Therapeutic Vaccination for Closed Head Injury

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ABSTRACT

Closed head injury often has a devastating outcome, partly because the insult, like other injuries to the central nervous system (CNS), triggers self-destructive processes. During studies of the response to other CNS insults, it was unexpectedly discovered that the immune system, if well controlled, provides protection against self-destructive activities. Here we show that in mice with closed head injury, the immune system plays a key role in the spontaneous recovery. Strain-related differences were observed in the ability to harness a T cell-dependent protective mechanism against the effects of the injury. We further show that the trauma-induced deficit could be reduced, both functionally and anatomically, by post-traumatic vaccination with Cop-1, a synthetic copolymer used to treat patients with multiple sclerosis and found (using a different treatment protocol) to effectively counteract the loss of neurons caused by axonal injury or glutamate-induced toxicity. We suggest that a compound such as Cop-1 can be safely developed as a therapeutic vaccine to boost the body’s immune repair mechanisms, thereby providing multifactorial protection against the consequences of brain trauma.

Key words: brain injury; autoimmune neuroprotection; strain differences; CNS inflammation; EAE-susceptibility; Cop-1 (Glatiramer acetate)

INTRODUCTION

INJURIES TO THE CENTRAL NERVOUS SYSTEM (CNS) may be grouped according to whether the damage predominantly affects the white matter (axons) or the gray matter (cell bodies or soma) (Schwartz et al., 1999). The outcome of a CNS insult depends not only on the extent of the primary injury but also on the amount of secondary degeneration that subsequently develops (Harrop et al., 2001; Yoles and Schwartz, 1998). Neurons that are directly damaged will inevitably die, regardless of the location (white or gray matter) of the lesion. The relatively rapid process of primary death is followed by the secondary degeneration of adjacent neurons that escaped the initial insult. The primary death of neurons and of supportive cells causes an increase in the concentrations of potentially toxic physiological substances such as glutamate and reactive oxygen and nitrogen species, creating a hostile environment for the neighboring neurons (Beattie et al., 2000; Schwartz and Yoles, 2000; Shohami et al., 1997, 1999; Tymianski and Tator, 1996). In addition, at the site of injury alterations of blood flow and im-

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pairment of energy metabolism and ionic homeostasis occur (Assaf et al., 1999; Mautes et al., 2001; Preston et al., 2001). These factors, among others, mediate the spread of secondary neurodegeneration. Treatments that prevent this propagation of damage are described as neuroprotective.

The CNS of mammals is characterized by “immune privilege,” a unique evolutionary status that restricts immune cell invasion of the intact CNS (Neumann, 2000; Streilein, 1995; Streilein et al., 2000). The finding that memory T cells patrol the healthy CNS (though they do not normally accumulate there) indicates that the barrier to immune cells is not absolute (Neumann et al., 1998; Wekerle et al., 1996). After injury, however, the disrupted blood–brain barrier (BBB) (Chen et al., 1996) freely allows the entry of lymphocytes and other immune cells, which then accumulate in the damaged tissue (Moalem et al., 1999a; Stahel et al., 2000). Inflammation of uninjured CNS tissue is seen in patients with autoimmune diseases such as multiple sclerosis (MS) and in rodents with experimental autoimmune encephalomyelitis (EAE), an animal model for MS (Bar-Or et al., 1999; O’Connor et al., 2001). Until quite recently, it was generally believed that immune invasion of the damaged CNS adversely affects recovery by promoting secondary degeneration (Dal Canto et al., 2000; Onuki et al., 2001; Popovich et al., 1997, 1999). Immune suppression was accordingly thought to be neuroprotective. However, immunosuppressive treatment was found to be effective, if at all, only when applied immediately after the trauma, becoming progressively less effective with time (Benton et al., 2001; Oudega et al., 1999; Solberg et al., 1999; Yoon et al., 1999).

