

**Efectes de l'FK506 en la regeneració després de reseccions  
nervioses severes reparades mitjançant autoempelts o tubs  
de silicona**

*Effects of FK506 on nerve regeneration and reinnervation  
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*Effects of continuous administration of FK506 on  
regeneration through silicone guides in a 6 mm gap in the  
sciatic nerve of the mouse (addenda)*

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**ABSTRACT:** We compared the effects of FK506 administration on regeneration and reinnervation after sciatic nerve resection and repair with an autologous graft or with a silicone tube leaving a 6-mm gap in the mouse. Functional reinnervation was assessed by noninvasive methods to determine recovery of motor, sensory, and sweating functions in the hindpaw over 4 months after operation. Morphometric analysis of the regenerated nerves was performed at the end of follow-up. The nerve graft allowed for faster and higher levels of reinnervation in the four functions tested than silicone tube repair. Treatment with FK506 (for the first 9 weeks only) resulted in a slight, although not significant, improvement of the onset of reinnervation and of the maximal degree of recovery achieved after autografting. The recovery of pain sensibility and of the compound nerve action potentials in the digital nerves, which directly depend on axonal regeneration, showed better progression with FK506 than reinnervation of muscles and sweat glands, which require reestablishment of synaptic contacts with target cells. The myelinated fibers in the regenerated nerve showed a more mature appearance in the FK506-treated rats. However, FK506 showed a marginal effect in situations in which regeneration was limited, as in a silicone tube bridging a 6-mm gap in the mouse sciatic nerve. In conclusion, treatment with FK506 improved the rate of functional recovery after nerve resection and autograft repair.

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## EFFECTS OF FK506 ON NERVE REGENERATION AND REINNERVATION AFTER GRAFT OR TUBE REPAIR OF LONG NERVE GAPS

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**F**ollowing severe peripheral nerve injuries resulting in loss of continuity and an interstump gap, the preferred method of repair consists of autologous nerve grafting.<sup>32</sup> The use of an autologous graft has some disadvantages, such as the need of a second surgical step, loss of donor nerve function, limited supply of donor nerves, and the mismatch between nerve and grafts. The use of nerve allografts has not been en-

couraging because regeneration is low or absent due to immunological rejection.<sup>2,9,40,56</sup> Immunosuppressive therapy is needed to prevent graft rejection, but its secondary complications (e.g., nephrotoxicity) usually overcome the benefits, and rejection occurs after discontinuation of immunosuppression with deleterious effects on the regenerated axons and the functional recovery achieved.<sup>2,9,56</sup> Tubulization (the implantation of a biocompatible nerve guide to bridge the gap) is an alternative to the repair of transected nerves. Tube repair provides physical guidance to the regenerating axons, prevents axonal escape into the surrounding tissues, and creates a local microenvironment whereby trophic factors from the injured nerve may become concentrated and facilitate axonal growth. However, the success of

**Abbreviations:** AG, autologous graft; CMAP, compound muscle action potential; CNAP, compound nerve action potential; dpo, day postoperation; FK, FK506; FKBP, FK506 binding protein; FRI, functional recovery index; PP, pinprick test; SFI, sciatic functional index; SG, sweat gland; SIL, silicone tube

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simple tubulization is dependent upon the length of the gap, usually failing when bridging relatively long gaps (i.e., 6 mm in the mouse<sup>8,29,41</sup> or 15 mm in the rat<sup>34,46,55</sup>).

The development of therapeutic agents that can augment the rate of nerve regeneration and the degree of functional restitution after injury is important. By accelerating axonal regeneration, the consequences of denervation on target organs (muscle atrophy, loss of sensory receptors, denervation hypersensitivity) would be diminished and functional recovery (following reinnervation) would be more efficient. Procedures that increase the regeneration and reinnervation rate such as nimodipine treatment<sup>1</sup> or surgical repair with predegenerated grafts,<sup>7</sup> have been shown not only to shorten the time until muscle and sensory reinnervation but also to reduce polyinnervation and misdirected reinnervation.

FK506 is a potent immunosuppressant used primarily for the prevention of rejection after solid-organ transplantation.<sup>19,44,48</sup> The immunosuppression caused by FK506 is primarily mediated by binding to the intracellular immunophilin FK506-binding protein-12 (FKBP-12) and subsequent calcineurin inhibition, which in turn causes inhibition of cytokine synthesis and immunosuppression.<sup>45</sup> In addition, immunophilin ligands have promising actions on the nervous system. In different experimental models,<sup>21,22</sup> FK506 has been shown to enhance neurite outgrowth *in vitro*,<sup>28,35</sup> and to exert neuroprotective effects in degenerative diseases,<sup>11,31</sup> ischemic insults,<sup>47</sup> and spinal cord injuries.<sup>5,37</sup> Furthermore, treatment with FK506 has been reported to enhance the rate of axonal regeneration in the rat after crush injury to the sciatic nerve,<sup>24–26,33,54</sup> and in a peripheral nerve grafted into the injured spinal cord.<sup>53</sup> Less consistent, however, have been studies examining more severe injuries repaired by nerve grafting.<sup>4,15,17</sup> Nevertheless, FK506 has recently been found to speed functional recovery in two successful hand transplantations.<sup>16,30</sup> In the present study, we evaluated the effect of FK506 administration on regeneration and reinnervation of distal target organs after a 6-mm gap resection of the mouse sciatic nerve in two repair models: autologous nerve graft, which allows for effective regeneration in all cases; and silicone tube repair, in which regeneration fails in most cases in the mouse with a 6-mm gap (thus, a distance considered limiting for regeneration through silicone tubes in this experimental model).<sup>8,29,41</sup>

## MATERIALS AND METHODS

**Surgical Procedure and Treatment.** Female Swiss OF1 mice, aged 2.5 months, were divided in four

groups: untreated autograft repair (AG;  $n = 8$ ), FK506-treated autograft repair (AG+FK;  $n = 5$ ); untreated silicone tube (SIL;  $n = 12$ ); and FK506-treated silicone tube (SIL+FK;  $n = 5$ ). Operations were performed under pentobarbital anesthesia (60 mg/kg intraperitoneally) in all four groups. First, the saphenous nerve was cut in the femoral space and a long segment of the distal stump removed to prevent regeneration and collateral reinnervation of the denervated sciatic territory. The sciatic nerve was then exposed at the mid thigh, transected at a constant point 45 mm from the tip of the third digit, and a segment of the distal stump resected to leave a final gap of 6 mm. In the two AG groups, the resected nerve segment was autografted between the stumps with two 10-0 monofilament epineural sutures at the proximal and distal suture lines. In the two SIL groups, the nerve stumps were fixed 1 mm inside the ends of a silicone tube (1 mm i.d. and 0.25 mm wall thickness; Dicoinsa, Barcelona, Spain) using one 10-0 suture stitch at each end, leaving a measured interstump gap of 6 mm. Once implanted, the tubes were filled with sterile saline solution. The wound was sutured and disinfected with povidone-iodine. To avoid autotomy after denervation, animals were pretreated with amitriptyline.<sup>38</sup> The experimental procedures were approved by the ethics committee of our institution. Mice in groups AG+FK and SIL+FK were given subcutaneous injections (5 days/week) in the back of FK506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) diluted in saline solution at a dosage of 5 mg/kg<sup>54</sup> beginning on the first day postoperation (dpo) until 5 weeks. The dose was reduced to 3 mg/kg for the following 4 weeks. Drug administration was discontinued after a total of 9 weeks of the 4-month study due to limited availability of the compound.

