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The Effects of FK506 on Dorsal Column Axons Following Spinal Cord Injury in Adult Rats: Neuroprotection and Local Regeneration

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There is considerable evidence that immunophilin ligands can promote the regeneration of axons in peripheral nerves and act as neuroprotective agents in the CNS. We have examined the effects of FK506 and GPI 1046 on the responses to partial transection of ascending spinal dorsal column axons at T9, in some cases combined with crush of one sciatic nerve. FK506 (0.5 or 2.0 mg/kg) and GPI 1046 (10 or 40 mg/kg) was administered subcutaneously immediately after surgery and five times a week thereafter. Some animals received methylprednisolone (MP) (two subcutaneous doses of 30 mg/kg) in addition to, or instead of, FK506. After survival times of 1–12 weeks, dorsal column axons were labeled transganglionically with cholera toxin B-HRP. There was massive axonal sprouting at the lesion sites in animals with sciatic nerve injury and immunophilin ligand treatment. In FK506-treated animals a few severed sensory axons regenerated for up to 10 mm rostral to the lesion. Of greater significance, 30% of 71 FK506-treated animals had spared axons in the dorsal column, extending to the nucleus gracilis, versus 8% of 50 control animals ($P < 0.05$), showing that FK506 reduces the likelihood of axonal destruction due to secondary injury. A combination of FK506 and MP afforded greater protection than MP alone ($P < 0.05$), but axonal survival was not affected by sciatic nerve crush, dose of FK506, or survival time after injury. GPI 1046 ($n = 11$) did not promote axonal survival. Thus FK506 protects axons from secondary injury following spinal cord trauma, and in this experimental model, its neuroprotective effect is greater than that of MP. © 1999 Academic Press

Key Words: **FK506; immunophilin ligands; spinal cord injury; axon regeneration; neuroprotection.**

INTRODUCTION

The immunosuppressant drugs FK506 and cyclosporin A, which are routinely used to prevent allograft rejection following transplant surgery (42, 45, 48), bind to intracellular proteins called immunophilins. FK506 binds to several FK506 binding proteins (FKBPs), of which FKBP12 appears to be the most important in the nervous system. FKBP12 forms complexes with a number of other proteins (reviewed in 47) including calcineurin, calcium release channels, and receptors for growth factors. A third immunophilin ligand, GPI 1046 (3-(3-pyridyl)-1-propyl (2*S*)-1-(3,3-dimethyl-1,2-dioxopentyl)-2-pyrrolidinedinecarboxylate), binds to FKBP-12 but does not affect calcineurin and is not an immunosuppressant (47, 49, 51). An indication that immunophilins might be involved in roles other than as intermediaries in immunosuppression is given by the fact that FKBP12 is more highly concentrated in peripheral nervous tissue and in the central nervous system (CNS) than in the immune system (47, 50). In fact, evidence has been emerging for some years that immunophilin ligands have protective effects on neurons both *in vivo* and *in vitro* (7, 11, 13, 16, 24, 25, 33–35, 53, 58; reviewed in 47) and may also be capable of promoting axonal regeneration (17–20, 30, 49, 56). FK506 binds to FKBP12 to form a complex which inhibits calcineurin (28). This, in turn, would be expected to increase the amount of the active form of the growth-associated protein GAP-43 in neurons. Furthermore, there has been one remarkable claim for the effectiveness of cyclosporin A in promoting structural and functional repair in the spinal cord (38, 52) and another report that FK506 improves the functional outcome of spinal cord injury produced by photothrombosis, through an as yet undefined mechanism which may involve an increase in the expression of GAP-43 (31, 32).

In this study we have investigated the effects of immunophilin ligands on the survival and regeneration of axons following injury to the fasciculus gracilis in the lower thoracic region of the spinal cord in adult rats. This tract contains the central axons of a population of large and medium-sized dorsal root ganglion (DRG) neurons, which extend along the length of the spinal cord to the nucleus gracilis in the medulla oblongata. The influences of a number of variables on the outcome of treatment with immunophilin ligands were also investigated. These variables included the dose of FK506 and GPI 1046, adjuvant therapy with methyl-

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prednisolone, sciatic nerve crush, and time of assessment after injury. The ascending axons of the fasciculus gracilis offer particular advantages for studying axonal injury and regeneration in the CNS: they form a compact unilateral bundle, many of them can be easily labeled by injecting tracers into the sciatic nerve (transganglionic labeling), and the regenerative potential of the neurons can be increased by injuring the sciatic nerve (8, 39, 40). Although the study was undertaken in the hope of finding significant effects of immunophilin ligands on axonal regeneration in the spinal cord, the major findings concern a neuroprotective effect on the ascending dorsal column axons.

MATERIALS AND METHODS

Surgical Procedures

All animal care and surgical procedures were approved and licensed under UK Government legislation on animal welfare and experimental procedures. Female Sprague–Dawley rats (weight 150–200 g) were deeply anesthetized with an inhaled mixture of 1.5% halothane, 3% nitrous oxide, and 1.5% oxygen. The surgical site was shaved and cleaned with antiseptic solution. An incision was made along the back and a laminectomy was performed at the T8–9 level to expose the dura, which was then widely opened by first making a hole with a needle and then enlarging it with microsurgical scissors. A small hole was made in the pia on the dorsal surface of the cord using a fine needle and this was enlarged across the midline using microsurgical scissors, at the same time cutting the dorsal vein. After hemostasis had been achieved, a fine needle (25 gauge) was swept across the dorsal aspect of the cord two or three times to a depth of 1.5 mm with the aim of transecting the dorsal columns, which at this level are formed predominantly by the fasciculus gracilis dorsally and the corticospinal tract ventrally. In four animals a much larger spinal cord lesion was made using a Decker microrongeur (1 × 5-mm straight cup; Johnson and Johnson, New Bedford, MA) attached to a stereotaxis frame, which removed the dorsal aspect of the cord to a depth of 2 mm. In some animals with standard lesions, the wound in the dorsal columns was covered with a thin layer of Silastic, fixed in place with Histoacryl tissue glue (B. Braun Melsungen AG, Germany). The muscle around the wound was closed with 3.0 silk sutures (Ethilon, UK), and the skin along the back was clipped together with Mitchel clips. In a subset of these animals the left sciatic nerve was exposed in the thigh and crushed with fine forceps at the same operating session. This procedure is known to enhance the regenerative responses of DRG cells (8, 39, 40) and has been shown recently to promote the regeneration of dorsal column axons following spinal cord injury (37 and S. Neumann and C. J. Woolf, personal communication). All rats received 1 mg of flunixin meglumie (Finadyne; supplied by Schering-Plough Animal Health, UK) and amoxycillin (SmithKline Beecham, UK) administered subcutaneously.