An increasing body of recent evidence suggests that under certain conditions, immune interaction with the injured CNS has a neuroprotective effect (Hammarberg et al., 2000; Schwartz and Moalem, 2001; Schwartz, 2001a,b). In rats subjected to partial crush of the optic nerve or contusive injury of the spinal cord, passive transfer of T cells specific to CNS myelin-associated self-antigens (“autoimmune” T cells) results in long-lasting protection from secondary degeneration (Butovsky et al., 2001; Hauben et al., 2000; Kipnis et al., 2000; Moalem et al., 1999b). This protection occurs even though transfer of these T cells may induce transient EAE (Antel and Owens, 1999; Ben-Nun et al., 1996). It was shown, however, that the autoimmune T cells inducing the neuroprotective response need not be pathogenic, as the response can be safely induced by T cells reactive to a harmless modified peptide of myelin proteins (Fisher et al., 2001; Hauben et al., 2001; Kipnis et al., 2000; Moalem et al., 1999b; Schori et al., 2001a). Further studies have shown that neuroprotection after optic nerve crush injury or after direct exposure of retinal ganglion cells (RGCs) to glutamate-mediated toxicity can also be conferred by vaccination with copolymer 1 (Cop-1), a compound that cross-reacts with myelin basic protein (MBP) (Kipnis et al., 2000; Schori et al., 2001a).

More recent studies have shown that immune neuroprotection is not merely the result of an experimental manipulation but is the body’s physiological response to trauma in experimental models of white or gray matter injuries (Schwartz and Kipnis, 2001; Schwartz and Moalem, 2001; Yoles et al., 2001). Autoimmune T cells are evoked spontaneously by CNS trauma and are controlled by naturally occurring regulatory CD4+CD25+T cells, which prevent the development of autoimmune diseases (Kipnis et al., 2002a; Schwartz and Kipnis, 2002). A possible explanation for the puzzling dichotomous effect of autoimmune T cells was provided by studies showing that the ability to manifest a spontaneous autoimmune response to CNS trauma is closely correlated with the individual’s ability to resist autoimmune disease development (Kipnis et al., 2001; Lundberg et al., 2001; Schwartz and Kipnis, 2001). Rats or mice that are susceptible to the development of EAE lack an efficient mechanism for controlling the spontaneous ability to evoke an injury-induced autoimmune response. Consequently, their T cell-mediated autoimmune response to injury is ineffective in terms of neuronal protection but is amenable to boosting by passive or active immunization.

Cop-1 is a synthetic amino acid polymer (4.7–11 kDa) composed of the amino acids L-alanine, L-lysine, L-glutamic acid, and L-tyrosine, in a molar ratio of 4.2:3.4:1.4:1.0 (Weiner, 1999). It was originally designed to induce EAE, thereby simulating MBP, but was found to be non-encephalitogenic and even to suppress MBP-induced EAE (Weiner, 1999). It also proved useful in the treatment of multiple sclerosis (Farina et al., 2001; Neuhaus et al., 2000). Cop-1 blocks chronic relapsing EAE induced in an (SJL/J × Balb/c/OLA) F1 mouse model by application of mouse spinal cord homogenate or encephalitogenic peptides of proteolipid protein (Aharoni et al., 1998). It binds with the relevant major histocompatibility complex proteins and leads to the activation of T suppressor cells, which are triggered by determinants common to Cop-1 and MBP (Aharoni et al., 1997, 1999; Sela, 1999; Teitelbaum et al., 1999).

In the present study, we examined the effect of immune system activation on the outcome of closed head injury (CHI). We found strain-related differences in the spontaneous ability of mice to fight off the consequences of CHI. We further found, by actively immunizing mice with Cop-1, that the immune response evoked by Cop-1 is neuroprotective, effectively reducing the spread of damage caused by the brain trauma.
MATERIALS AND METHODS

Animals

Inbred male adult C57Bl/6J and Balb/c/OLA mice (8–12 weeks old) were supplied by the Animal Breeding Center of the Weizmann Institute of Science. Mice were housed in a light- and temperature-controlled room and handled according to the regulations formulated by IACUC (Institutional Animal Care and Use Committee). Animals were matched for age in each experiment.

Antigens

Cop-1 (Copaxone®) was purchased from Teva Pharmaceuticals (Petah Tikva, Israel).

Immunization

Mice were injected with 100 μg of Cop-1 emulsified in an equal volume of complete Freund’s adjuvant (CFA) containing 5 mg/mL of Mycobacterium tuberculosis H37 RA (Difco). Control mice were injected with phosphate-buffered saline (PBS) emulsified in CFA. The emulsion (total volume 0.2 mL) was injected intramuscularly into the flank.