**Functional Evaluation.** Functional reinnervation of target organs in the hindpaw was tested at several intervals after denervation up to 4 months by means of a battery of noninvasive neurophysiological tests.<sup>38</sup> Regeneration of large myelinated nerve fibers was assessed by nerve conduction tests. With the animals under light anesthesia (30 mg/kg intraperitoneally of pentobarbital), the sciatic nerve was stimulated percutaneously through a pair of needle electrodes near the sciatic notch and the compound muscle action potential (CMAP) recorded from plantar muscles with microneedle electrodes. The active electrode was inserted into the third metatarsal space and the reference electrode in the tip of the fourth digit. The evoked CMAP was amplified and displayed on a digital oscilloscope (Tektronix

420, Tektronix, Wilsonville, OR) at settings appropriate to measure the amplitude from baseline to the maximal negative peak and the latency from stimulus to the onset of the first negative deflection. For sensory nerve conduction tests, the active electrode was placed at the medial side of the base and the reference electrode placed at the tip of the fourth toe (near the digital nerves). Compound nerve action potentials (CNAPs), evoked by proximal electrical stimulation of the sciatic nerve, were displayed on the oscilloscope to measure their amplitude from baseline to peak and latency to onset. Reinnervation by small nerve fibers was evaluated by testing sympathetic sudomotor and nociceptive responses. Sweating was stimulated by injection of pilocarpine nitrate (5 mg/kg subcutaneously). Ten minutes later a silicone material (Elasticon, Kerr Co., Romulus, MI) was applied over the plantar surface of the hindpaw. As the material hardened, it retained the impressions made by the sweat droplets emerging from individual sweat glands (SGs). The number and distribution of SG impressions were determined under a dissecting microscope using transillumination. Recovery of pain sensitivity was tested by light pricking with a needle under a dissecting microscope in five areas, from the most proximal pawpad to the tip of the second digit on the plantar aspect of the denervated paw. A pinprick (PP) score was assigned from no response (0), reduced or inconsistent responses (1), to normal reaction (2) in each area tested in comparison with responses in the contralateral intact paw.

For normalization of the data, values obtained after operation were expressed as a percentage of

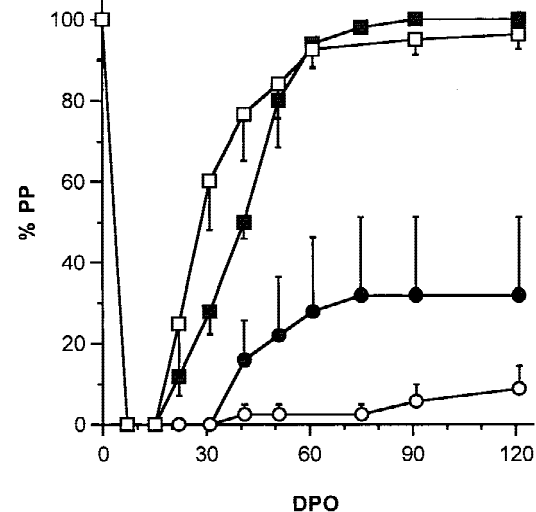
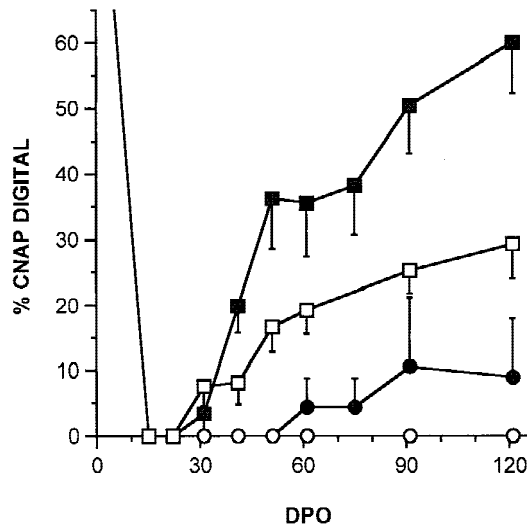
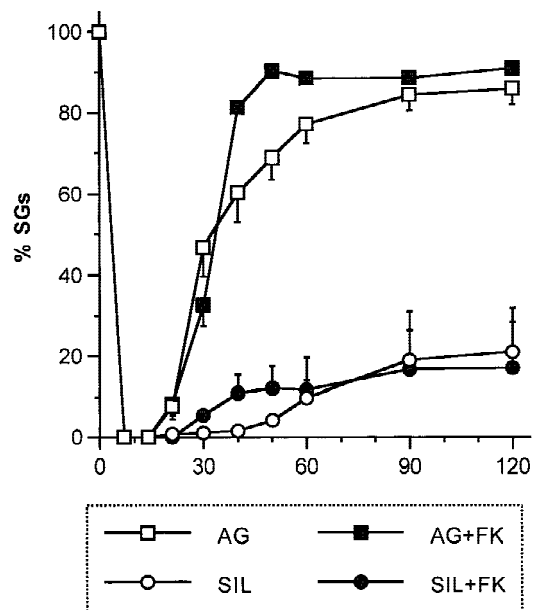
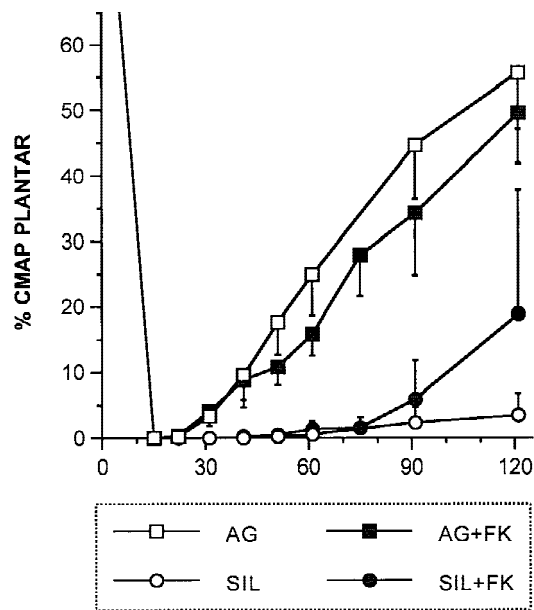
preoperative values for each individual mouse and plotted against time. For each functional parameter indicative of the degree of reinnervation (CMAP and CNAP amplitudes, number of SGs, and PP score), we determined the day of the first response after denervation (when there was no reinnervation, arbitrary values of 150 days were assigned) and the percentage of maximal recovery achieved during follow-up (with zero values for mice without reinnervation), and calculated an overall functional recovery index (FRI) representing the area under the reinnervation curve. These calculations (see Table 1) include all operated mice in each group, taking into account both the rate of success and the degree of recovery achieved. With respect to the onset of reinnervation, plots in Figures 1 and 2 give an indication of the time when functional recovery started in any animal of the group, whereas values in Table 1 refer to the mean day for all animals, including those without recordable reinnervation. The degree of recovery shown in the figures and by the maximal percentage in Table 1 also includes values from all mice, with zero values for those without responses.