Preparation and Administration of Drugs

FK 506 was obtained from Fujisawa Ireland Ltd (Ireland), 1 mg of FK 506 was dissolved in 200 μl of absolute ethanol, and this solution was mixed with Intralipid (Pharmacia Laboratories Ltd, UK) in the ratio of 2 to 3 vol. Individual animals were given either low- or high-dose regimes (0.5 or 2.0 mg/kg), by subcutaneous injection five times per week, starting within 30 minutes of surgery. The treatment regime and dosage was continued until the animal was killed. Control animals received injections of vehicle alone.

GPI 1046 was supplied by Guilford Pharmaceuticals (Baltimore, MD); 12 mg was dissolved in 20 μl of absolute ethanol and, on the day of administration, was mixed with Intralipid in the ratio 1 to 14 vol. Individual animals were given either low- or high-dose regimes (10 or 40 mg/kg) as described above for FK506. Control animals were treated as for the FK506 group.

Methylprednisolone sodium succinate (MP) was obtained from Upjohn (U.S.A.); it was dissolved in sterile distilled water at a concentration of 30 mg/ml. Each animal received two doses of 30 mg/kg by subcutaneous injection. The first dose was administered within 30 min of surgery and the second and final dose was administered 24 h later.

Transganglionic Labeling, Fixation, and Histochemical Processing

After survival periods of 1 to 12 weeks the animals were again anesthetized with halothane as described above, and the left sciatic nerve was exposed in the thigh and injected with 0.02 mg of cholera toxin subunit B conjugated to horseradish peroxidase (CT-HRP, List Biological Laboratories, U.S.A.) dissolved in 1 μl of distilled water. Three to four days later the animals were overdosed with intraperitoneal pentobarbitone sodium 0.5–1 ml (Sagatal, Rhone-Merieux, Ireland). The heart was exposed and the right atrium was opened; a cannula was inserted into the left ventricle and the rat was perfused with approximately 400 ml of 0.1 M phosphate buffer at room temperature, followed by 500–750 ml of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at room temperature. The spinal cord was exposed and the segmental level was determined with reference to the entry of nerve roots from the sciatic nerve. The entire spinal cord and brain stem were removed and stored overnight at 4°C in 30% sucrose. Blocks were cut from different levels of the spinal cord and brain stem and then frozen in OCT (Miles Inc., U.S.A.).

Horizontal longitudinal or transverse sections were cut at a nominal thickness of 50μm on a cryostat and placed in serial order in reaction wells containing 0.1 M phosphate buffer. The free-floating sections were then reacted with 3,3′,5,5′-tetramethylbenzidine (TMB) to visualize transganglionically transported label. Briefly, 0.3 g of sodium nitroferricyanide was dissolved in 280 ml of distilled water and 15 ml of sodium acetate buffer (pH 3.3). A solution of 0.015 g of TMB in 7.5 ml of absolute alcohol was prepared. The two solutions were then mixed in the ratio 1:39 (TMB:sodium nitroferricyanide). Sections were immersed in this mixture at 4°C for a period of 20 min, following which 0.01% hydrogen peroxide was added in a ratio of 1 part in 25. After an additional 5 min this mixture was replaced by a new solution containing all three reagents in the same proportions as given above and this process was repeated at 5-min intervals over the course of a 30-min reaction period. At the end of this time the sections were rinsed several times in sodium acetate buffer and then air-dried, dehydrated, and coverslipped.

Treatment Groups

Three series of experiments were carried out. In the first series (Series I) 78 rats were divided into three main treatment groups: Group 1 animals were treated with FK506, Group 2 animals were treated with GPI 1046, and Group 3 animals (controls) received vehicle alone (Table 1). Subgroups of FK506- and GPI 1046-treated animals received either high- or low-dose regimes and in each group some animals also received MP (see Table 1). Survival periods before analysis varied between 1 and 12 weeks. Analysis of the material in the first series was not carried out "blind." Because of the possibility of observer bias, a second series of experiments (Series II) was performed, in which 54 rats underwent the same surgical procedures as those in the first series, but were randomly assigned to either a group treated with FK506 (low dose only) in precisely the same regime as used in the first series or a group which served as vehicle-only controls. In each group some animals were also treated with MP (see Table 1). The survival period for Series II animals was 7–15 days after injury. Analysis of the material was carried out on coded slides by the first author who was unaware of the treatment category of the animals from which the slides were derived. In addition 4 additional animals (Series III) treated with low-dose FK506 and sciatic nerve crush had larger lesions made using biopsy forceps, as described above. The results of these four experiments were analyzed separately from those of Series I and II.

TABLE 1

Treatment Details

	Treatment group	Main treatment and dose	No. of animals	Additional treatment +MP/−MP
Series I	1	FK506 0.5 mg/kg	31	14/17
		FK506 2.0 mg/kg	12	3/9
	2	GPI 1046 10 mg/kg	6	0/6
		GPI 1046 40 mg/kg	5	3/2
	3	Controls; vehicle only	24	11/13
		Total	78	
Series II		FK506 0.5 mg/kg	28	16/12
		Controls; vehicle only	26	13/13
		Total	54	
Series III		FK506 0.5 mg/kg	4	0/4
		Total, all groups	136	

Note. All 136 animals had dorsal column injury (in some cases with simultaneous sciatic nerve injury (not shown in table) and were transganglionically labeled with CT-HRP from an injection into the left sciatic nerve. Series I animals were not randomized; Series II animals were analyzed blind. Series III animals had a large lesion of the dorsal columns. FK506 and GPI 1046 were given at two dose levels (low and high) in Series I but at only one level (low) in Series II and III. MP, methylprednisolone.