Brain Injury

Experimental CHI was inflicted using a weight-drop device (Chen et al., 1996). Mice were anesthetized with ether, and anesthesia was confirmed by loss of pupillary and corneal reflexes. Following a midline longitudinal incision, the skin was retracted and the skull exposed. The left anterior frontal area was identified and a tipped Teflon cone was placed approximately 1 mm lateral to the midline in the mid-cortical plane. The head was fixed, and a 75-g weight was dropped on the cone from a height of 18 cm, resulting in a relatively severe focal injury to the left hemisphere, corresponding to a value of 4–6 on the human scale (range, 3–15) of the Glasgow Coma Score (Beni-Adani et al., 2001). The mice received supportive oxygenation with 100% O₂ for no longer than 2 min and were then returned to their cages.

Neurological Severity Score

The clinical status of the injured mice was evaluated according to a set of criteria for testing reflexes and motor functions. According to this method, motor ability, balancing, and alertness are evaluated by the mouse’s performance of ten motor and behavioral tasks, yielding a neurological severity score (NSS). One point is awarded for failing to perform a particular task (Table 1). Sham-operated mice typically score zero, as they are able to perform all the tasks. The severity of the injury is reflected by the NSS at 1 h; values obtained thereafter are usually lower, as the clinical status spontaneously improves during the recovery period. We evaluated the NSS at 24 h, 48 h, and 7 days after the injury in one experiment, and at 14, 19, and 27 days in another. The difference (defined as ΔNSS) between the NSS values at 1 h and at any time point thereafter reflects the extent and speed of recovery of the tested mice. Thus, higher ΔNSS values denote better recovery. This is a useful tool for the evaluation of drug efficacy or genetic manipulation (Beni-Adani et al., 2000; Panikashvili et al., 2001; Tehranian et al., 2002).

Magnetic Resonance Imaging

The MRI procedure was based on a protocol used previously (Assaf et al., 1999) for the same model. Six weeks after the injury, mice (n = 5) were anesthetized and scanned in vivo in a Bruker 4.7T Biospec scanner. T2-weighted and diffusion-weighted images were obtained from nine axial slices, each 1 mm thick, covering the entire brain. The acquisition parameters are as recorded in Figure 1.

MRI Analysis

The MRI analysis was designed to quantify the volume of damage by automated recognition of affected areas (Fig. 1a). In defining a damaged area in an image, some technical problems are encountered, such as different amplifications for different slices, non-uniform intensities within a slice, and background noise. To overcome these problems we applied the following image normalization pre-processing procedure for the T2-

<table>
<thead>
<tr>
<th>Table 1. Neurological Severity Score (NSS) for Mice with Closed Head Injury</th>
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<tr>
<td><strong>Symptom</strong></td>
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<tr>
<td>Presence of mono- or hemiparesis</td>
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<tr>
<td>Inability to walk on a beam 3 cm wide</td>
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<tr>
<td>Inability to walk on a beam 2 cm wide</td>
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<tr>
<td>Inability to walk on a beam 1 cm wide</td>
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<tr>
<td>Inability to balance on a beam 0.5 cm wide</td>
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<tr>
<td>Inability to balance on a round stick 0.5 cm in diameter</td>
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<tr>
<td>Failure to exit a 50-cm-diameter circle within 2 min</td>
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<td>Inability to walk straight</td>
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<tr>
<td>Loss of startle reflex</td>
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<td>Loss of seeking behavior</td>
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<td>Maximum total</td>
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For each failed task, the mouse receives 1 point. A score of 10 reflects failure in all tasks, and 0 reflects success in all tasks.
weighted images (the entire process was performed without utilizing user-defined thresholds or expert-defined regions). Using the c-means algorithm we clustered the intensities within each slice, thereby defining a threshold for elimination of low-intensity (i.e., low SNR) pixels. By block-processing each slice, we obtained a two-dimensional surface describing the global non-uniformities in the intensities (Fig. 1b). This curved surface was bicubically interpolated and subtracted from the image, to reduce the non-uniform component of the image (Fig. 1c). To allow application of a common threshold at a later stage, the intensities within each slice were fitted to the normal distribution, and transformed to normalized probabilities (Fig. 1d). A morphological low-pass filter was used to smooth data with minimal smoothing of the edges. Following this pre-processing, the images were further processed to locate “patches” that correspond to hyperintense areas of damaged tissue. The boundaries of patches were defined by the use of a derivative operator (Fig. 1e). The resulting boundaries were segmented, using a c-means–defined threshold. Isolated points and free-end lines were removed and the resulting patches were labeled (Fig. 1f). Patch areas were calculated and summed to obtain an estimated volume. Inter-slice accumulation was calculated for interconnected patches only, starting from a single reference point manually defined in a damaged site. Areas of damage in the T2-weighted images were qualitatively compared to the areas in the diffusion-weighted images.