The walking track test was carried out prior to surgery, and at 90 and 120 dpo to assess the recovery of locomotor function. The plantar surface of the hindpaws was painted with acrylic paint and the mice allowed to walk along a narrow corridor with white paper on its base. The print length, and the distances between the first and fifth toes and the second to fourth toes, were measured with a precision device on footprints of the operated and intact paws. The three parameters were combined in the sciatic func-

**Table 1.** Mean onset day of reinnervation, maximal percentage of reinnervation, and functional recovery index for each function tested and the average for 4-month follow-up after lesion and repair of a 6-mm gap in the four groups studied (mean  $\pm$  SEM).

	<i>n</i>	SGs	PP	CMAP plantar	CNAP digital	Mean
Onset (day)						
AG	8	25.5 $\pm$ 1.7	27.8 $\pm$ 2.5	28.7 $\pm$ 1.5	46.0 $\pm$ 7.3	32.0 $\pm$ 2.4
AG + FK	5	22.8 $\pm$ 1.8	25.6 $\pm$ 2.2	29.2 $\pm$ 1.8	39.0 $\pm$ 2.0	29.1 $\pm$ 1.4
SIL	12	109.2 $\pm$ 12.8*†	137.7 $\pm$ 8.6*†	140.2 $\pm$ 8.5*†	151.0 $\pm$ 0.0*†	134.5 $\pm$ 6.4*†
SIL + FK	5	84.0 $\pm$ 27.1*†	107.0 $\pm$ 26.9*†	129.0 $\pm$ 22.0*†	133.0 $\pm$ 18.0*†	113.2 $\pm$ 18.2*†
Maximal (%)						
AG	8	86.0 $\pm$ 3.9	96.2 $\pm$ 3.7	55.6 $\pm$ 8.5	29.3 $\pm$ 5.3	66.8 $\pm$ 3.1
AG + FK	5	91.4 $\pm$ 1.1	100.0 $\pm$ 0.0	50.7 $\pm$ 8.3	60.4 $\pm$ 7.5*	75.6 $\pm$ 1.1*
SIL	19	20.7 $\pm$ 7.5*†	5.8 $\pm$ 4.2*†	3.5 $\pm$ 3.3*†	0.0 $\pm$ 0.0*†	7.3 $\pm$ 3.2*†
SIL + FK	5	20.3 $\pm$ 14.3*†	30.0 $\pm$ 20.0*†	18.9 $\pm$ 18.9	10.6 $\pm$ 10.6†	19.9 $\pm$ 15.4†
Functional recovery index						
AG	8	7175 $\pm$ 424	8392 $\pm$ 564	2979 $\pm$ 571	1903 $\pm$ 254	5112 $\pm$ 309
AG + FK	5	7796 $\pm$ 107	8000 $\pm$ 312	2373 $\pm$ 495	3507 $\pm$ 472*	5419 $\pm$ 158
SIL	19	1107 $\pm$ 422*†	250 $\pm$ 223*†	134 $\pm$ 117*†	0 $\pm$ 0*†	369 $\pm$ 176*†
SIL + FK	5	1278 $\pm$ 898*†	2193 $\pm$ 1430*†	465 $\pm$ 465*†	495 $\pm$ 495*†	1108 $\pm$ 802*†

\**P* < 0.05 vs group AG; †*P* < 0.05 vs group AG + FK.



**FIGURE 1.** Percentage of the amplitude of the CMAP of plantar muscles and the CNAP of digital nerves over time in groups of mice with sciatic nerve resection of a 6-mm gap and repair with a nerve autograft (AG) or a silicone tube (SIL) treated or not with FK506.

tional index (SFI),<sup>13</sup> adapted to mice, to quantify changes in the walking pattern.

**Morphological Evaluation.** After follow-up, animals were reanesthetized, the operated nerve dissected from surrounding tissues, and a long segment including several millimeters proximal and distal to the graft harvested. The regenerated nerves were fixed in glutaraldehyde-paraformaldehyde (3%–3%) in 0.1 mol/L cacodylate buffer (pH 7.4, 4 h, 4°C), and cut into two pieces to enable transverse sections at the middle of the tube or graft and at the

**FIGURE 2.** Percentage of the number of reactive SGs and the PP score over time in groups of mice with sciatic nerve resection of a 6-mm gap and repair with a nerve autograft (AG) or a silicone tube (SIL) treated or not with FK506.

distal nerve. Samples were postfixed in OsO<sub>4</sub> (2%, 2 h), dehydrated through an ethanol series, and embedded in Epon. Transverse semithin sections (0.5 μm) of the entire nerve were stained with toluidine blue and examined under light microscopy. Images were acquired with an Olympus DP10 camera to a PowerMacintosh G3 computer (Apple Computer, Cupertino, CA) and printed at a final magnification of ×200 for measurement of the cross-sectional area of the nerve, and ×2600 for morphometric analysis. Morphometric evaluations (myelinated axon counts, axon and fiber diameters and areas, and the g ratio) were made from prints of systematically selected

fields with the aid of a computer-linked digitizing tablet and appropriate software designed for Macintosh.<sup>29</sup> When there was no nerve regeneration at the cross-sectional level, zero values were entered for nerve area and number of myelinated fibers, but the sample was not used for further morphometric analysis.

All data are expressed as the mean  $\pm$  SEM. Statistical comparisons between groups were made by the Kruskal–Wallis test followed by the Mann–Whitney *U*-test, and the chi-square test for the proportion of success. Differences were considered significant at *P* < 0.05.

## RESULTS

The animals treated with FK506 did not show any apparent secondary effect from the drug. No infections or unexpected deaths occurred during follow-up.

**Functional Results.** The first CMAPs recorded from plantar muscles reappeared, as polyphasic potentials of small amplitude and long latency, in all the mice with AG repair, with a similar average onset of about 29 days in both groups. During the following months, their latency shortened to close to normal and amplitude progressively increased to reach mean final values of 55% in group AG and 51% in group AG+FK with respect to preoperative values (Fig. 1 and Table 1). In tubulized mice, plantar CMAPs were recorded in only two animals of group SIL and in one of group SIL+FK. The mean final amplitude was considerably higher (19%) in the latter than in the former (4%). Digital CNAPs were recorded earlier in mice of group AG+FK (mean 39  $\pm$  2 dpo) compared to mice of group AG (46  $\pm$  7 dpo) (Fig. 1). Maximal responses elicited were about 30% in group AG and 60% in group AG+FK (Table 1). Only one mouse in group SIL+FK, and none of group SIL, showed sensory nerve reinnervation during follow-up.