Analysis of Material

Serial longitudinal (horizontal) sections through the lesion site and dorsal columns rostral to the lesion and transverse sections through the upper thoracic spinal cord and through the dorsal column nuclei were systematically examined for the presence of transganglionically labeled axons, using bright and dark field microscopy. Particular attention was given to trying to identify terminal regions of labeled axons in the spinal cord. In addition, lesion size was assessed by making camera lucida drawings of dark field images of the lesion at its maximum extent, allowing a calculation of the maximum length and width of the lesion.

Differences between the frequencies of labeled axons in different treatment subgroups were compared using contingency tables and a χ^2 test or, if numbers were too small, a one-tailed Fisher's exact test (FET). Nonparametric statistics were used to calculate the median and interquartile range (IQR) of lesion size in different treatment groups, and differences were analyzed using the Mann–Whitney test.

RESULTS

In all, 153 animals were used in this study of which 136 yielded useful results; 9 were discarded because of inadequate transganglionic labeling and 8 animals died postoperatively. Of the latter only 1 of the deaths, due to a urinary infection in an animal receiving FK506, was a likely consequence of drug treatment; all others were due to surgical complications or were unexplained. Death rates in different treatment groups did not differ significantly. Animals in all groups nor-

mally recovered rapidly from surgery and showed apparently normal locomotion (except for the left hind limb in rats with a sciatic nerve crush). Bundles of transganglionically labeled axons (and possibly individually labeled axons) were visualized with clarity in this material, particularly in dark field (Fig. 1). Although there could be uncertainty over the identity of some labeled structures in the lesion site there was no ambiguity in the identification of labeled axons in the dorsal columns and dorsal column nuclei, particularly in the case of longitudinal horizontal sections (Figs. 1A–1E, 2, and 3).

Series I

In all groups of animals the ascending dorsal column axons could be traced to the caudal margin of the lesion. In cases involving sciatic nerve crush and/or treatment with an immunophilin ligand, large numbers of axonal sprouts could be seen in the lesion site, where some apparently terminated. The largest numbers of sprouts within the lesion site were seen in animals with both a conditioning (sciatic nerve) injury and immunophilin ligand treatment; in animals without a sciatic nerve crush, or immunophilin ligand treatment, most labeled axons appeared to have retracted or died back from the lesion site. There were two patterns in the arrangement of labeled axonal sprouts at the lesion site; they were either present as a disordered tangle or they formed bundles which appeared to be orientated at 90° to the longitudinal axis of the cord and were occasionally found to be growing out of the cord (Fig. 1A). However, axons growing out from the cord were not observed in animals in which Silastic had been glued over the injured dorsal columns to isolate the wound from surrounding tissues.

In the great majority of animals without immunophilin ligand treatment, no labeled axons were found to extend rostral to the lesion site. In groups treated with FK506, some labeled axons were observed to extend from a few hundred micrometers to 5 mm or more and very rarely up to 10 mm rostral to the lesion site. Such axons generally displayed swollen or bulbous terminal regions (Figs. 2 and 3). The possibilities that appearances such as those illustrated in Figs. 2 and 3 represent varicosities along the course of longer regenerating axons, or of spared axons, rather than terminal regions were considered but eliminated by careful through-focus microscopic examination of serial sections. Labeled regenerating axons which had extended more than a few hundred micrometers rostral to the lesion site were not common and were found both within the degenerating dorsal columns (Figs. 2 and 3) and, more often, in abnormal rostral positions. Thus they were occasionally observed in gray matter (not illustrated) or more commonly in or immediately below the meninges (Fig. 1B) or in dorsal roots rostral to the lesion (Fig. 1C). Labeled fibers in the dorsal roots rostral to the lesion could not be traced through the dorsal root entry zone into the cord. It is therefore probable that they entered the roots directly from the lesion site or grew along the surface of the cord in an ectopic position and subsequently into and along dorsal roots (but not beyond the dorsal root entry zone). The number of labeled fibers found on the surface of the spinal cord and in the dorsal roots around and rostral to the lesion was always much smaller than the number of axons in the lesion site. The sprouting response was much less marked in vehicle controls and least in vehicle control animals in which the sciatic nerve was not crushed.

In addition, in some animals we observed labeled axons that extended along the entire dorsal column. We interpret these long rostrally projecting sensory axons as spared fibers (i.e., axons not destroyed by the initial injury; see Discussion). In 30% of FK506-treated animals in Series I, spared dorsal column axons were found extending through the lesion cavity (Fig. 1D), along the ipsilateral dorsal column (Fig. 1E), to terminate unilaterally at their normal destination in the nucleus gracilis of the medulla oblongata (Figs. 1F and 4). In the remaining 70% of FK506-treated subjects there were no spared fibres present within the degenerating dorsal columns. In contrast, only 8% of control animals were found to have spared fibers. None of the animals treated with GPI 1046 had spared fibers. The difference between FK506- and GPI 1046-treated animals was significant ($P < 0.05$, FET). Regenerating axons with terminal swellings in the dorsal columns more than 300μm or so rostral to the lesion site were only detected in animals in which spared fibers were present and were usually observed in close association with small bundles of or individual spared axons (Fig. 3).

Series II

Among the 54 animals entered into the randomized protocol, spared fibers were found in 29% of those treated with FK506, in contrast to 8% of controls. This difference was significant ($P < 0.05$, one-tailed FET).

Since the results in Series I and Series II were essentially the same, data from both groups were combined to give an overall result for all 71 animals treated with FK506 and all 50 control animals (Tables 2 and 3). The difference between FK506-treated and control groups was clearly significant ($P < 0.01$, χ^2 test). Although spared fibers were more frequent with a lower dose of FK506 (31% compared to 25% with the higher dose; see Table 2), the difference did not reach statistical significance.

