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Research Report

Nerve degeneration is prevented by a single intraneural apotransferrin injection into colchicine-injured sciatic nerves in the rat

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ABSTRACT

In this work, we have immunohistochemically analyzed the effects of single injections of apotransferrin (aTf) on the expression of myelin (myelin basic proteins [MBPs]) and axonal (protein gene product 9.5 [PGP 9.5] and β_{III}-tubulin [β_{III}-tub]) proteins in colchicine-injected and crushed sciatic nerves of adult rats. A protein redistribution was seen in the distal stump of injured nerves, with the appearance of MBP- and PGP 9.5-immunoreactive (IR) clusters which occurred earlier in crushed nerves (3 days post-injury [PI]) as compared to colchicine-injected nerves (7 days PI). β_{III}-tub-IR clusters appeared at 1 day PI preceding the PGP 9.5- and MBP-IR clusters in colchicine-injected nerves. With image analysis, the peak of clustering formation was found at 14 days PI for MBP and at 3 days PI for β_{III}-tub in colchicine-injected nerves. At 28 days of survival, the protein distribution patterns were almost normal. The intraneural application of aTf, at different concentrations (0.0005 mg/ml, 0.005 mg/ml, 0.05 mg/ml, 0.5 mg/ml), prevented nerve degeneration produced by colchicine, with the appearance of only a small number of MBP- and β_{III}-tub-IR clusters. However, aTf was not able to prevent clustering formation when the nerve was crushed, a kind of injury that also involves necrosis and blood flow alterations. The results suggest that aTf could prevent the colchicine effects by stabilizing the cytoskeleton proteins of the nerve fibers, avoiding the disruption of the axonal transport and thus the myelin degeneration. Transferrin is proposed as a complementary therapeutic avenue for treatment of cytotoxic nerve injuries.

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Abbreviations:
β-III-tub, βIII-tubulin
aTf, apotransferin
MBP, myelin basic protein
PGP, protein gene product
IR, immunoreactive
LI, like immunoreactivity
PI, post-injury
WD, Wallerian degeneration
SCs, Schwann cells
MAP, microtubule-associated protein
STOP, stable tubule only peptide
DRG, dorsal root ganglia
CNS, central nervous system
PBS, phosphate-buffered saline
SEM, standard error of the mean

1. Introduction

When a peripheral nerve is injured, the nerve fibers degenerate and there is a breakdown of the myelin sheath, a process called Wallerian degeneration (WD) (Buss and Schwab, 2003). WD can also be activated by injection of colchicine or other neurotoxins into the nerve (Angevine, 1957; Cliffer et al., 1998; Singer and Steinberg, 1972) or can also be triggered by defects in myelin (Frei et al., 1999; Garbern et al., 2002) or oxygen delivery (Oosthuyse et al., 2001).

Nerve degeneration has been extensively described for traumatic–ischemic injuries. Myelin sheath is broken down and fragmented into ellipsoids (Kidd et al., 1996). Schwann cells (SCs) isolate these myelin ellipsoids by surrounding them with a thick vimentin-rich layer and then proliferate (Chaudhry et al., 1992; Fernández-Valle et al., 1995; Ide, 1996; Liu et al., 1995; Ramón y Cajal, 1928; Stoll et al., 1989). This clustering process of myelin into ellipsoids consists in packaging of myelin proteins and can be easily monitored by immunohistochemistry (Pesini et al., 1999; Setton-Avruj et al., 2002). Myelin protein immunoreactivity in clusters increases with the progress of this process (Setton-Avruj et al., 2002). Afterwards, hematogenous phagocytic cells invade the whole nerve, enter SC tubes and uptake and degrade myelin proteins into small fragments (Avellino et al., 1995; Beuche and Friede, 1984; Brück, 1997; Dailey et al., 1998; Hirata et al., 1999; Setton-Avruj et al., 2002; Stoll and Müller, 1999).

Colchicine interferes with the formation and maintenance of microtubules that constitute axonal cytoskeleton, producing a transport blockage of molecules from the soma to the target tissue and vice versa which ultimately causes WD (Ginn and Peterson, 1992; Singer and Steinberg, 1972). There are several factors that are normally interchanged between SCs and axons. Cell cytoskeleton is presumed to play a crucial role in the communication between these cells (Corfas et al., 2004). Furthermore, the normal cytoskeleton structure and its stabilization are crucial in the maintenance of nerve function (Amos, 2004).

Transferrin, a transmembrane glycoprotein which introduces iron into the cell, is also considered a trophic factor (Mescher and Munaim, 1988). Transferrin is expressed in SCs of myelinated nerves (Lin et al., 1990). It has been published that a single intracranial injection of apotransferrin (aTf) into 3-day-old rats produces an increase in the myelination process as well as in the levels of cytoskeletal proteins such as actin, tubulin and microtubule-associated proteins (MAPs) including STOP (stable tubule only peptide) (Escobar Cabrera et al., 1994; Escobar Cabrera et al., 1997; Escobar Cabrera et al., 2000).

Transferrin has also been found in axolotl sciatic nerves, and it becomes concentrated over 20-fold in the nerve during limb regeneration (Kiffmeyer et al., 1991). Furthermore, transferrin undergoes anterograde fast transport in axons and is released distally at growth cones (Mescher and Kiffmeyer, 1992).

In addition, increases in overall tubulin synthesis in peripheral neurons have been documented after axotomy (Jiang and Oblinger, 1992). Moreover, evidence obtained using cDNA clones for in situ hybridization has suggested that some tubulin isotypes may be functionally more important than others during axonal regeneration. For example, preferential increases in the expression of the class II and III β tubulin mRNAs occur in dorsal root ganglia (DRG) neurons after peripheral axotomy, while little or no change in the levels of the class I and IV β tubulin mRNAs occurs (Hoffman and Luduena, 1996; Moskowitz and Oblinger, 1995; Wong and Oblinger, 1990). Although the neuron-specific isotype β_{III}-tub has been studied during development (Jiang and Oblinger, 1992), degeneration and regeneration in animal models showing many changes in its synthesis and transport (Moskowitz and Oblinger, 1995), no evidence exists about its behavior after a peripheral nerve lesion and its behavior after transferrin application.