Sudomotor responses returned beginning at 21 dpo in both AG groups. The total number of reactive SGs increased with a higher slope and achieved maximal recovery earlier in group AG+FK than in group AG, although the final levels (91% and 86%) were not significantly different (Fig. 2 and Table 1). In groups with tube repair reinnervation of SGs was detected in a few mice and followed a similar course during follow-up, with maximal values of about 20% of controls. The nociceptive responses were found to start at a mean of 28 and 26 dpo in both AG groups, increasing with a high slope to a maximal response close to normal by 90 dpo (Fig. 2). In the SIL groups, responses to pinprick were consistently, although

not significantly, higher in group SIL+FK than in group SIL.

The autologous nerve graft allowed successful regeneration in all mice, with an earlier onset of reinnervation and a higher maximal recovery than in tubulized nerves for all functions tested (Table 1). When groups with AG repair were compared, FK506 treatment produced an acceleration of the onset of reinnervation by 3 days and a slight increase (10%) of the mean levels of reinnervation. With respect to groups repaired with an SIL tube, the proportion of regenerated animals was similarly low. The mouse regenerated in group SIL+FK showed better target reinnervation than the two mice regenerated of group SIL, although differences were not significant because of the low number of responses and thus the high variability inherent in this model.

The footprint analyses did not show noticeable recovery of the walking pattern despite the presence of muscle reinnervation. The SFI remained at values around -100 in all groups, without significant changes between results at 90 and 120 dpo (Table 2). However, the SFI and the individual measurements were slightly better in groups with AG than in groups with SIL tube repair.

**Morphological Results.** Microscopic dissection at the injury site showed a well-formed regenerated nerve through the autograft in all mice of groups AG and AG+FK. There was mild fibrous tissue around the two suture sites. On the other hand, there was a regenerated nerve inside the chamber in only two mice of group SIL and in one of group SIL+FK (Table 3). In these successfully regenerated cases, the tube was covered by a thin fibrous capsule and the regenerated nerve was located at the center of the tubes and was of smaller caliber than the normal nerve. In one more mouse in both SIL groups, some axons had grown through the fibrous capsule but there was no intratubular regenerated nerve (as shown using light microscopy), and these were likely

**Table 2.** Results of the walking track tests performed at 4 months postoperation, expressed as the sciatic functional index comparing the operated hindlimb and the normal contralateral hindlimb (mean  $\pm$  SEM).

Group	<i>n</i>	Sciatic functional index	
		90 dpo	120 dpo
AG	8	-104.8 $\pm$ 2.6	-105.5 $\pm$ 3.6
AG + FK	5	-89.5 $\pm$ 6.7	-90.5 $\pm$ 5.6*
SIL	10	-112.5 $\pm$ 6.1	-106.3 $\pm$ 5.9
SIL + FK	5	-116.1 $\pm$ 7.5 <sup>†</sup>	-102.9 $\pm$ 11.2

\**P* < 0.05 vs group AG; <sup>†</sup>*P* < 0.05 vs group AG + FK.

**Table 3.** Number and percentage of mice with any degree of functional reinnervation (at least in two of the tests performed) during follow-up and with a regenerated nerve and pinch test positive distally in the different groups studied.

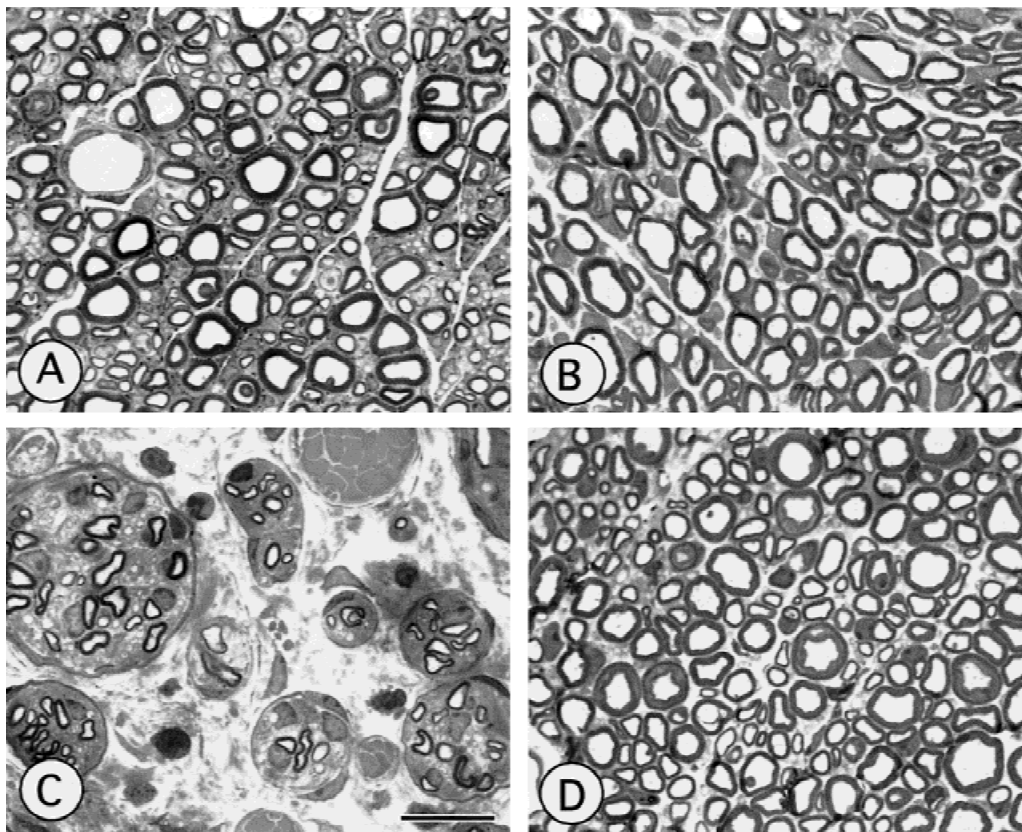
Group	<i>n</i>	Reinnervation	Regenerated nerve
AG	8	8 (100%)	8 (100%)
AG + FK	5	5 (100%)	5 (100%)
SIL	12	3 (25%)*†	2 (17%)*†
SIL + FK	5	2 (40%)*†	1 (20%)*†

\**P* < 0.05 vs group AG; †*P* < 0.05 vs group AG + FK (chi-square test).

responsible for the limited reinnervating responses in SGs and PP tests. In the remaining mice, there was no continuity between the nerve stumps.

Light microscopy sections taken at the middle of the graft demonstrated a regenerated nerve, with good architecture and numerous myelinated and unmyelinated axons grouped in small nerve fascicles (Fig. 3). In the samples regenerated in SIL tubes, the nerve was of smaller size, with a fibroblast outer layer surrounding a central core of myelinated and unmyelinated axons grouped in smaller fascicles (Fig. 3).