The administration of MP had no significant effect on the frequency of spared fibres (21 vs 20%; Table 3). Furthermore, when treatment groups were further subdivided, it was apparent that both FK506 alone and

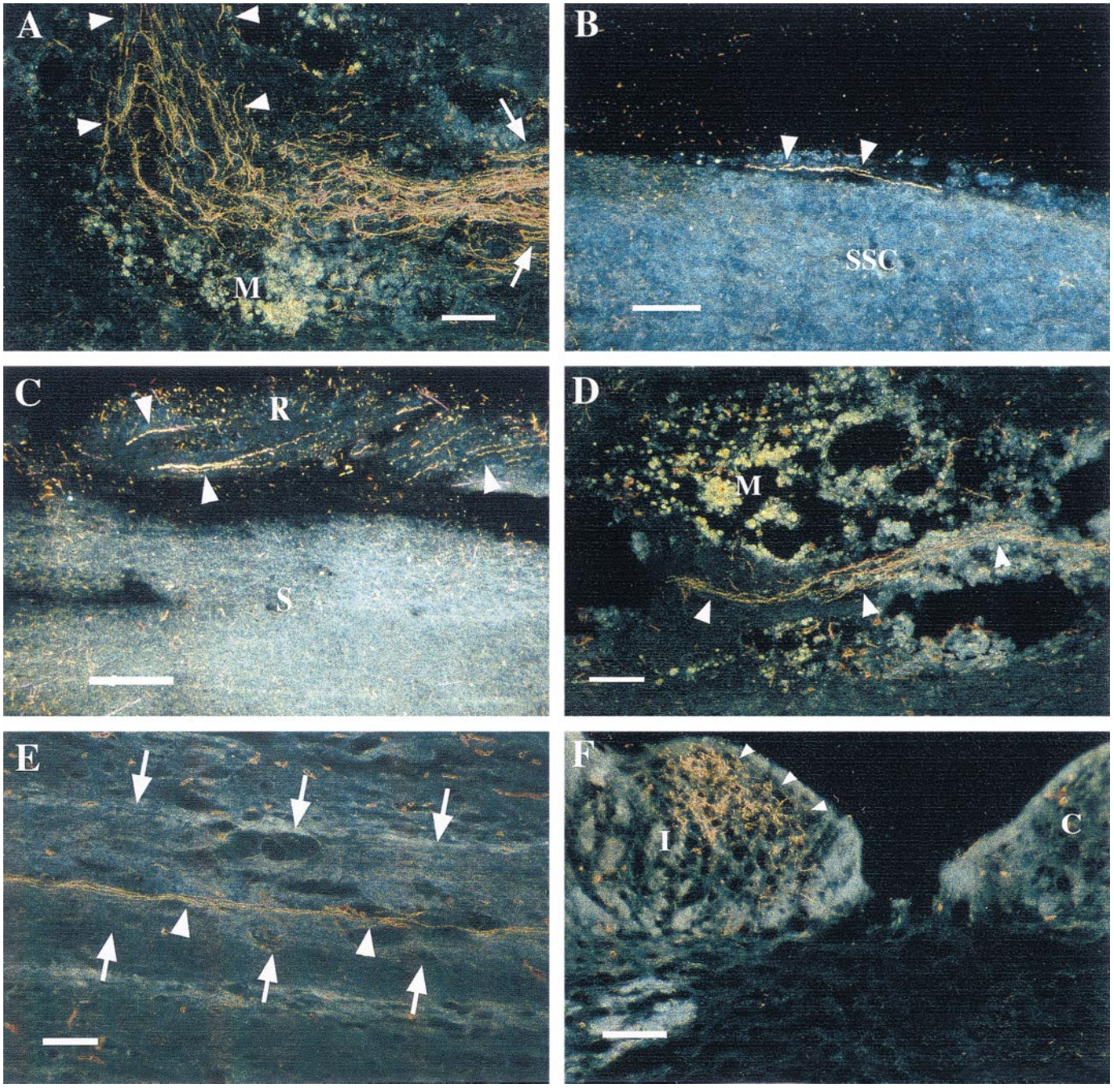

FIG. 1. Dark field photomicrographs of longitudinal horizontal (A–E) or transverse (F) sections of adult rat spinal cord 2–8 weeks after bilateral dorsal column injury and 2 days after unilateral injection of CT-HRP into the sciatic nerve to label dorsal column axons on the left side. All of the illustrations are from animals treated postoperatively with FK506. (A) Regenerating axons extending from the caudal dorsal column (arrows) into a lesion cavity containing debris-laden macrophages (M) and then out of the lesion (between arrowheads) toward the surface of the spinal cord. Three weeks after dorsal column injury and sciatic nerve crush. (Bar, 100μm.) (B) Labeled axons (arrowheads) in an ectopic position 2 mm rostral to the lesion, on the surface of the thoracic cord, probably within the meninges. SSC, superficial part of the degenerating dorsal column of the spinal cord. Three weeks after injury. (Bar, 100 μm.) (C) Ectopic axons within a thoracic dorsal root (R), close to the dorsal root entry zone, 2 mm rostral to the lesion. S, superficial part of the degenerating dorsal column. Two weeks after dorsal column injury and sciatic nerve crush. (Bar, 200 μm.) (D) Labeled axons (arrowheads) passing through a large lesion cavity containing debris-laden macrophages. Three weeks after injury. (Bar, 100 μm.) (E) Spared sensory fibers (arrowheads) approximately 8 mm rostral to the lesion within the degenerating dorsal columns (outlined by arrows). The labeled axons are confined to the left side. Eight weeks after injury. (Bar, 50 μm.) (F) Labeled axons and terminals (arrowheads) in the nucleus gracilis ipsilateral to the CT-HRP-injected sciatic nerve. Four weeks after injury. (Bar, 100 μm.)