In this work, the effect of intraneural injections of colchicine, using three different concentrations, was studied. In order to characterize the WD process promoted by colchicine, a survival time curve was done. In addition, the possible effect of aTf upon the WD process was analyzed after single injections of this protein into crushed (mechanical injury) or colchicine-injected (chemical injury) sciatic nerves, and the patterns of distribution of specific markers of myelin (MBPs) and axon (β_{III}-tub and PGP 9.5) were evaluated by immunohistochemistry.

2. Results

In colchicine-injected rats, MBP-like immunoreactivity (-LI) and β_{III}-tub-LI were organized in ellipsoid clusters which follow a fiber orientation pattern. The number of IR clusters progressively increased, reaching a peak with the highest concentration of colchicine used (Figs. 1a–c). In saline-injected and contralateral nerves (not shown), a normal pattern of myelin and nerve fibers organization was observed. When the area of IR clusters was measured, a corresponding increase was evidenced (Fig. 1d).

The analysis of the effect of colchicine over time showed the appearance of MBP and PGP 9.5-IR clusters at 7 days post-injury (PI) (Figs. 2a–d). However, with PGP 9.5, a slight increase in the intensity of the signal was evidenced already at 3 days (Fig. 2b). In contrast, β_{III}-tub-IR clusters reached its peak at 3 days PI (Fig. 4b), progressively recovering a fibrillar normal pattern at 14 days. At 14 days PI, MBP clusters changed in shape, adopting a round appearance, and there was a loss of nerve bundle pattern of the mark in the nerve (Fig. 2e). PGP 9.5-IR clusters instead decreased as compared to 7 days. Concomitantly, newly formed PGP 9.5-IR axons were seen (Fig. 2f). Later on, at 28 days, there was a decrease in the number and in the size of MBP-IR clusters within the nerve, showing an almost normal pattern of myelin organization (Fig. 2g). A similar pattern was detected with PGP 9.5-LI (Fig. 2h). Image analysis evidenced that the peak of immunostained area for MBP-IR clusters was reached at 14 days PI (Fig. 3).

With double immunostainings (Figs. 4a–c), the changes in expression of β_{III}-tub- and MBP-LI could be followed in parallel. When 5 mM colchicine was injected alone, the appearance of both β_{III}-tub- and MBP-IR clusters was observed (Fig. 4b). However, after the simultaneous injection of 5 mM colchicine and 0.5 mg/ml aTf, only a small number of β_{III}-tub-IR clusters appeared at 3 days PI and only a slight change in MBP-LI was observed (Fig. 4c).

In experiments where colchicine was injected into the nerve together with aTf at all concentrations used (0.0005 mg/ml, 0.005 mg/ml, 0.05 mg/ml, 0.5 mg/ml), only a small number of MBP-IR clusters were observed at 7 days PI (Figs. 5a–d), with the frequent presence of MBP-IR granulated cells. These MBP-IR cells were identified as mast cells by their meta-chromatic properties using toluidine-blue staining. When 5 mM colchicine was injected in different animals, MBP-IR clusters appeared (Fig. 5e). In aTf-injected sciatic nerves, a normal pattern of myelin and nerve fibers organization was seen.

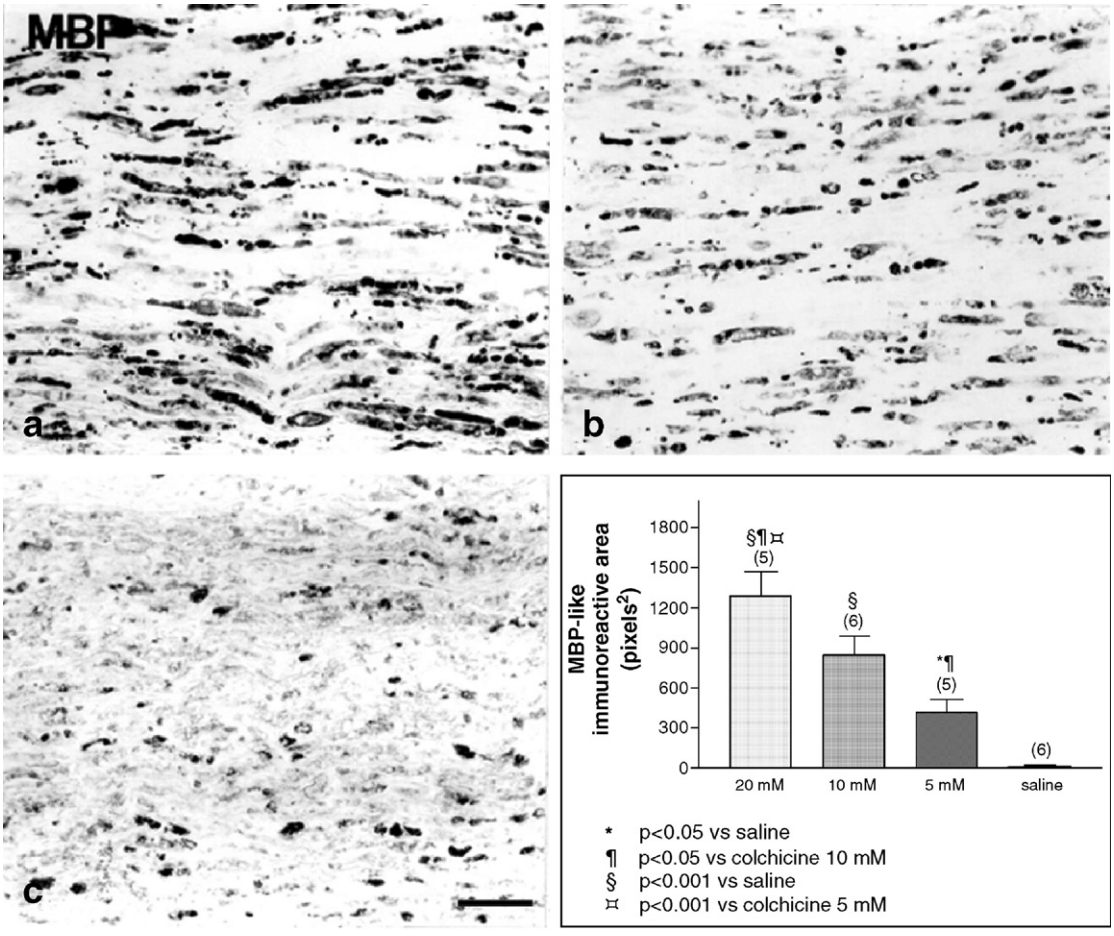

Fig. 1 – Panels a–c show micrographs of MBP immunostained sections from animals injected with 20 mM (a), 10 mM (b) and 5 mM (c) colchicine into the sciatic nerve following a survival time of 7 days. Scale bar: 40 μm. These results were measured by quantitative densitometry, and data are shown in panel d as the mean ± SEM.