The quantitative morphometric analysis (Table 4) showed that groups with a nerve autograft achieved a good degree of regeneration, with a near normal number of myelinated fibers regenerated through the graft. At distal nerve sections, group AG had slightly more myelinated fibers than group AG+FK. There were no significant differences between these two groups regarding the mean size and degree of myelination of the regenerated fibers. The histogram distribution of myelinated fiber diameters was skewed to the left in comparison with control nerves, indicating that regenerated fibers had a reduced caliber (Fig. 4). The proportion of very small fibers (<2 μm in diameter) was lower in group AG+FK (2%) than in group AG (7%) at midgraft as well as at the distal nerve level. In the scattergram plots of g ratio to axonal perimeter (Fig. 4), a population of fibers at the upper left corner in group AG indicates the presence of immature, small axons with a thinner-than-normal myelin sheath for their size, whereas the distribution in group AG+FK was close to normal. Furthermore, the presence of fibers in the lower left corner in group AG (absent in group



**FIGURE 3.** Transverse semithin sections of regenerated nerves taken at the midpoint of the graft or tube 4 months after sciatic nerve resection and repair, leaving a 6-mm gap, in a representative animal of groups AG (A), and AG+FK (B), in the animal with better outcome of the two regenerated from group SIL (C), and in the one animal regenerated from group SIL+FK (D). Bar = 15.6 μm.

**Table 4.** Morphological parameters of the regenerated nerve at medial and distal levels of the graft or tube in the four groups studied (mean  $\pm$  SEM).

Group	n	Nerve area (mm <sup>2</sup> )		No. of myelinated fibers	
		Middle	Distal	Middle	Distal
AG	8	0.156 $\pm$ 0.015	0.175 $\pm$ 0.031	4538 $\pm$ 403	4003 $\pm$ 431
AG + FK	5	0.143 $\pm$ 0.011	0.153 $\pm$ 0.011	4026 $\pm$ 197	3195 $\pm$ 330
SIL	12	0.020 $\pm$ 0.015*†	0.009 $\pm$ 0.006*†	146 $\pm$ 133*†	20 $\pm$ 18*†
SIL + FK	5	0.011 $\pm$ 0.011*†	0.030 $\pm$ 0.030*†	328 $\pm$ 328*†	388 $\pm$ 388*†

Group	n	Axonal diameter ( $\mu$ m)		g ratio	
		Middle	Distal	Middle	Distal
AG	4	2.68 $\pm$ 0.06	2.53 $\pm$ 0.15	0.64 $\pm$ 0.02	0.61 $\pm$ 0.01
AG + FK	5	2.70 $\pm$ 0.06	2.70 $\pm$ 0.09	0.68 $\pm$ 0.01	0.66 $\pm$ 0.01
SIL	2	2.00 $\pm$ 0.17	1.94 $\pm$ 0.44	0.67 $\pm$ 0.05	0.70 $\pm$ 0.02
SIL + FK	1	3.19	2.81	0.69	0.69

All animals were included for nerve area and number of myelinated fibers, whereas only animals with a nerve regenerated inside the tube were included for axonal diameter and g-ratio calculations.  
\* $P < 0.05$  vs group AG; † $P < 0.05$  vs group AG + FK.

AG+FK and controls) indicates the presence of small axons with a thicker-than-normal myelin sheath for their size suggesting immature radial growth (atrophic fibers). In the groups repaired by tubulization, the mean size of the regenerated cable and the number of myelinated fibers were significantly reduced compared to AG groups.

## DISCUSSION

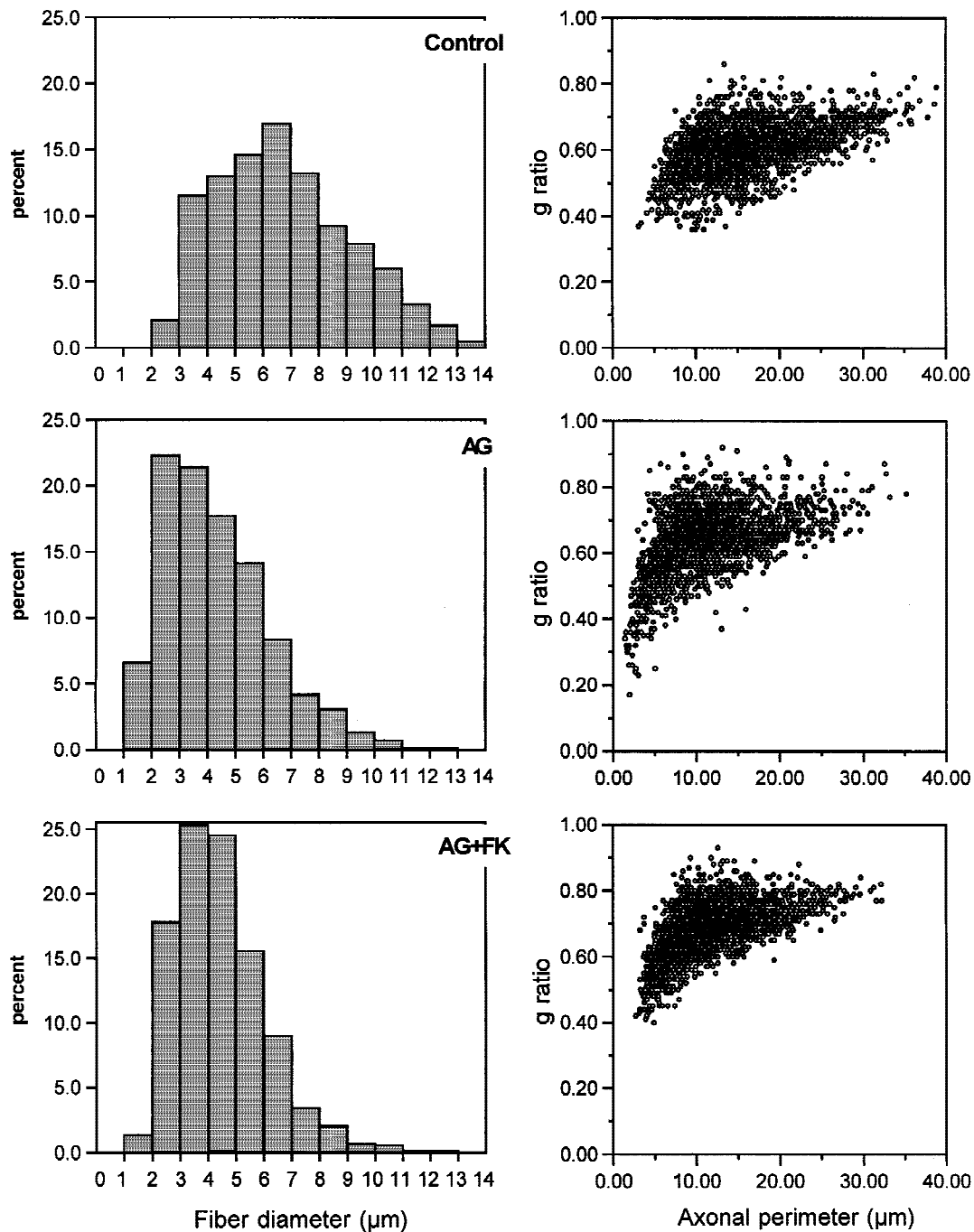
Tubulization repair is an effective repair method that allows axonal regeneration after nerve injuries that result in relatively short gaps, as shown in assays in rodents<sup>38,43</sup> and in primates.<sup>3,14</sup> However, whereas in a nerve graft regenerating axons find aligned endoneurial tubules containing Schwann cells as a source of trophic factors, successful regeneration after tubulization depends on the formation of a new extracellular matrix scaffold, over which blood vessels, fibroblasts, and Schwann cells migrate and form a new nerve structure.<sup>55</sup> This implies a delay in axonal elongation, and failure of regeneration if the nerve stumps are not able to provide an initial regenerative cable with enough diffusible and cellular trophic elements inside the tube. Therefore, regeneration fails through long gaps most likely because the regenerative capabilities of the nerve stumps have been exceeded and Schwann cells are not able to provide a permissive environment for axonal elongation.<sup>36,42,57</sup>