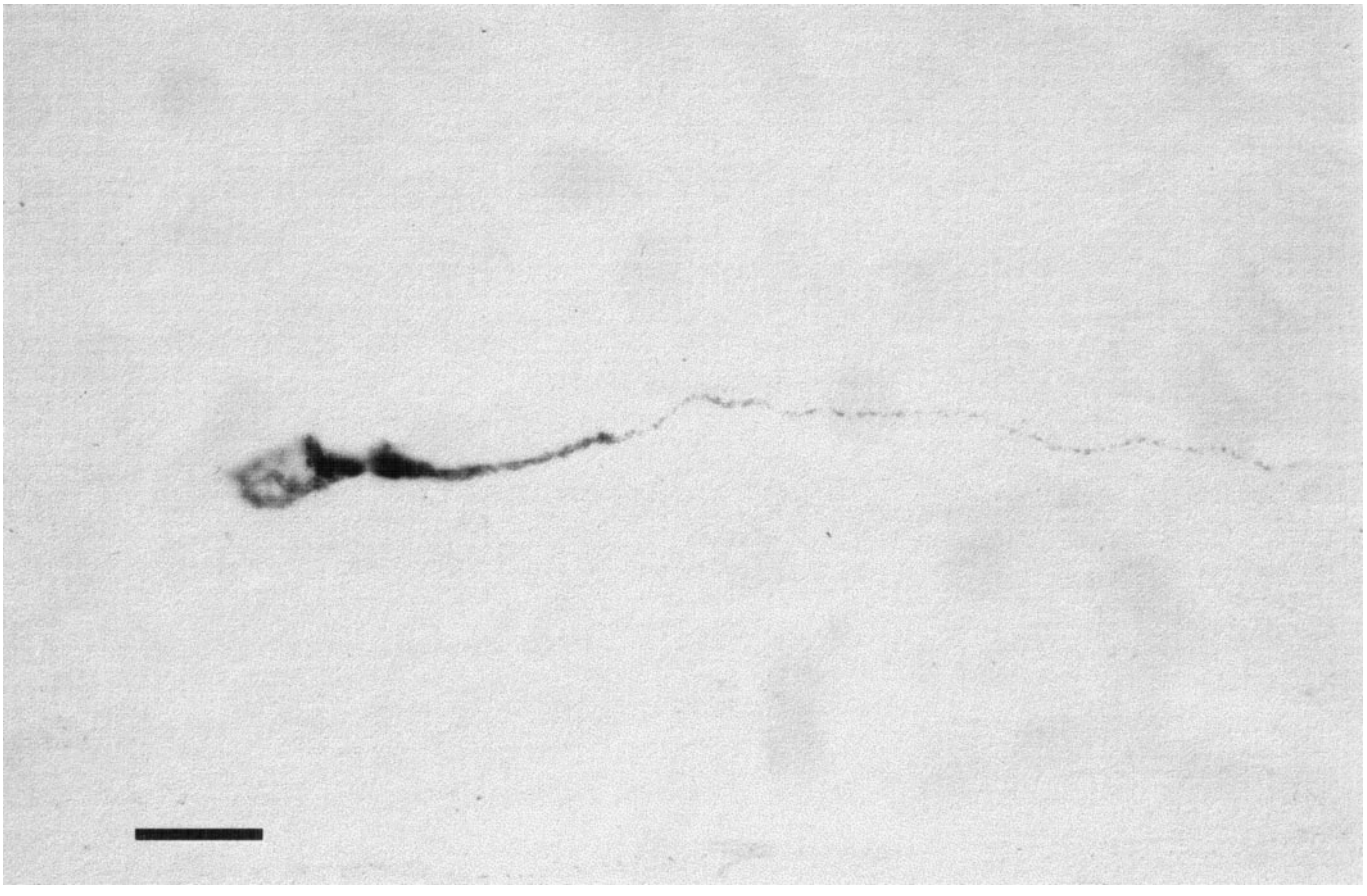

FIG. 2. The swollen terminal of a regenerating axon growing in a rostral direction within a degenerating dorsal column. Four weeks after dorsal column injury and sciatic nerve crush with FK506 treatment. (Bar, 25 μm.)

FK506 in combination with MP resulted in a higher percentage of animals having spared fibers than did MP treatment alone (Table 3).

Animals assessed at different survival periods after dorsal column injury were classified into three groups: those examined at 15 days or less, those examined at 16–40 days, and those examined at more than 40 days after operation. The percentage of animals with spared fibers did not change significantly in groups with progressively longer survival times, whether or not treated with FK506. Even in the earliest survival period, spared fibers could be traced for the entire length of the rostral dorsal columns and there was no evidence of a "front" of axons terminating in the cord rostral to the site of transection; indeed regenerating axons (terminal regions of labeled axons) were extremely rare more than 5 mm beyond the lesion. Similarly there was no evidence that sciatic nerve crush influenced the percentage of animals with spared fibers (Table 2), in contrast to local regeneration immediately rostral to the lesion site, which was contingent on peripheral nerve injury. Whereas FK506 treatment was clearly associated with the presence of spared fibers, whether or not FK506 had been administered appeared to make no difference to lesion size, defined as the product of the maximum caudorostral length and maximum width of the lesion. The median area of lesions in FK506-treated rats was 3.63 mm^2 (interquartile range 1.8 to 5.7 mm^2), and in control rats, 4.65 mm^2 (interquartile range 3.4 to 5.6 mm^2) (Mann–Whitney test, $P = 0.4$). A similar analysis provided no evidence that lesion size was significantly affected by whether or not MP had been administered.

Series III

In none of the four animals in which larger spinal cord lesions had been made using biopsy forceps was evidence of spared fibers found.

DISCUSSION

The main finding of this study is that FK506 treatment promoted the survival of ascending dorsal column axons following spinal cord injury, whereas GPI 1046 and MP had no such effect. In addition, FK506 treat-