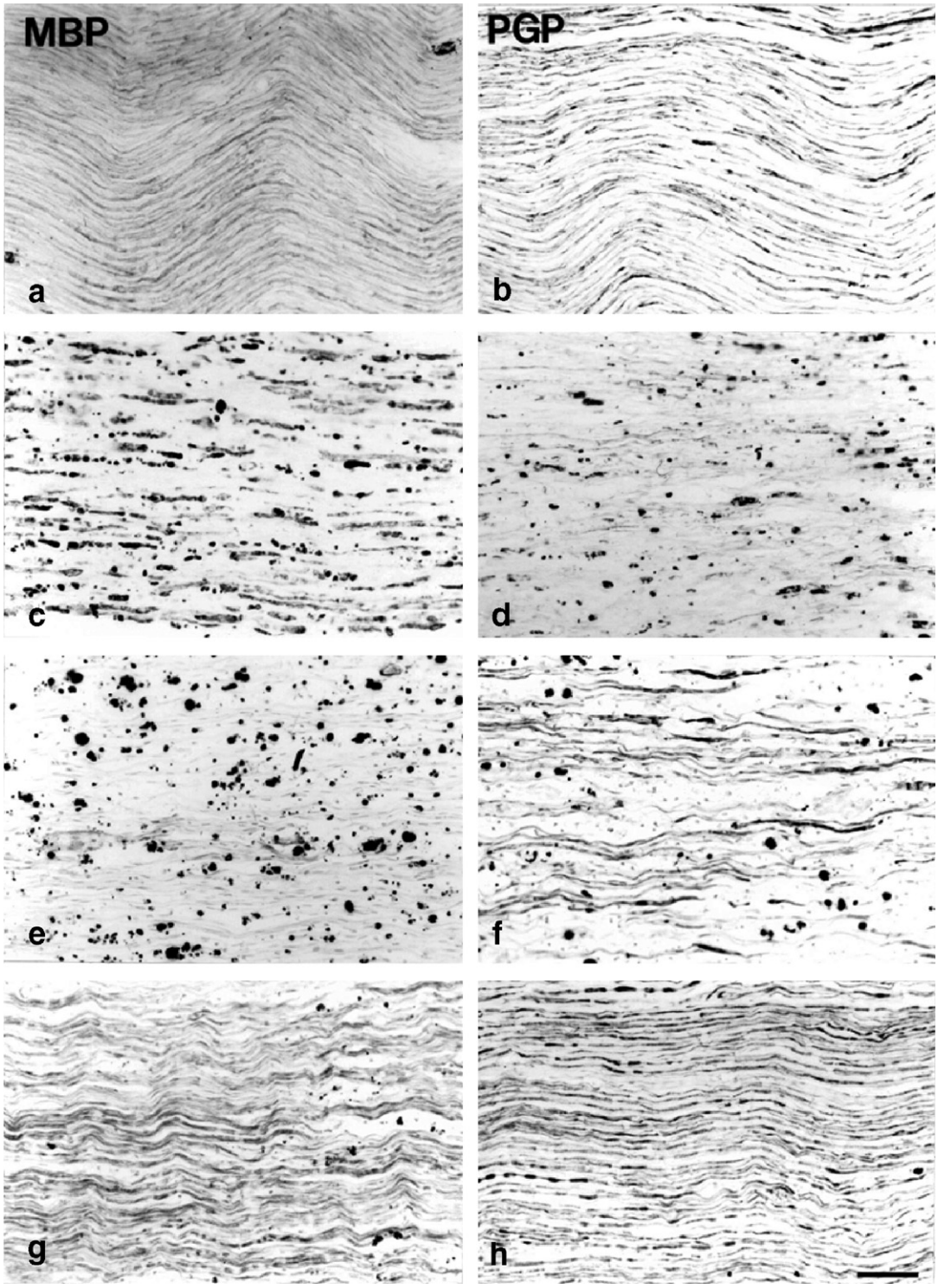

Fig. 2 – Micrographs of MBP (a, c, e, g) and PGP 9.5 (b, d, f, h) immunostained sections after 10 mM colchicine injection into the sciatic nerve, at 3 (a, b), 7 (c, d), 14 (e, f) and 28 (g, h) days post-injury. Scale bar: 40 μm.

Image analysis of immunostained areas corresponding to MBP-LI was significantly lower in colchicine + aTf-injected nerves as compared to colchicine-injected nerves and did not differ from saline or aTf-injected nerves (Figs. 4d and 5f).

Immunostaining for PGP 9.5 showed an almost normal pattern in colchicine + aTf-injected nerves (Figs. 6a–d) as well as in aTf-injected nerves (Fig. 6f). In contrast, in colchicine-injected nerves (5 mM), there was a high number of clusters displaying the label (Fig. 6e).

In the proximal zone of crushed sciatic nerves, 3 and 7 days PI, MBP-LI followed a normal pattern of myelin organization (not shown), whereas in the distal zone the

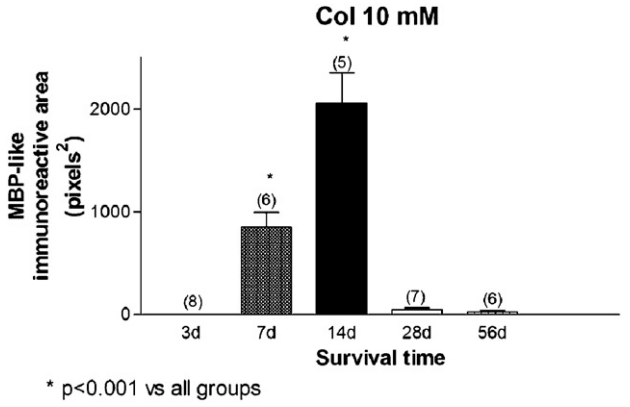

Fig. 3 – Effect of 10 mM colchicine injection into the sciatic nerve on the area of MBP immunostaining in the distal region of the nerve, 3 to 56 days post-injury. The measurements were made by quantitative densitometry, and data are shown as the mean ± SEM.

appearance of MBP-IR clusters was detected at 3 (not shown) and 7 days PI (Fig. 7a). When a single injection of 2 μl of 0.05 mg/ml or 1 mg/ml aTf was performed immediately before crushing, no changes were observed in MBP-LI (Fig. 7c). PGP 9.5-IR clusters were also observed in the distal region of sciatic nerves with or without the addition of aTf at both concentrations used (Figs. 7b, d). Image analysis confirmed the lack of significance between these experimental groups (Fig. 8).