FK506 and other mimetic drugs seem to be efficient in accelerating regenerative outgrowth, and thus increasing the rate of regeneration, as demonstrated after injuries that allow successful spontaneous regeneration, such as crush and section and

graft repair. FK506 also enhances regeneration in a tubulized 10-mm gap in the rat sciatic nerve.<sup>4</sup> However, FK506 has a more limited effect on situations where regeneration is limited, as shown in the tubulized 6-mm gap in the mouse sciatic nerve. Similarly, after dorsal column transection in the rat spinal cord, FK506 stimulated massive sprouting of injured axons and promoted sparing of axons not severed in the initial injury by reducing the extent of secondary injury, but showed a limited effect for promoting regeneration, as only a few axons were seen regenerated beyond the lesion over short distances.<sup>5</sup> Although it is possible that the reduced degeneration in this latter model influenced the degree of regeneration, the ability of FK506 to promote regeneration may be selective for neuronal subtypes. For example, FK506 is able to promote regeneration of rubrospinal neurons in a peripheral nerve graft,<sup>53</sup> but not following chronic denervation.<sup>50</sup>

The present results demonstrate that, following a severe peripheral nerve injury made by resecting a nerve segment, autologous nerve graft repair allows for better regeneration and faster and higher levels of reinnervation than repair with silicone tubes. The use of an autograft ensures success of regeneration in all animals, whereas tubulization fails in most cases when bridging a long gap, such as a 6-mm gap in the mouse.<sup>8,29,41</sup> Treatment with FK506 resulted in a slight, albeit nonsignificant, improvement of the onset of reinnervation and of the maximal degree of functional recovery achieved during a 4-month post-operation follow-up. The recovery of pain sensibility and of the CNAP in the most distal digital nerves, which directly depend on axonal regeneration,





**FIGURE 4.** Left column: frequency distribution (percent) of myelinated fiber diameters in the regenerated nerve at the distal level in groups AG and AG+FK compared to control nerves. The histograms of injured nerves show a distribution skewed to the left, more marked in group AG than in group AG+FK. Right column: scattergrams of g ratio to axon perimeter for the myelinated axons at the midgraft level compared to the normal nerve.

showed better progression with FK506 than reinnervation of muscles and SGs, the latter two parameters requiring reestablishment of new synaptic contacts with target cells. The regenerated myelinated fibers showed a more mature morphometric profile in

mice treated with FK506, a finding that is consistent with an enhanced rate of regeneration.<sup>29</sup>

The reason that FK506 only marginally affected nerve regeneration in the present study may relate to at least three factors: duration of treatment; dose;

and species (size of the animal). First, it has been shown that FK506 is most efficacious if given during the entire regenerative period.<sup>24</sup> Accordingly, in previous studies after sciatic nerve crush<sup>26,33</sup> or resection with autograft repair,<sup>15,17</sup> rats were treated with FK506 for the entire duration (weeks) of the study. In our longer study (months), FK506 withdrawal was necessitated by the limited supply of the drug. Nevertheless, withdrawal of FK506 administration was started at a time when reinnervation was established in all mice given an autograft, and it is important to note that we did not find any decline of the level of target reinnervation achieved. This is in contrast to the decline in progression of recovery and the development of morphological signs of rejection after withdrawal of FK506 in rats treated by nerve allograft,<sup>9</sup> where FK506 was used solely as immunosuppressant therapy. To stimulate axonal regeneration in cases where there is no immunological compromise, it is possible that FK506 may be effective, although not maximally, if given only during a short time after injury,<sup>37</sup> therefore avoiding the complications of long-term immunosuppression. Thus, the present results are consistent with the previous finding that treatment during only the first 9 days after nerve crush significantly increased the caliber of the regenerating nerve compared with controls, although it was less effective than treatment during the entire 18 days of study.<sup>24</sup> However, this time period was probably not long enough to ensure steady reinnervation of distal targets after a crush injury in the rat sciatic nerve.

Second, the ability of FK506 to increase nerve regeneration *in vivo* is dose-dependent, with the best results obtained at a daily dose of 5 mg/kg in the rat.<sup>54</sup> Thus, we selected this dosage in order to test if FK506 would improve regeneration in more severe injury models, and if it could allow regeneration through a tubulized 6-mm gap where regeneration fails in most cases.<sup>8,29</sup> However, given the bell-shaped dose-response curve observed in the rat,<sup>54</sup> it is possible that this is not the optimal dose in the mouse. Thus, an adequate dose comparison study needs to be conducted in the mouse for the nerve graft model. One possible explanation for the relative variability of the results of studies of experimental nerve injuries is the variety of models and testing methods used. After autologous graft repair in the rat tibial nerve, Doolabh and Mackinnon<sup>15</sup> demonstrated that FK506 administered at a low dose (1 mg/kg per day intraperitoneally) expedited recovery of the walking track pattern in about 1 week with respect to controls or rats treated with cyclosporine

A, but later there were no differences between the groups. The histomorphometric analysis performed at 7 weeks postgrafting showed greater myelinated fiber density in FK506-treated animals, although by 10 weeks there were no differences between groups. Similarly, a higher axonal count was found in FK506-treated rats (0.3 or 0.6 mg/kg subcutaneously) than in untreated animals 2 weeks after autograft repair, but there were no significant differences by 6 weeks.<sup>17,18</sup> The higher mean levels of reinnervation and the slightly greater size of myelinated fibers that we found at the end of the 4-month study in group AG+FK than in group AG suggest that the higher dose regime used may be more effective, supporting the need for a dose-response characterization in the mouse.

Third, because an acceleration in the rate of nerve regeneration would manifest as a greater increase in regenerative distance with time, the net effect would be less apparent in smaller species of animals and along the length of shorter nerves. Thus, an appreciable increase in functional recovery by FK506 would be expected to be less significant in the mouse than the rat sciatic nerve.

