (Meng et al., 2009; van Strien et al., 2010) or SOCS-3 (Karlsen et al., 2001; Crespo et al., 2002) suppresses expression of the iNOS gene.

IL-4 is another Th2-derived cytokine that could also contribute to the down-regulation of iNOS gene expression. This cytokine has been reported to inhibit iNOS gene expression at a transcriptional level. This inhibition is mediated by IL-4′s ability to block the production of interferon-γ response factor-1 (IRF-1) protein (Morris et al., 2009), a critically important transcriptional element that enhances expression of IFNγ-inducible genes such as iNOS.

Future studies should be designed with the aim of investigating the exact mechanisms by which INDP is inhibiting the expression of the gene encoding for iNOS. NO production could also be decreased as a result of the competition for L-arginine between iNOS and arginase. The aforementioned Th2-prevalent phenotype could be enriching the microenvironment with IL-4, a cytokine that, in addition to inhibiting iNOS gene expression, could also diminish the production of NO by another pathway. IL-4 increases arginase activity by inducing its production (Louis et al., 1999). Because L-arginine is the substrate for both iNOS and arginase, a plausible mechanism of IL-4-mediated inhibition of NO synthesis could also be via depletion of L-arginine through increased arginase activity.

On the other hand, a previous study showed that IFNγ, a relevant cytokine released by Th1 lymphocytes, could reduce the amount of NO produced by glial cells (Shaked et al., 2005). This finding could seem contradictory to our previous Th2 phenotype hypothesis; however, it is relevant to mention that both Th1 and Th2 phenotypes (although the latter predominates) could be participating in promoting neuroprotection (Butovsky et al., 2005). With regard to the role of Th1 lymphocytes, it has been shown that these cells and their main cytokine (IFNγ), are fundamental players in the beneficial effect provided by protective autoimmunity (Kipnis et al., 2002). IFNγ increases the expression of MHCII molecules in microglial cells (Butovsky et al., 2005). This effect facilitates the communication of these cells with T lymphocytes, allowing the activation of microglia under a neuroprotective phenotype, which is characterized by the release of a scarce amount of NO (Shaked et al., 2005). IFNγ could also be capable of inducing iNOS gene expression (Chesrown et al., 1994); however, the present work found a significant reduction in the expression of this gene. This observation suggests that, in some way, the microenvironment induced by protective autoimmunity and the limited concentrations of IFNγ are sufficient to induce the protective phenotype in microglia and not cause an increase in iNOS gene expression. This notion should be studied further.

The present study also showed that the decline in levels of NO is an effect induced only by A91- or Cop-1-specific T lymphocytes. This observation is supported by the results obtained in our studies performed in vitro. We observed that lower NO synthesis was a consequence of a peptide-specific response, i.e., in the pres-

ence of a non-CNS peptide (IP), T cells (nonactivated cells) were not capable of inhibiting NO production. This finding confirms that INDP and not an unrelated antigen induces a specific response (protective autoimmunity), which is capable of promoting protective effects after SC injury.

The exact role that NO plays after neural injury is a topic that remains under investigation. Some studies have shown that NO could be protective and even necessary to ameliorate tissue damage after injury (Zhuang et al., 2009); however, others have provided evidence on the pathogenic effects of this compound (Dawson and Dawson, 1998; Liu et al., 2000). It seems that the dual role of NO depends on its concentration at the site of injury. The excessive production of NO, specifically via iNOS, can lead to neurotoxicity. In fact, the enhanced formation of NO has been implicated in the pathogenesis of SC injury (Kwak et al., 2005). A reduction in the amount of NO could contribute to lessening tissue damage by decreasing lipid peroxidation and other detrimental processes such as apoptosis.

It is of relevance to mention that the results observed in the present study were obtained from animals resistant to EAE. In these animals, the beneficial action of protective autoimmunity is better than in EAE-susceptible ones (Kipnis et al., 2001). Previous reports have shown that INDP promotes beneficial effects even in EAE-susceptible animals (Hauben et al., 2001), but it is feasible to expect that our therapy could also be reducing the production of NO and iNOS gene expression in these animals. In fact, a recent finding in our laboratory demonstrated that INDP reduces lipid peroxidation in Lewis rats (unpublished data).

Further studies are needed to understand better the mechanisms by which protective autoimmunity promotes neuroprotection. The present work provides some elements for this understanding, at least the inhibitory effect of protective autoimmunity on lipid peroxidation. INDP induces a significant reduction of iNOS gene expression and, as a consequence, diminishes NO production.

Because NO is only one of the reactive species participating in lipid peroxidation, future studies will be directed toward investigating the effect of protective autoimmunity on the release of other free radicals (i.e., superoxide anion). The results of the current study encourage the design of future investigations directed toward understand the mechanism of action of INDP. In the same way, we wish to establish the best conditions for the use of INDP as a therapeutic approach in SC injury and other neurodegenerative disorders (i.e., cerebral ischemia, traumatic brain injury, Alzheimer's disease, Parkinson's disease, etc.). This work also has the goal of sparking the search for new and more effective peptides that can be used with no risk of an autoimmune disease.

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