Control experiments rendered negative stainings when primary or secondary antibodies were omitted.

3. Discussion

In this paper, we have shown the effect of colchicine-injected into the sciatic nerve upon the distribution of MBP-LI and two neural markers β_{III}-tub- and PGP 9.5-LIs in a dose-dependent manner. The results show the reorganization of these proteins in clusters, which display a specific pattern following several survival times. Interestingly, the pattern observed for MBP was similar to that described in a WD model obtained by ligation of the sciatic nerve (Setton-Avruj et al., 2002). Furthermore, the results indicate that the colchicine effect may be counteracted when it is co-injected together with aTf.

Injections of 2 μl of colchicine (5–20 mM) into the sciatic nerve induce WD, evidenced by axonal (β_{III}-tub and PGP 9.5 immunostaining) and myelin sheath (MBP immunostaining) breakdown and removal distally to the site of injection. Changes in MBP-LI during clustering process are much

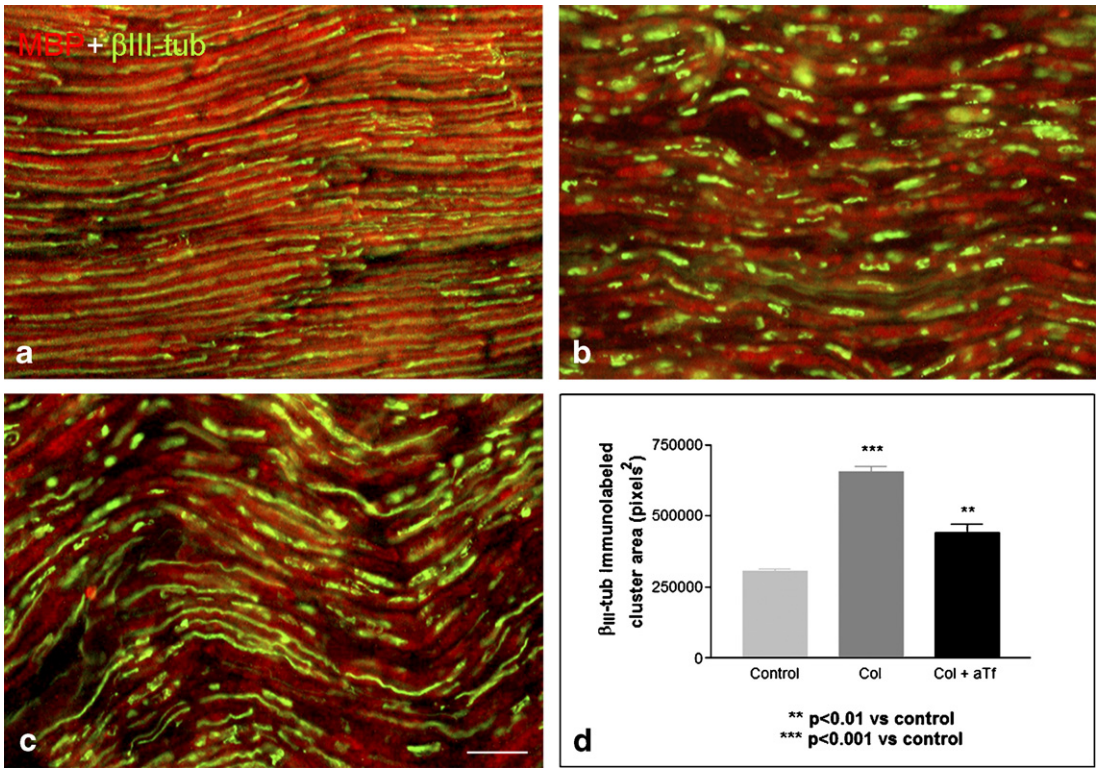

Fig. 4 – Immunofluorescence micrographs of sections of the sciatic nerve from a control (a) and 3 days after injection with 5 mM colchicine (b) and with 5 mM colchicine plus apotransferrin at 0.5 mg/ml concentration (c) double stained for β_{III}-tub (FITC, green) and MBP (TRITC, red) shown in merged staining color. Scale bar: 40 μm. (d) Image analysis of β_{III}-tub immunostaining in the distal part of the sciatic nerve after 5 mM colchicine injection (col) and 5 mM colchicine plus 0.5 mg/ml apotransferrin (col + aTf) injection, 3 days post-injury. The measurements were made by quantitative densitometry, and data are shown as the mean ± SEM.