At present, the cellular localization of FK506's nerve regenerative effect is unclear. However, the ability of FK506 and its nonimmunosuppressant derivatives to increase neurite outgrowth in neuronal culture lines and primary neuronal cultures<sup>28,35,49</sup> indicates a direct neuronal action. Furthermore, analysis of the rate of nerve regeneration revealed an increase in growth with time without an alteration in the delay to onset for nerve regeneration.<sup>54</sup> Thus, acceleration in the rate of axonal regeneration by FK506 is likely due to a direct effect on the axotomized neurons to speed elongation but not enhance regenerative sprouting. However, a possible additional action to accelerate wallerian degeneration also needs to be considered. The Schwann cell and its basal lamina are crucial components in the environment through which regenerating axons grow to reach their peripheral targets. During wallerian degeneration, Schwann cells from the distal stump proliferate, help inflammatory infiltrating cells to eliminate debris, and upregulate the synthesis of trophic and tropic factors.<sup>52</sup> In fact, regeneration fails or is delayed in situations where wallerian degeneration is slowed.<sup>39,51</sup> In contrast, the use of predegenerated nerve grafts increases the axonal regeneration rate.<sup>12,41</sup> In this context, it may be relevant that FK506 was reported to decrease the myelin debris found in the distal nerve at early times postoperation with FK506 treatment.<sup>17,18</sup> However, FK506 was not

found to affect the amount of macrophages infiltrating the injured nerve tissue.<sup>19,37</sup> Although FK506 stimulates proliferation of Schwann cells cultured from predegenerated nerves,<sup>18</sup> the significance of this finding is unclear because the effect was observed using a very high (100- $\mu$ mol/L) concentration, known to actually inhibit neurite outgrowth.<sup>10,19</sup>

A number of molecular mechanisms have been proposed to explain the regeneration-promoting effects of FK506. Binding to FKBP-12 and inhibition of calcineurin increases the phosphorylation of several substrate molecules, including GAP-43, a protein that plays an important role in neural plasticity and is highly expressed in the regenerative growth cones.<sup>6</sup> However, the demonstration that nonimmunosuppressant derivatives of FK506 do not inhibit calcineurin, yet speed nerve regeneration,<sup>28,49</sup> and that FK506 elicited neurite elongation in hippocampal neuronal culture of FKBP-12 knockout mice<sup>23</sup> suggested that these effects of FK506 are not mediated by binding to FKBP-12 but dependent on its interaction with FKBP-52. A model has been proposed<sup>20–22</sup> whereby binding to FKBP-52 leads to a conformational change, resulting in dissociation of the mature steroid receptor complex (containing FKBP-52, *hsp*-90, and p23). Downstream effectors mediating enhancement of nerve regeneration may include increased expression of *c-jun* and GAP-43<sup>27,37</sup> and stimulation, via *hsp*-90 activation, of the MAP kinase/ERK2 pathways—the latter suggesting a possible convergence with signal transduction pathways for neurotrophic factors. Thus, dose–response studies should be conducted not only for FK506 but also for its nonimmunosuppressant FKBP binders, as well as for *hsp*-90 binding compounds, to determine the combination of graft and compound that achieves maximal efficacy in nerve transection models.

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## EFFECTS OF CONTINUOUS ADMINISTRATION OF FK506 ON REGENERATION THROUGH SILICONE GUIDES IN A 6 MM GAP IN THE SCIATIC NERVE OF THE MOUSE.

### MATERIAL AND METHODS

#### *Surgical procedure and treatment*

The sciatic nerve was exposed as described previously and a 6 mm segment was resected.

The gap was repaired with a silicone guide, 8 mm long, 1 mm i.d. and 0.25 wall thickness (Dicoinsa, Barcelona, Spain) (Navarro et al., 2001).

The 10 animals were treated with 5 mg/kg/day of FK506 (Fujisawa Pharmaceutical, Osaka, Japan) during all the study (120 days).

#### *Functional and morphological evaluation*

The methods of assessment were the same described in the previous study (Study 3, Navarro et al., 2001).

For comparison, the results of this group (SIL+FKcont) are shown in comparison with those obtained in groups with a silicone tube repair that were untreated (group SIL) or treated with FK506 under discontinuous dosage during the first 90 days postoperation (group SIL+FKdisc), that are shown also in Study 3.

Table 1. Results of functional tests performed during 120 days follow-up in the groups of mice repaired with a silicone guide and either treated or not with FK506 during the entire study.

<b>Onset</b>	+rg/n	CMAP pl	CNAP d	PP	SGs	Mean
<b>SIL</b>	2/12	139±8.5	150±0	137±9	109±13	138±7
<b>SIL+FK cont</b>	2/10	126±16	126±16	124±17	123±18	127±15
<b>SIL+FK disc</b>	1/5	129±22	133±18	107±26.9	84±27.1	113.2±18.2
<b>RM</b>	+rg/n	CMAP pl	CNAP d	PP	SGs	Mean
<b>SIL</b>	2/12	3.5±3.3	0±0	5.8±4.2	10.0±6.9	7.5±3.4
<b>SIL+FK cont</b>	2/10	4.7±3.3	9.2±6.8	21.1±14.0	12.4±9.6	11.8±7.9
<b>SIL+FK disc</b>	1/5	18.9±18.9	10.6±10.6	30±20	20.3±14.3	19.9±15.4
<b>IRF</b>	+rg/n	CMAP pl	CNAP d	PP	SGs	Mean
<b>SIL</b>	2/12	134±132	0±0	250±223 <sup>a</sup>	590±406	240±4
<b>SIL+FK cont</b>	2/10	230±158	678±491	1539±1018	1080±731	779±516
<b>SIL+FK disc</b>	1/5	465±465	495±495	2193±1430	1278±898	1108±802

## RESULTS

### Functional results

The success of regeneration in this group was poor; only 2 of the 10 mice of the group SIL+FK showed distal target reinnervation at the end of follow up. This was a similar proportion to that found in untreated mice (group SIL of Study 3). In the animals with reinnervation, the first CMAPs recorded from plantar muscles were polyphasic potentials of small amplitude and appeared early in the FK506-treated animals than in the untreated group. No CNAPs of digital nerves were recorded in the SIL group, whereas the two animals with reinnervation in group SIL+FK reached considerably good levels of CNAP amplitude. The nociceptive and sudomotor responses of treated animals reappeared earlier than in the two regenerated of the untreated group. Table 1 shows the mean results for the onset and the degree of recovery of group SIL+FK compared with group SIL.

### Morphological results

In the two animals of the group SIL with successful reinnervation there was an important amount of fibroblasts and connective tissue surrounding small fascicles of myelinated and unmyelinated regenerative fibers. In comparison, the two animals with regenerated nerve in group SIL+FK showed a more homogeneous aspect, with a higher density of regenerated fascicles and about 2500 myelinated fibers at mid-guide and the half in the distal nerve (Table 2).