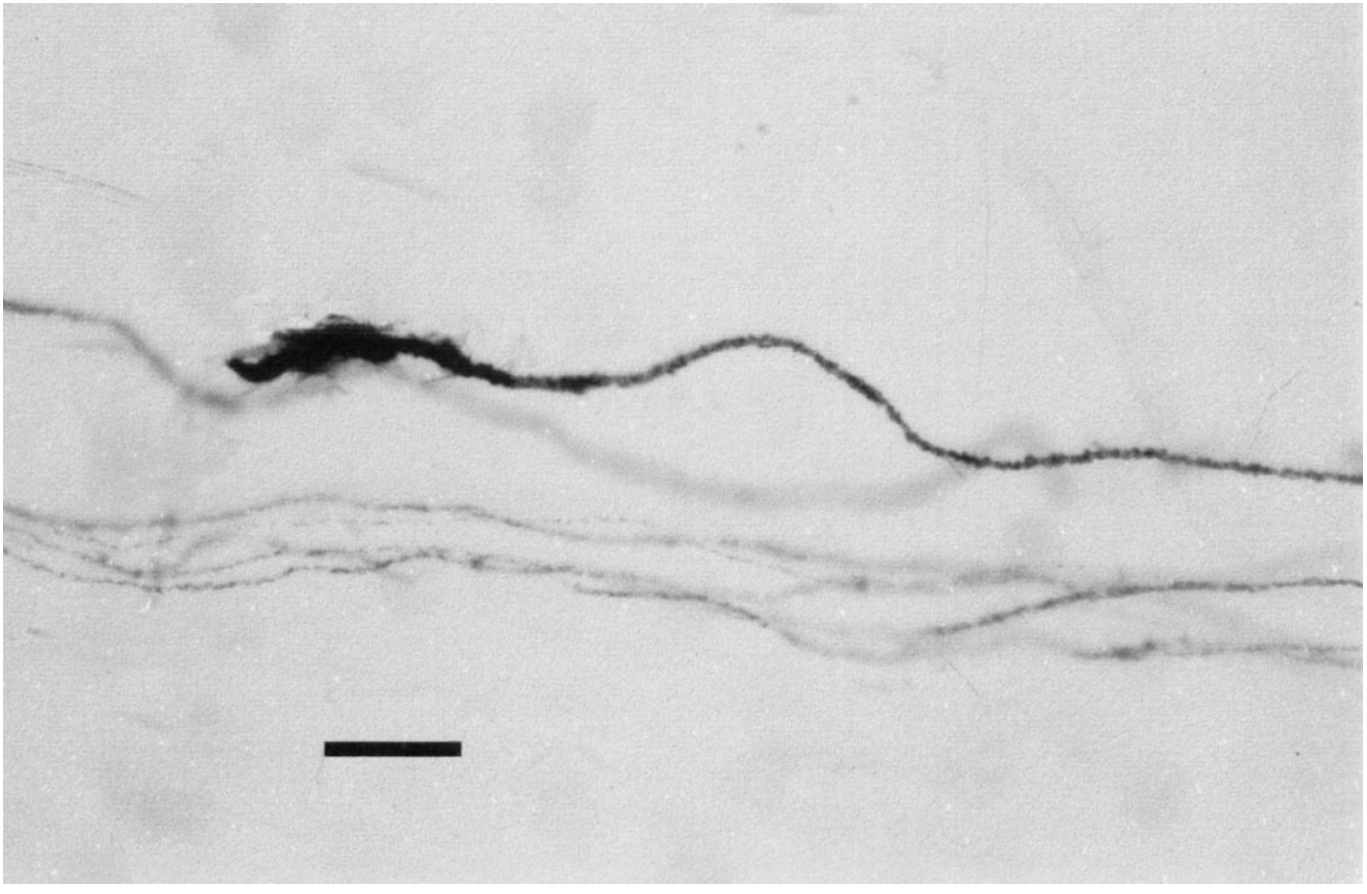

FIG. 3. The swollen terminal of a regenerating axon within a fascicle of several long rostrally projecting sensory fibers that are presumably spared 4 weeks after dorsal column and sciatic nerve injury with FK506 treatment. (Bar, 25 μm.)

ment, in combination with a sciatic nerve crush, promoted massive axonal sprouting at the lesion site and limited regeneration of severed primary sensory axons rostral to the lesion site.

Long Rostrally Projecting Labeled Axons in the Dorsal Columns Represent Spared Axons

Labeled axons extending rostrally along the dorsal columns were most commonly found in animals treated with FK506. We initially interpreted them as being regenerated axons but that is improbable for the following reasons. (i) A front of regenerating axon terminals within the rostral dorsal columns was not seen in any experiment. (ii) Long, rostrally projecting labeled axons were present even at 7–15 days after injury (Fig. 4). Such axons extended approximately 5–6 cm from the site of the lesion to the nucleus gracilis in the medulla. Even if the latent period is discounted (i.e., the time required for the initiation of the sprouting response and the regeneration of axons across the lesion), rates of axonal elongation up to 8 mm/day would be required for this result to be explained by regeneration of severed axons. Such rates of growth are more rapid even than those of the fastest regenerating peripheral nerve fibers (29, 55). It might be argued that FK506 treatment not only promotes regeneration but also increases the rate of regeneration of the dorsal column axons. Indeed, FK506 treatment does increase the growth rate of peripheral axons following sciatic nerve crush (17, 18, 20, 57). However, the maximum rate of regeneration of FK506-stimulated peripheral axons is still only 5.1 mm/day. It is therefore extremely unlikely that regenerating axons could have reached the DCN, 5–6 cm from the lesion site, within 7 days. (iii) When animals were classified according to the length of their postoperative survival (Table 2) there was no progressive increase in the percentage with long rostrally projecting labeled axons or in the numbers of labeled fibers within individual animals, as might be expected if such fibers were regenerated/regenerating axons. (iv) The long labeled rostrally projecting fibers were consistently straight in course and unilateral in distribution. If these axons were regenerated, a less orderly and lateralized pattern would be expected. (v) Finally, such fibers were not present in any of the four animals in which larger lesions had been made, involving the

TABLE 2

Summary of Effects of FK506 and Treatment Variables

	FK506 Series I + Series II		Controls Series I + Series II	
	No. of animals	No. (%) with LRS fibers	No. of animals	No. (%) with LRS fibers
Dose				
High	12	3 (25%)	50	4 (8%)
Low	59	18 (31%)		
Postoperative survival				
15 days or less	35	9 (26%)	32	3 (9%)
16–40 days	25	9 (36%)	9	1 (11%)
More than 40 days	11	3 (27%)	9	0
Sciatic nerve injury				
Unilateral crush	54	16 (30%)	35	3 (9%)
No crush	17	5 (29%)	15	1 (7%)

Note. Because the group of animals treated with a high dose of FK506 was small, the apparent difference between the low- and high-dose groups is not statistically significant. Grouping of the FK506-treated and control animals into three arbitrary postoperative survival subgroups (15 days or less; 16–40 days; and more than 40 days) shows that the percentage of animals with spared fibers did not increase at longer survival times. Whether or not an animal had received a unilateral sciatic nerve crush appeared not to affect the percentage of animals with spared fibers but did affect sprouting and regeneration through and beyond the lesion site which only occurred in animals with sciatic nerve crush (data not shown).

TABLE 3

Effects of Supplementing FK506 Treatment with Methylprednisolone

Treatment groups	No. in group	Percentage with spared fibers	Significance
All experiments (N = 121)			
+MP	57	21	n.s.
−MP	64	20	
Subcategories			
FK506 + MP	33	33	
MP alone	24	4	$P < 0.05$ (FET)
FK506	38	26	$P < 0.05$ (FET)

Note. The results summarized in this table are taken from all Series I and II animals except the GPI 1046 treatment groups. The effects of methylprednisolone (MP) are first compared across the entire group; the difference in the percentage of animals in the MP-treated and nontreated animals is not significant (χ^2 test). Another indication that MP has relatively little effect in this model of spinal cord injury is that MP treatment had no significant effect on lesion size (data not shown). In the bottom half of the table, the effects of MP alone are compared with the effects of MP + FK506 and FK506 alone. In both cases the difference with respect to the percentage of animals with spared fibers is significant. FET, Fisher's exact test.