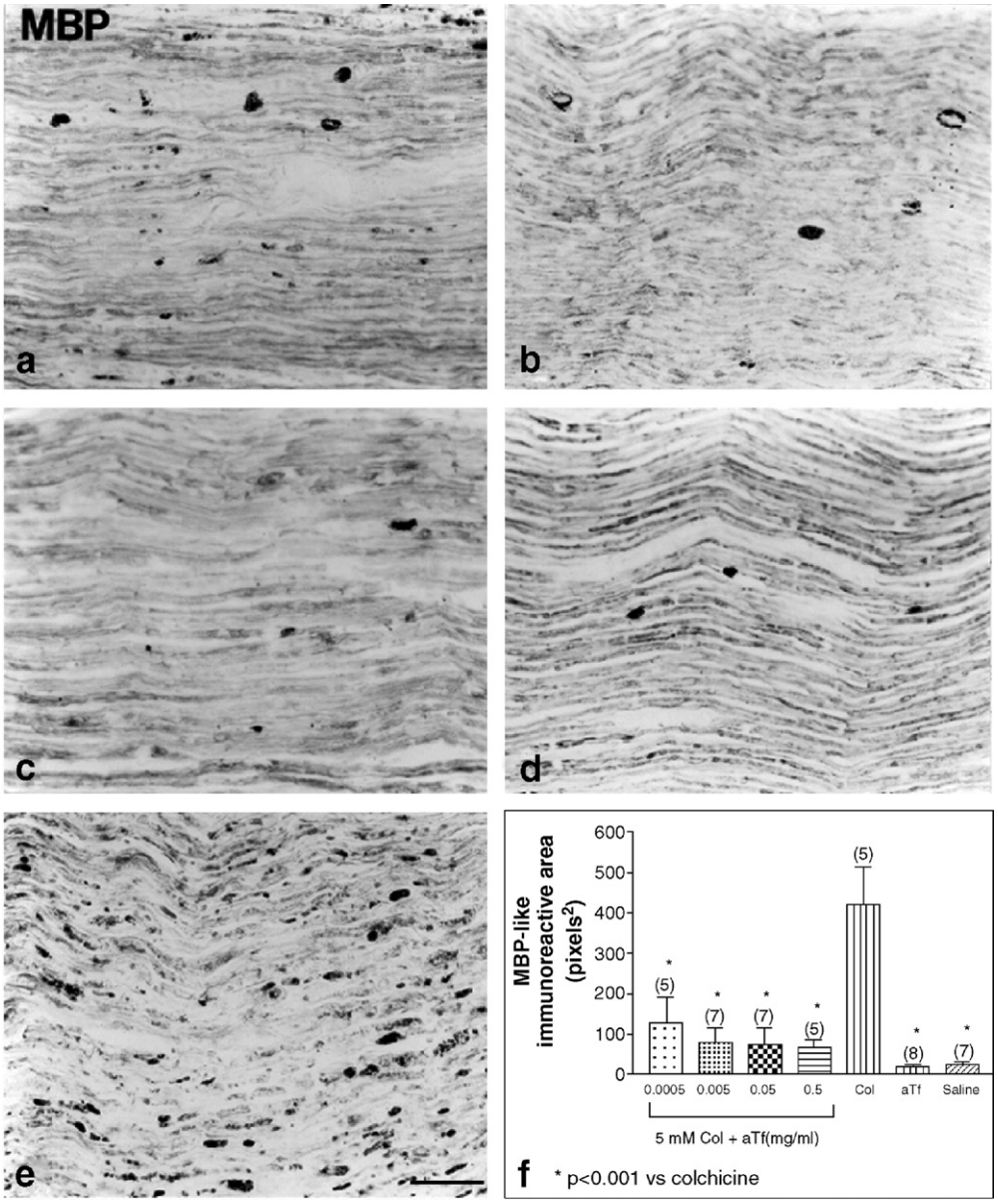

Fig. 5 – Panels a–e show micrographs of MBP immunostained sections of sciatic nerves after injection with 5 mM colchicine plus apotransferrin at different concentrations [0.0005 mg/ml (a), 0.005 mg/ml (b), 0.05 mg/ml (c), 0.5 mg/ml (d) and with 5 mM colchicine alone (e)]. Scale bar: 40 μm. These results were measured by quantitative densitometry, and data are shown in panel f as the mean ± SEM.

stronger when compared to PGP 9.5-LI and are likely caused by protein modifications, which result in increased MBP-epitope exposure. Moreover, the β_{III}-tub-LI showed a change in the pattern of distribution distal to the site of injection. A loss of β_{III}-tub-IR fibrillar pattern was observed concomitantly with the appearance of β_{III}-tub-IR clusters.

The appearance of MBP-IR clusters was shown to be dose-dependent, with an increase in their numbers with the higher doses of colchicine used.

The first signs of a PGP 9.5- and β_{III}-tub-IR clustering process, which is a sign of axonal degeneration (Geisert and Frankfurter, 1989; Li et al., 1997; Wilkinson et al., 1989), in colchicine-injected animals were observed from 3 days of survival and onwards, whereas the MBP-IR clusters first appeared at 7 days PI. These results suggest the need for a degeneration of axons prior to a degenerative process in myelin, thus giving support to the idea that fiber demyelination and axonal degeneration are two inseparable parts of peripheral axonopathies (Liu et al., 1995).

At 14 days PI, numerous apparently newly regrowth PGP 9.5-IR axons could be found across the nerve, whereas numerous MBP-IR rounded clusters remained present (Figs. 3e and f). In fact, our results suggest that a complete degeneration process must take place in order to allow the subsequent regeneration. The presence of macrophages and the action of SCs promoting a degenerative process give further support to this hypothesis (Avellino et al., 1995; Brück, 1997; Fernández-Valle et al., 1995; Hirata et al., 1999).