The analysis outlined above has a few drawbacks. It is insensitive to tissue elimination due to necrosis, and the calculated volume takes into account only those damaged areas that are in contact with the site of primary injury.

Statistical Analysis

ΔNSS values are expressed as means ± SEM and were analyzed using the non-parametric Mann-Whitney U test.

RESULTS

Spontaneous Neurological Recovery after Closed Head Injury Varies among Strains and Is Correlated with Resistance to Autoimmune Diseases in the CNS

Mice of two strains (n = 20 per group) differing in their ability to withstand the consequences of axonal injury or glutamate toxicity (Kipnis et al., 2001; Schori et al., 2001b) were subjected to a well-controlled head injury. Uniformity of the insult was verified by the similarity of NSS values obtained 1 h after the insult. Thereafter, follow-up of the spontaneous recovery, measured by the improvement in neurological status (expressed as ΔNSS), revealed that the Balb/c/OLA (resistant) mice recovered better than the C57Bl/6J (susceptible) mice (p < 0.05; Fig. 2). This finding is in line with the previously observed recovery of rodents from other CNS insults (Kipnis et al., 2001).
Vaccination with Cop-1 Improves the Functional Outcome of CHI

Our previous findings in models of white matter (axonal) insult and of toxic glutamate insult to gray matter (myelin-free neurons) demonstrated that an immune-mediated protective response can be safely boosted by the use of Cop-1. Cop-1 vaccination apparently circumvents the tissue-specificity barrier to therapeutic vaccines after a CNS insult (Schori et al., 2000; Schori et al., 2001a). Mice of each strain were divided into three groups (Fig. 3) and then subjected to CHI. In both strains, mice that were vaccinated, 7 days prior to injury, with Cop-1 emulsified in CFA showed better functional outcome than control mice that were untreated or were injected with PBS emulsified in CFA. The two strains differed significantly, however, in the rate of recovery. In Balb/c/OLA mice, ΔNSS was significantly greater in the Cop-1–treated than in PBS-treated mice at 2 and 7 days after injury (Fig. 3A; p < 0.05 and 0.01, respectively; Mann-Whitney U test). In contrast, the recovery of C57Bl/6J mice, whether vaccinated with Cop-1 or treated with PBS, was much slower (Fig. 3B). Immunization of naïve mice with Cop-1 emulsified in CFA did not affect their behavioral scores (data not shown).

Since pre-immunization is not a relevant therapeutic approach in cases of brain injury it was of interest to determine whether immunization performed after the insult was also effective. Immediately after CHI, Balb/c/OLA mice were immunized with Cop-1 in CFA, or injected with PBS in CFA, or not treated, and their clinical recovery was followed for 7 days (Fig. 4A). One week after injury and treatment, the difference in brain injury out-
come between the groups became significant (Fig. 4B; \( p < 0.01 \)). To verify the clinical findings, three mice chosen randomly from each group were evaluated, 6 weeks after CHI, by magnetic resonance imaging (MRI). The results showed that the damaged areas in the Cop-1–vaccinated mice were significantly smaller (2.1 ± 0.75 mm\(^3\); mean ± SD) than in the PBS-treated mice (5.1 ± 1.43 mm\(^3\); Fig. 4B). The areas of damage demonstrated in diffusion-weighted images overlapped those seen in T2-weighted images (Fig. 5). As expected, in the T2-weighted images the overall intensity of the injured left hemisphere was greater than that in the right hemisphere, despite manipulation of the images to minimize intensity non-uniformities. In agreement with other studies using the same CHI model (Beni-Adani et al., 2001), we obtained hyperintense damaged areas in the non-injured right hemisphere, possibly reflecting secondary degeneration.