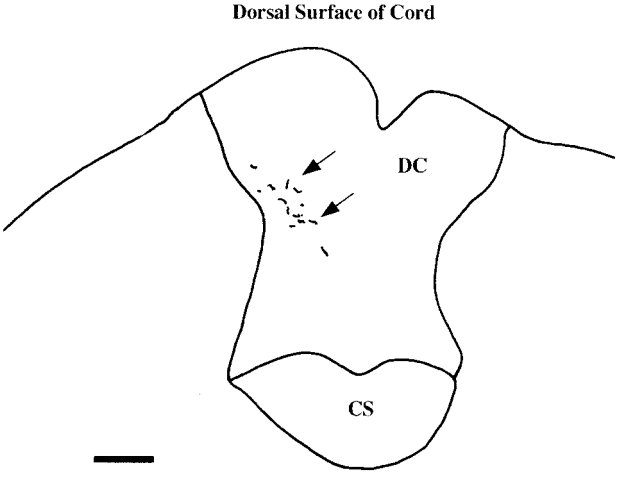

FIG. 4. Line drawing of a transverse section of the dorsal portion of upper thoracic spinal cord showing long rostrally projecting labeled fibers (arrows) at an intermediate depth within the dorsal columns 2 weeks after injury with FK506 treatment. Such fibers might have been spared by being pushed aside as the needle was swept across the dorsal columns. In other cases long rostrally projecting sensory fibres were present in the deepest parts of the dorsal columns, presumably spared because the incision did not extend to the ventral extremity of the dorsal columns. CS, corticospinal tract; DC, dorsal column. (Bar, 100 μm.)

complete destruction of the dorsal columns at the injury site. Regenerating axons might be expected to grow around or across even large lesions but spared axons could not be present in such experiments.

Immunophilin Ligands Promote Axonal Sprouting in Spinal Injuries but Not Long-Distance Regeneration

It therefore appears that the immunophilin ligands used in this study have two distinguishable effects on injured dorsal column axons. First, FK506 and to a lesser extent GPI 1046 stimulate postinjury sprouting of injured axons and in the case of FK506 at least, a limited amount of regenerative growth beyond the lesion, but only of relatively few axons and only over relatively short distances. This finding stands in contrast to a previous study (38, 52) in which cyclosporin A, another immunophilin ligand, was reported to promote long-distance regeneration of corticospinal axons in the spinal cord. The rare labeled axons with terminal swellings identified in the dorsal columns more than a few millimeters rostral to the lesion were found only in association with spared fibers extending all the way to the nucleus gracilis. This observation suggests that the spared fibers may have provided some form of support to the regenerating axons. Nonetheless, the results suggest that the spinal cord around the lesion site, in particular, the degenerate dorsal columns, does not constitute a permissive environment for axonal regeneration; the dorsal column axons were induced to regenerate vigorously by sciatic nerve injury combined

with immunophilin treatment, but few of the axons reentered the spinal cord tissue rostral to the lesion. A strong sprouting response by injured neurons does not appear to be capable of producing long-distance regeneration of axons in the cord beyond the lesion. The enhanced sprouting response produced by FK506 in our experiments may be partly the result of similar processes to those which produced enhanced recovery after photothrombotic lesions (32). Increased GAP-43 activity as a result of the inhibition of calcineurin would be expected to enhance axonal sprouting (1). However, the conditioning sciatic nerve crush, which combined with FK506 treatment to produce maximal sprouting, would be expected to greatly increase the expression of GAP-43 mRNA in the primary sensory neurons we have studied, irrespective of drug treatment (8). The regenerating axons were arranged as an apparently disorderly network in the lesion site except where they formed bundles orientated toward the surface of the cord. Presumably there is no tropic attraction to the rostral side of the lesion (unlike the situation in severed peripheral nerves) but such an attraction may exist toward structures such as dorsal roots at the cord surface. We do not know where the axons which apparently grew out from the lesion site ended. Relatively few labeled axons were found on the meninges or in dorsal roots rostral to the lesion, but it is possible that axons could have been attracted to dorsal roots near the lesion site and grown in larger numbers centrifugally toward the dorsal root ganglia.

FK 506 Promotes Axonal Sparing Following Spinal Injury

The second and very significant effect of FK506 is to promote the sparing of axons not severed in the initial injury. We hypothesize that in some animals the dorsal column lesions were incomplete, but that in the absence of FK506 treatment the axons that escaped transection were usually destroyed by the secondary degenerative changes in the tissue surrounding the site of primary trauma. Secondary degeneration following an initial lesion has been reported in many studies (2, 3, 10, 14, 15, 59, 60, 61) and our finding that the median axial length of lesions produced by the transverse linear incision was over 3 mm suggests that significant secondary injury did indeed occur in our experiments. Furthermore, spared axons could be traced through the lesion, surrounded on all sides by clear evidence of pathology, suggesting that the secondary injury extended the boundary of the lesion well beyond the initial incision.

Spared fibers were significantly more common among the FK506-treated animals than in the controls; 30% of FK506-treated animals (21 of 71 animals in Series I and II) showed spared fibers versus 8% (4 of 50 control animals in Series II and II). Not only is this result highly significant in itself but it almost certainly underrepresents the effect of FK506 on axonal survival, for the following reason. It is axiomatic that for an axon-sparing effect of FK506 to be demonstrated, the initial lesion of the dorsal column must be incomplete (in accordance with which are the uniformly negative findings in the Series III rats with undoubtedly complete lesions). Among the 71 FK506-treated animals it is highly probable that some would have had complete dorsal column transections, thus precluding the possibility that drug treatment could reduce secondary axonal destruction. The proportion of such animals is unknown but if even a small number was included among the 71 treated animals, the percentage of treated animals showing an effect of FK506 on survival of spared axons would be much higher than 30%.