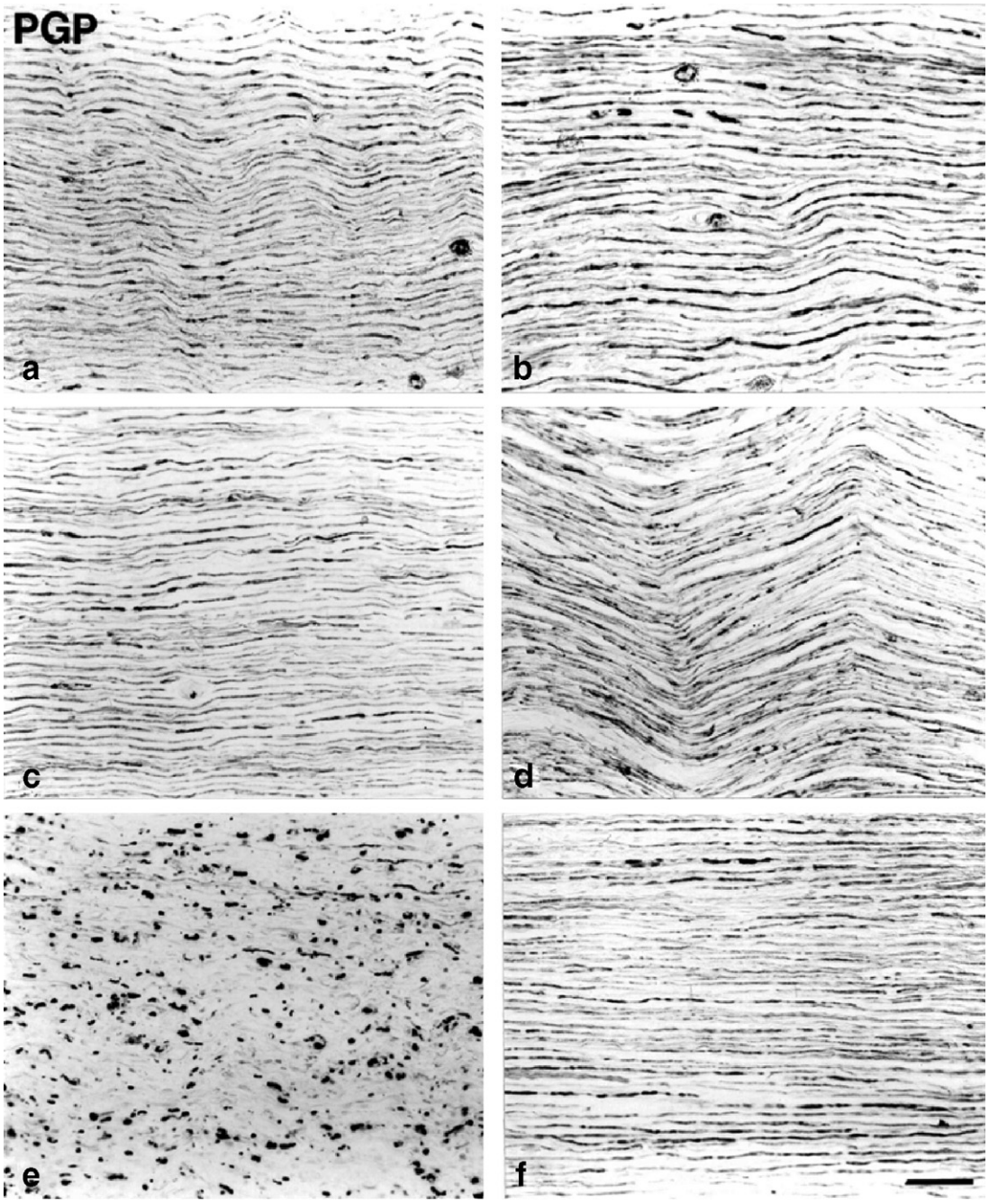

Fig. 6 – Micrographs of PGP 9.5 immunostained sections of sciatic nerves after injection with 5 mM colchicine plus apotransferrin at different concentrations [0.0005 mg/ml (a), 0.005 mg/ml (b), 0.05 mg/ml (c), 0.5 mg/ml (d)] and with 5 mM colchicine (e) or 1 mg/ml apotransferrin (f) alone. Scale bar: 40 μm.

At 28 days, the images of colchicine-injected or crushed sciatic nerves were very similar and almost normal in the patterns of PGP 9.5, β_{III}-tub and MBP organization.

In our work, using 10 mM concentration of colchicine, the peak of appearance of MBP clusters, quantified by measuring the MBP-immunostained area, was detected at 14 days of survival. Instead, in animals with ligation of the sciatic nerve, the peak was found at 7 days PI (Setton-Avruj et al., 2002). In addition, the immunostained areas in sciatic nerves after a 10 mM colchicine injection were lower than in ligated or crushed nerves. The signs of WD appeared earlier in the sciatic nerve when subjected to ligation (Setton-Avruj et al., 2002) or crushing as compared to colchicine injection. This is perhaps the result of a more dramatic change of the nerve which also undergoes an important necrosis and alteration of blood flow, with an earlier increase in calcium ions and proteolytic enzyme activation, due to a mechanical injury (Banik, 1992; George et al., 1995; Martínez and Canavarro, 2000; Wang et al., 2000).

In a model of fast axonal transport disruption in vivo, without blood flow alteration (Wu et al., 1994), a down-regulation in the expression of P_0 mRNA in the distal region of the nerve after 3 days of survival, without the appearance of

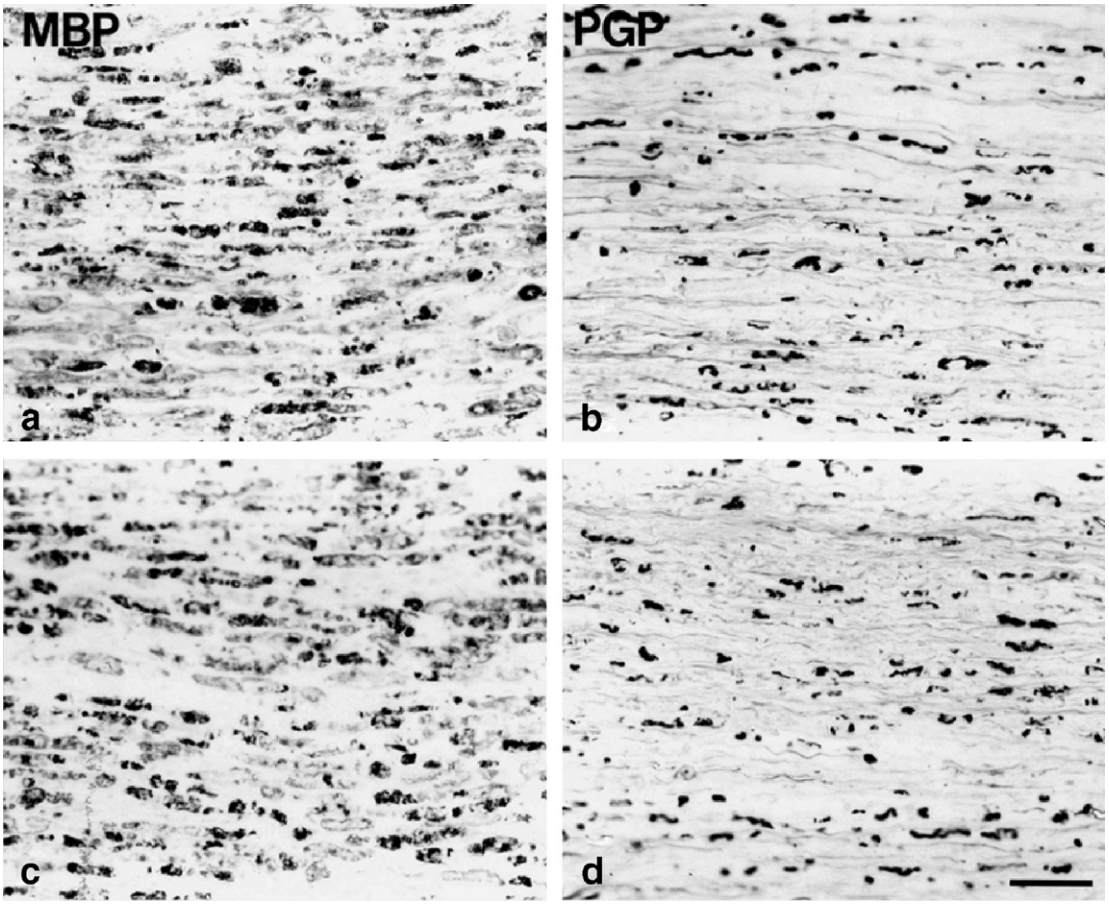

Fig. 7 – Micrographs of MBP (a and c) or PGP 9.5 (b and d) immunostained sections of crushed sciatic nerves (a, b) or nerves subjected to crush + 0.05 mg/ml apotransferrin injection (c, d) at 7 days post-injury. Scale bar: 40 μm.