Using MRI techniques and H&E staining in the same experimental model, we previously demonstrated that the best correlation with diffusion-weighted images at all time points was obtained with CHI-induced histological damage visualized 1 week after the injury. This was true for both rats (Assaf et al., 1999) and mice (Beni-Adani et al., 2001).

**DISCUSSION**

The results of this study showed differences between two mouse strains in their ability to withstand the consequences of closed head trauma. On the basis of our previous experience with optic nerve injury and glutamate toxicity, we attribute this difference to differences in the ability of the mouse T cells to mediate a protective immune response (Kipnis et al., 2001; Schori et al., 2001b). After CNS injury and challenge with any myelin antigens, a process of T cell–dependent immune neuroprotection appears to be spontaneously elicited in a strain that is genetically resistant to the development of CNS autoimmune diseases, but evoked only to a limited extent in inherently susceptible strains (Kipnis et al., 2001; Schwartz and Kipnis, 2002). The findings of this study thus suggest that the functional outcome of head trauma is similarly affected by genes that are associated with control of the immune response.

Recent findings suggest that the ability to manifest a protective autoimmune response after a CNS insult is controlled by naturally occurring regulatory CD4\(^+\)CD25\(^+\) T cells (Kipnis et al., 2002a; Schwartz and Kipnis, 2002). Inactivation of these regulatory T cells or vaccination with a suitable antigen can augment the spontaneous neuroprotective response, which—even in individuals capable of manifesting a beneficial autoimmunity—is relatively weak at best, and needs to be boosted. Immunization with Cop-1 in this study indeed resulted in better recovery from CHI in both strains.

Active immunization with the synthetic copolymer Cop-1 in this study resulted in immune neuroprotection in both of the tested mouse strains. Clinical recovery, as expressed by improvement in NSS, was significantly more rapid and more substantial in mice pre-immunized with Cop-1 than in PBS-treated mice. The treatment was ef-
effective even when applied after the injury, suggesting that there is a clinically relevant therapeutic window for immune-mediated neuroprotection after CHI.

Cop-1 vaccination was previously shown to confer neuroprotection in the rat or mouse model of optic nerve crush injury, in rat or mouse RGCs directly exposed (by intraocular injection) to glutamate-mediated toxicity, and after neuronal degeneration in a glaucoma-like rat model of ocular hypertension (Kipnis et al., 2000; Schori et al., 2001a). The results of the present study showed that immunization with Cop-1 is followed by a decrease in the propagation of damage induced by CHI. The mouse strain better able to withstand injurious conditions (Balb/c) responded rapidly to the Cop-1 vaccination, and 24 h after the trauma we could already detect a difference between the Cop-1-vaccinated and the control groups. In C57Bl/6J mice, the strain less resistant to injurious conditions, the response to the treatment was weaker and slower; nevertheless, once a steady state was reached significantly more neuroprotection was evident in the Cop-1–immunized mice than in the PBS-treated controls. By analyzing the MRI images, we were able to delineate the regions of damaged tissue and estimate the volume of the central site of damage. MRI images are closely correlated with histological data from spinal cord injury models (Hauben et al., 2000; Nevo et al., 2001). Even allowing for inaccuracies, the calculated results showed clear differences between Cop-1–treated mice and controls. Inaccuracies in assessment were mainly due to underestimation of the damaged areas.

Studies of T cell–based neuroprotection after optic nerve insult and spinal cord injury have shown that, after an injury, T cells home to the site of damage (Butovsky et al., 2001; Moalem et al., 1999a). These findings led to the suggestion that in order to exert their neuroprotective effect, T cells have to home to the lesion site and become activated there by their specific antigens, which are presented to them by the relevant antigen-presenting cells. Recent data obtained in our laboratory suggest that this local immune response is Th1-dependent (Kipnis et al., 2002b), and that it serves to activate resident microglia, thereby increasing their capacity for buffering glutamate and for general phagocytosis (Shaked et al., unpublished data). It also increases the production of neurotrophic factors by microglia or other resident cells (Barouch et al., 2002; Moalem et al., 2001). In seeking a way to boost a protective effect of autoimmune T cells, a number of compounds were tested, among them Cop-1. T cells specific to Cop-1 were found to be present in the undamaged CNS, similarly to T cells specific to myelin antigens (though in smaller quantities) (Kipnis et al., 2000; Aharoni et al., 2002). Moreover, after local damage (caused for example by injection of glutamate), treatment with Cop-1 was found to increase the infiltration of immune cells into the lesion site (Schori et al., 2001a). It thus appeared that Cop-1 could safely simulate the effect of myelin antigen in augmenting the relevant local immune response.