Mechanisms by Which FK506 May Produce Axonal Sparing

The mechanisms for this protective effect are obscure. It is possible that the effect of FK506 on axonal sparing could be indirect, resulting from its immunosuppressant actions, since spinal injury may be associated with an immune response that is reduced by immunosuppression (38, 52). In the present study, however, a dose of 0.5 mg/kg elicited a sparing effect whereas such a dose was not sufficient to prevent immune rejection of neuronal transplants in rats (41). Another possible indirect route for an effect on axonal sparing could be via an effect on glia. Unfortunately, little is known of the levels of FKBP12 in glia or the effects of FK506 on glial cells. It is also possible and indeed more likely that the enhanced ability of axons to survive secondary injury might be a direct effect, i.e., one mediated by the binding of FK506 to FKBP12 within neurons. A direct protective action on axons might be expected to increase axonal survival or improve functional outcome (31) while having a lesser effect on lesion size, and indeed we found that lesion size did not differ significantly between control and FK506-treated rats, consistent with our finding that the effects of FK506 on lesion size are less marked than on axonal sparing or indeed on functional outcome. It is questionable, however, whether such measurements, in peroxidase-reacted horizontal sections, would have been capable of detecting relatively small difference in lesion size.

GPI 1046 did not appear to protect axons from secondary injury in our study, at doses which have been reported by others to stimulate axonal sprouting within the brain (51). This would suggest that a reduction in calcineurin phosphatase activity may be important in mediating the protective effects of FK506 on dorsal column axons, since the GPI 1046/FKBP12 complex lacks a calcineurin-binding effector domain. Such a mechanism would be compatible with the finding that calcineurin is colocalized with FKBP12 within the CNS

(50). Moreover, rapamycin, which competes with FK506 for a common binding site on FKBP12 but does not inhibit calcineurin activity, blocks the neuroprotective actions of FK506 on the cerebral cortex following ischemic injury (44). A number of molecular mechanisms have been proposed to result in cellular neuroprotective effects of FK506. Calcineurin inhibition might protect neurons by increasing the phosphorylation of substrate molecules such as GAP-43, which is well established as playing an important role in axon regeneration in the PNS and CNS (45), or by preventing the dephosphorylation of nitric oxide synthase, thereby diminishing its catalytic activity (11) and reducing the formation of nitric oxide (NO), which is thought to be a key mediator of glutamate-induced toxicity (12). Furthermore, NO itself can also increase the formation of other free radical species (27) leading to further cellular damage, and moreover FK506 is known to reduce superoxide formation in neutrophils (36). Recently evidence has emerged that *in vitro* protective effects of FK 506 on neurons might be mediated by a reduction in the active form of the nuclear protein c-Jun (6, 23). Previous studies have suggested that cyclosporin A has a protective effects on neurons (26, 33, 54), provided that it is given in adequate doses to ensure sufficient concentrations within the CNS.

Axonal Sparing in Experimental Spinal Cord Injury

The neurological effects of spinal cord injury are caused, in large part, by damage to long-tract axons caused by the initial trauma and exacerbated by secondary effects. The concept that drug therapy might reduce such axonal loss is fundamental to most pharmacological approaches to minimize the consequences of spinal cord injury. However, many studies have only examined the numbers of surviving neurons or lesion size, sometimes predominantly involving the gray matter. Others have demonstrated that treatment can increase the numbers of spared myelinated axons (2, 3), but have not distinguished between protective effects on the entire neuron and the axon alone. Since transected dorsal column axons originate from medium to large DRG neurons, which can survive axonal injury even without treatment (8, 21), the effects of FK506 on axonal sparing must be independent of viability of the cell body. To our knowledge, evidence for this basic distinction in the possible effects of neuroprotective drugs has never been presented or considered in the context of spinal cord injury. The concept of treatment-induced axonal sparing is also pertinent to the study of neuroregeneration since the histological and functional outcomes of these two neurobiological processes are potentially similar. Axonal sparing and regeneration must therefore be meticulously distinguished if regeneration over a long distance is to be demonstrated, particularly since experimental strategies using grafts to promote regeneration commonly involve the administration of immunosuppressive drugs.

Clinical Implications

Axonal transection is a critical factor in many neurological conditions including head injury and strokes of the internal capsule, as well as spinal cord injury. Any drug treatment that reduced axonal loss would clearly have the potential to be useful in the treatment of these conditions as well. Several issues, however, remain to be addressed. Although we have studied only one population of axons within the spinal cord and have no data to confirm that the spared fibres are fully functional, these findings raise hopes that the FK506 could be used in the treatment of spinal injury in humans. We remain uncertain, however, about such issues as how soon after injury the first dose of FK506 needs to be administered to produce a protective effect, nor do we know the optimum duration of treatment or the optimum dose. At present, MP is the only drug routinely administered after acute spinal cord injury (4, 5, 22). Previous laboratory studies of the effects of MP on experimental spinal cord injuries have produced strong but not entirely consistent evidence of its ability to reduce the secondary degeneration that follows the primary lesion (2, 9, 14). We could find no evidence that MP enhances axonal sparing; our results suggest that the combination of FK506 and MP is significantly more effective than MP alone in protecting axons, but not significantly more effective than FK506 alone. However, one of the possible explanations of the variability of the results of studies of experimental spinal cord injury is the variety of models used. The model we have used in the present study was initially chosen to investigate the possible effects of immunophilin ligands on axonal regeneration and may not be capable of, or suitable for, detecting all types of neuroprotective effects. Furthermore, MP would have been more likely to produce a neuroprotective effect when given in multiple doses by intravenous administration within the first 24 h (5, 59); although our experiments produced no evidence of a neuroprotective effect of MP, they were not designed to do so.

Immunosuppressants are already commonly prescribed to prevent allograft rejection and their pharmacology is therefore well established (43). Although in this study FK506 was administered throughout the postoperative period of up to 12 weeks, in order to stimulate regeneration, there is already experimental evidence that spinal cord function is improved by only three doses of FK506 given within the first 48 h after injury (31, 32) and it is, therefore, likely that the complications of long-term administration may be avoidable. Furthermore, a temporal therapeutic window appears to exist, since intravenous FK506 will reduce cortical damage in a model of cerebrovascular accident

when administered 60–120 min after the injury, although it will not do so if administration is delayed until 180 min after injury (7). The findings of this study with regard to the neuroprotective effects of FK506 following spinal cord injury may have potentially important clinical implications in spinal cord injury, head injury, cerebrovascular accidents, and several other neurological conditions.

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