WD, was observed. It is worth mentioning that, in our experimental model, where the axonal transport of trophic factors is also blocked, at 3 days PI, there is little evidence of myelin degeneration process, which reaches its peak between 7 and 14 days. These results seem to indicate that in traumatic injuries other causes than a mere axonal blockage may play a major role in triggering myelin degeneration.

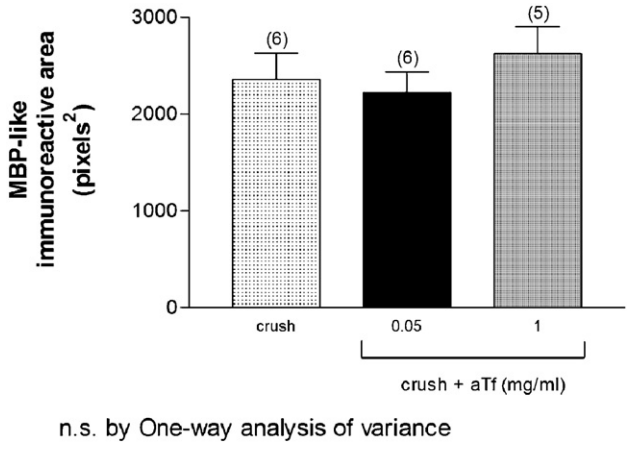

Fig. 8 – Image analysis of MBP immunostaining in the distal part of the sciatic nerve after crushing or crushing plus 0.05 or 1 mg/ml apotransferrin injection, 7 days post-injury. The measurements were made by quantitative densitometry, and data are shown as the mean ± SEM.

At all concentrations used, aTf prevented WD in the colchicine-injected sciatic nerves. The mechanisms involved in this process may in part involve the stabilization of the nerve fiber cytoskeleton proteins, such as β_{III}-tub. We have shown the degeneration pattern of β_{III}-tub after colchicine injection and its prevention when aTf is present. Moreover, experiments done in the central nervous system (CNS) may be valuable for the understanding of such effect. aTf administered intracranially to newborn rats produces a marked increase in myelin cytoskeleton proteins such as tubulin, actin and STOP and in the mRNA of tubulin and actin (Escobar Cabrera et al., 2000). Transgenic mice overexpressing the human transferrin gene also showed an increase in the immunoreactivity of certain cytoskeleton proteins (Marta et al., 2002). Taking into account the results previously described in the CNS, we can suggest that the aTf action may be explained through its stabilizing effect on β_{III}-tub and other cytoskeletal proteins of the axon and myelin sheaths which are affected by colchicine (Ginn and Peterson, 1992; Singer and Steinberg, 1972). It is worth noting that aTf could not prevent WD after crushing, which involves an additional ischemic compromise affecting other structural proteins of the nerve. Since there are preferential increases in the expression of

class II and III β tubulin mRNA isotypes in DRG after peripheral axotomy (Hoffman and Luduena, 1996; Moskowitz and Oblinger, 1995; Wong and Oblinger, 1990), a differential response of β tubulin isotypes to traumatic or cytotoxic injuries of the sciatic nerve could be expected. Thus, the study of expression of these isotypes in DRG and peripheral nerves could clarify the different response to aTf in traumatic or cytotoxic lesions of the sciatic nerve.

A possible mechanism for aTf prevention of colchicine effects may be the promotion of both transcription and translation of tubulin, actin and STOP followed by an increase in the levels and transport of the corresponding proteins (Hoffman and Luduena, 1996; Moskowitz and Oblinger, 1995; Wu et al., 1994). Furthermore, a local protective action of aTf over polymerized β_{III}-tub should not be underestimated. As a consequence, these proteins could stabilize the microtubular system, avoiding the disruption of the axonal transport induced by colchicine.

Studies done with cultured SCs demonstrated that serum withdrawal from the medium causes a dedifferentiation of SCs as evidenced by the increase in the levels of GFAP and $p75_{NTR}$ and a decrease in MBP and P_0 content. The supplementation of the serum-free medium with holotransferrin (hTf) or aTf + iron returns the cells to a phenotype similar to that of myelinating SCs (Salis et al., 2002). These results indicate that transferrin could avoid the formation of MBP- and PGP 9.5-IR clusters both by stabilizing the axonal transport through the upregulation of synthesis of the microtubule network and through the prodifferentiating effect of transferrin on SCs by promoting the increase in the myelinating phenotype.

The application of aTf in crushed nerves in a model of delayed WD such as the Wld^s mutant mouse could also be of interest (Coleman and Perry, 2002; Glass et al., 1993; Samsam et al., 2003). In fact, by delaying nerve degeneration, a time window may be obtained in which aTf would be able to stabilize the axonal cytoskeleton. Similar applications could bring promising therapeutic alternatives both in those brain and spinal cord injuries, in which axon degeneration does not happen instantaneously after injury and may instead take place hours later (Coleman and Perry, 2002). Furthermore, aTf could be a good candidate for complementary treatment of nerve cytoskeleton injuries such as those poli/myoneuropathies that take place after colchicine or vincristine treatment of many disorders, i.e. amyloid nephropathy, gout, renal or cardiac transplant patients and hematological neoplasm (Chauvenet et al., 2003; Kuncl and George, 1993; Quasthoff and Hartung, 2002; Rana et al., 1997).