From the results of all the studies carried out to date, it appears that the endogenous T cell response evoked by CNS insults has a similar function at the different sites of CNS damage. However, the antigenic specificity of the T cells that are evoked spontaneously may differ from one site to another (Schori et al., 2001a; Mizrahi et al., 2002). In the case of axonal injury, both the endogenous (insult-evoked) T cell response and the exogenous therapeutic T cells are directed to myelin-associated antigens (Kipnis et al., 2000, 2002b). The antigenic specificity of the endogenous T-cell response evoked by CHI has yet to be discovered. It seems, however, that regardless of
whether the insult is inflicted on the gray or the white matter, it can benefit from T cells specific to Cop-1. This compound, being a random copolymer, has a heterogeneous composition. The heterogeneity might explain why Cop-1-reactive T cells can function at different sites, unlike T cells specific to an antigen with a homogeneous molecular structure (Kipnis and Schwartz, 2002). It is also possible that Cop-1-reactive T cells might act as bystander modulators of the endogenous evoked response (Aharoni et al., 1998). Whatever the mechanisms operating in the present study, our results argue in favor of immune involvement in neuroprotection. Like previous results in the optic nerve and the spinal cord (Hauben et al., 2001; Hauben and Schwartz, 2003), the present findings would support the therapeutic use of immunomodulators rather than immunosuppressors, so as to maximize the benefit from the immune system rather than eliminate any possible immune-mediated effect (Schwartz and Kipnis, 2002). It is important to note, however, that the Cop-1 administration protocol used for patients with MS does not lead to neuroprotection. Studies are currently in progress to establish the optimal formulation and protocol required to safely boost the beneficial autoimmune response for neuroprotection.

It was postulated that the T cell-mediated protective response evoked by CNS insults is harnessed to assist local innate immune mechanisms to cope with the insult-induced secretion of self-destructive compounds (Nevo et al., 2003). This T cell-mediated response needs to be rigorously regulated in order to provide protection without risk of autoimmune disease induction. Vaccination with Cop-1 appears to provide a safe way both to regulate and to boost the response. A principal role of the immune system is to efficiently eliminate dead cells and cell debris, and in addition to remove viable cells at the periphery of the injury site, which would otherwise be targeted by the agents of self-destruction and hence become the starting-point of a second wave of self-destructive activity. Protective immunity provides a way to arrest this phase of degeneration, but at some cost.

The process of nerve degeneration is chaotic and involves the activity of numerous physiological compounds. Some of them, though normally essential for brain function (e.g., glutamate), become toxic when (as a result of the insult) their normal concentrations are exceeded. Pharmacological intervention aimed at reducing the toxicity of a particular compound is likely to be accompanied by an undesirable disruption of that compound’s normal functioning. Protective autoimmunity appears to be the body’s mechanism of coping with conditions of stress, such as those caused by closed head injury. Taken together with earlier findings, the results of this study further support the contention that the immune response evoked by CNS trauma is always at least potentially beneficial, but it needs to be properly regulated for the beneficial effect to be expressed. If properly regulated and suitably boosted, protective autoimmunity is therefore likely to provide a global therapeutic effect. Since Cop-1 has already been approved for clinical use, it would appear that there are no serious obstacles to its immediate development for the treatment of closed head injury.

ACKNOWLEDGMENTS

We thank S. Smith for editing the manuscript and A. Shapiro for animal maintenance. M.S. holds the Maurice and Ilse Katz Professorial Chair in Neuroimmunology. The work was supported by Proneuron Ltd. (Ness-Ziona, Israel) and, in part, by grants from the Glaucoma Research Foundation and the Alan Brown Foundation for Spinal Cord Injury (to M.S.).

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