4. Experimental procedures

4.1. *Colchicine dose curve*

Adult (200–300 g body weight) Wistar rats were anesthetized intraperitoneally with chloral hydrate (350 mg/kg), and the right sciatic nerves were exposed at the level of the thigh and injected with 2 μl of colchicine (Sigma; 5, 10 and 20 mM, in saline) 5 mm distal to the nerve branch cutaneus femoris caudalis. Sham animals injected with saline were also prepared. The injections were performed using a Hamilton 1801RN 10 μl syringe mounted on a stereotaxic frame, with the needle oriented towards the dorsal root ganglia. To identify the site of injection, a suture was made in the muscle nearby. The animals were then reanesthetized and perfused via the ascending aorta with 60 ml of 4% paraformaldehyde containing 0.2% picric acid in 0.1 M phosphate buffer (37 °C) and 250 ml of the same fixative, but cold (4 °C).

4.2. *Injection of colchicine and apotransferrin*

Following the procedures mentioned above, right sciatic nerves of adult rats were injected with 2 μl of a mixture of 5 mM colchicine and aTf (0.0005 mg/ml to 0.5 mg/ml, in saline). Sham animals injected with saline or aTf (1 mg/ml) alone were also prepared. The animals were allowed to survive for 7 days and reanesthetized and perfused as described above.

4.3. *Crushing of sciatic nerve*

The right sciatic nerve of adult rats was crushed under chloral hydrate anesthesia at the midthigh level for 5 s with jeweler's forceps. Immediately after the crush, 2 groups of animals were intraneurally injected with 2 μl of aTf at 1 mg/ml or 2 μl of aTf at 0.05 mg/ml. The animals were allowed to survive for 7 days and reanesthetized and perfused as described above.

4.4. *Immunoperoxidase procedure*

Immediately after perfusions, the right sciatic nerves were carefully dissected out and their proximal and distal ends were identified. The contralateral sciatic nerves were also obtained. All the nerves were immersed in the same fixative for 90 min and stored in 20% sucrose for 48 h. The tissues were then frozen and cut longitudinally with a cryostat (Microm, Zeiss) at 16 μm section thickness. The sections were mounted onto gelatin pre-coated glass slides, allowed to dry for at least 1 h, rinsed twice in PBS and dehydrated.

Sections were incubated overnight in a humid chamber at 4 °C with anti-MBP (1:1500, a gift from Dr. A. Campagnoni), anti-PGP 9.5 (1:3500, rabbit, Ultraclone, Isle of Wight, UK) or anti-β_{III}-tub (1:2000, mouse, Chemicon) antibodies, diluted in PBS containing 0.2% (w/v) bovine serum albumin, 0.03% Triton X-100 and 0.1% (w/v) sodium azide. The slides were rinsed twice in PBS and incubated at room temperature for 1 h with biotinylated secondary antibodies (1:200, Vector Lab., Burlingame, California, USA), rinsed twice in PBS and incubated with ABC (Vectastain Elite kit, Vector Lab.) for 1 h at room temperature. Peroxidase activity was demonstrated by reaction with 3,3′-diaminobenzidine using H_2O_2 and nickel salts for enhancement of the reaction product. After dehydration, the sections were coverslipped with synthetic Canada Balsam as mounting media.

4.5. *Immunofluorescence procedure*

After perfusion, the sciatic nerves of control and experimental animals were dissected out and identified as described above and cut at 14 μm thickness in a cryostat and processed for the indirect immunofluorescence technique (Coons, 1958). Briefly,

sections were incubated overnight in a humid chamber at 4 °C with mouse antiserum to β_{III}-tub (1:500, Chemicon) or rabbit antiserum MBP (1:400) antibodies, diluted in PBS containing 0.2% (w/v) bovine serum albumin, 0.03% Triton X-100 and 0.1% (w/v) sodium azide. Furthermore, double labelings were performed by incubating sections with a mixture of rabbit MBP and mouse monoclonal antibodies to β_{III}-tub. After rinsing in PBS, sections were incubated for 30 min at 37 °C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or goat anti-mouse (1:80, Jackson ImmunoResearch Laboratories, West Grove, PA). For double labeling, secondary antibodies were applied as a mixture of tetramethylrhodamine isothiocyanate (TRITC)-conjugated donkey anti-rabbit antiserum (Jackson ImmunoResearch Laboratories) and FITC-conjugated sheep anti-mouse (1:10, Amersham, Amersham, UK). The sections were rinsed and mounted in a mixture of glycerol and PBS (3:1) containing *para*-phenylenediamine (Johnson and Nogueira Araujo, 1981; Platt and Michael, 1983).

4.6. *Microscopy*

All sections were examined with a Nikon Eclipse E-800 photomicroscope either under bright field or fluorescence illumination using proper filter combinations for FITC and TRITC. Photographs were taken in Technical Pan Film (Eastman Kodak, Rochester, NY, USA) or Kodak TMAX-400 film and digitalized using a Nikon Scanner ORCA LS-2000 (Tokyo, Japan). A Nikon DXM 1200 digital camera (Tokyo, Japan) was also used.

Resolution, brightness and contrast of the digital or digitalized images were optimized using the Adobe Photoshop software (Adobe Systems Inc., San Jose, CA) and printed using and Epson Stylus Photo EX printer (Seiko Epson Corp., Nagano, Japan).

4.7. *Control experiments*

Controls were done incubating sections with a preimmune serum and incubation without the primary or secondary antibodies. In order to confirm the specificity of the antibodies, Western blot studies were done following previously published protocols (Setton-Avruj et al., 2002).

4.8. *Image analysis measurements*

Image analysis measurements were made following the procedure of Setton-Avruj et al. (2002) using a KS400 system (Kontron Elektronic/Zeiss) and a Hyper HAD Sony CCD IRIS black and white video camera mounted onto an Optiphot-2 Nikon microscope for image acquisition. Briefly, images were digitalized in a rectangular frame of 640 × 480 pixels using the 10× objective in the photo mode of illumination intensity. To adjust possible defects in the illumination of optical pathway, a low-pass image was produced for subtraction and background shading correction. After that, a gray value for image segmentation was interactively chosen. Defining a threshold gray level, all the pixels whose gray value informative content was lower or higher than the segmentation gray were set to white and the others set to black. Six to eight immunolabeled area values, each one of 122,500 pixels^2, were obtained per nerve and the mean value ± SEM was calculated for each nerve of the different experimental conditions tested.

For colchicine experiments, the immunostained area values of each nerve were obtained from images from 3 to 9 mm distal to the injection site. For crushing experiments, the images processed were from regions between 3 and 6 mm distal to the crush injury site.

Statistical analysis was carried out by applying one-way analysis of variance and Newman–Keuls multiple comparison post-test.

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