## DISCUSSION

As we shown in a previous study (Navarro et al 2001), FK506 has not the capacity to

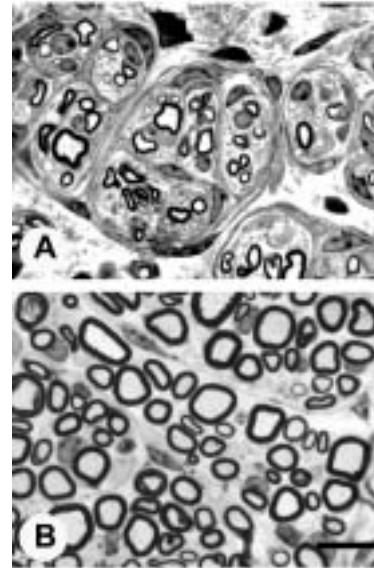


Fig 2. Cross-sections of regenerated nerves at mid-tube 4 months following sciatic nerve resection and tubulization repair with Silicone guides in (A) the best animal of the untreated group SIL, and (B) in one of the treated mice of group SIL+FK cont. Note the higher density and size of the regenerated fibers in the animal of SIL+FK group compared with the untreated animal. Bar =10 $\mu$ m.

improve regeneration through situations where the regeneration is limited, as in severe nerve transection repaired with silicone guides. The rate of succesful regeneration is not increased by the continuous administration for all the follow up, and it is only about 20%. However, the animals treated with FK506 that regenerate showed better functional recovery for the different parameters evaluated, and the presence of digital potenciales, absent in the

TABLE 2. Morphological analysis of regenerated nerves at the mid-point of the tube and distally 4-months following resection and tubulization repair of a 6-mm gap.

	Nerve cable	Nerve area (mm <sup>2</sup> )		N myelinated fibers	
		Medial	Distal	Medial	Distal
<b>SIL</b>	2/12	0.020±0.015	0.010±0.010	146±133	20±18
<b>SIL+FK cont</b>	2/10	0.023±0.016	0.012±0.030	638±418	320±210
<b>SIL+FK disc</b>	1/5	0.011±0.011	0.030±0.030	328±328	388±388

animals with regeneration in the control group. It seems that the capacity of FK506 to accelerate nerve regeneration is not enough to avoid the failure of regeneration across a long distance in a poor environment. Similarly, FK506 fails to increase axonal regeneration in chronically denervated nerves (Sulaiman et al. 2002), since it can not compensate a poor environment to sustain nerve regeneration.

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**Dosi-dependència de l'efecte immunosupressor de l'FK506  
sobre empelts nerviosos al·logènics o xenòlegs**

*Comparative dose-dependence study of FK506 on  
transected mouse sciatic nerve repaired by allografts or  
xenografts.*

*Udina E, Voda J, Gold BG, Navarro X.  
J Periph Nerv Syst 2003, 8:145-154.*



# Comparative dose-dependence study of FK506 on transected mouse sciatic nerve repaired by allograft or xenograft

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**Abstract** We evaluated the effects of FK506, at doses of 0.2, 2, and 5 mg/kg/day, on the response to nerve grafts implanted in outbred mice. A 6 mm long segment of the sciatic nerve was transected and repaired by autograft (the same segment resected), allograft (from another mouse), or xenograft (from a rat nerve). The regenerating nerves were harvested after 3 weeks and studied under light and electron microscope. Allografts of animals treated with the 5 mg/kg/day dose of FK506 appeared similar to those from autografts, demonstrating an equivalent number of myelinated fibers. In mice treated with the 2 mg/kg/day dose, regeneration was slightly hindered, as indicated by the reduced number of myelinated fibers. In contrast, in mice given a 0.2 mg/kg/day dose of FK506, allografts were not different from untreated allografts; both groups showed a marked rejection response with only few unmyelinated axons and no myelinated fibers. Xenografts showed a more severe rejection than allografts, with a marked inflammatory cell reaction throughout the graft. In contrast, in mice treated with the 5 mg/kg/day dose, xenografts exhibited a mild cell reaction and a greater number of regenerated myelinated fibers. In conclusion, effective axonal regeneration is achieved with FK506 administration at doses of 5 mg/kg/day through allografts and, partially, through xenografts.

**Key words:** allograft, FK506, immunosuppression, nerve regeneration, sciatic nerve, xenograft

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## Introduction

Severe lesions of the peripheral nerve resulting in significant tissue loss require graft reconstruction. Because the supply of autografts is limited, the development of artificial nerve guides using biomaterials represents a promising alternative (Rodríguez *et al.*, 2000). The production of artificial nerve guides, how-

ever, is still in its infancy. While allografts are readily available, they require immunosuppression to prevent rejection (Pollard and Fitzpatrick, 1973a; 1973b; Zalewski and Gulati, 1984a; 1984b; Mackinnon *et al.*, 1987; Bain *et al.*, 1988; Yu *et al.*, 1990). An additional complication is the formation of scar tissue due to the immunologic response, which would inhibit the growth of the axons through long grafts (Evans *et al.*, 1994). These difficulties can be partially overcome by pretreating the nerve grafts to decrease their antigenicity, albeit the final outcome is not as good as that obtained using fresh autografts (Evans *et al.*, 1994) due to the poor viability of Schwann cells (Gulati, 1988). In contrast, systemic administrations

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of immunosuppressive drugs, such as azathioprine and hydrocortisone (Pollard and Fitzpatrick, 1973b; Pollard et al., 1973; Mackinnon et al., 1987), cyclosporin A (Zalewski and Gulati, 1984b; Bain et al., 1988; Yu et al., 1990) or FK506 (Büttemeyer et al., 1995; Feng et al., 2001; Okajima et al., 2002), do achieve results close to those found using autografts.

FK506 and cyclosporin A are new immunosuppressive drugs that changed the course of solid organ transplantation because they inhibit specifically cellular immunity and are less aggressive than cytotoxic drugs (Janeway et al., 1999). Moreover, FK506 also has neuroregenerative and neuroprotective effects independent of its immunosuppressive activity (Gold et al., 1997; 1999; Gold, 2000; Guo et al., 2001). These dual actions make FK506 ideal for situations requiring both immunosuppression and nerve regeneration. Thus, there are reports of successful allogenic hand transplantation in patients immunosuppressed by FK506 (Dubernard et al., 1999; Jones et al., 2000; Margreiter et al., 2002), in whom the rate of nerve regeneration (assessed by Tinell's sign) was faster than expected. However, the optimal dosage needs to be determined in multiple experimental models to optimize its clinical use in this regard. In the present study, we examined the dose–response activity of FK506 in mice after repairing sciatic nerve transection using allografts or xenografts. We employed a range of FK506 dosages (0.2, 2, and 5 mg/kg/day) previously found to accelerate the rate of nerve regeneration in the mouse crush model (Udina et al., 2002), the 5 mg/kg/day dose being the maximally effective dose, as in the rat (Gold et al., 1994; 1995; Wang et al., 1997).

## Materials and Methods

### Surgical procedure

All surgical procedures were performed under pentobarbital-induced anesthesia (60 mg/kg intraperitoneally) in seven groups of 2.5-month-old female OF1 mice ( $n = 5$  per group). The sciatic nerve was exposed at the mid-thigh and transected at a constant point (45 mm from the tip of the third digit), a segment of the distal stump was resected to leave a final 6 mm gap, and a nerve segment was grafted between the proximal and distal stumps with two 10-0 monofilament (Ethilon-2888, Ethicon) epineurial sutures at each end. For the autograft group (the most ideal graft repair), the resected segment was sutured into the same nerve, preserving its fascicular and longitudinal orientation. For the allograft group, a nerve segment of the sciatic nerve was harvested from an OF1 mouse obtained from a different litter than that of the

recipient mouse. In the xenograft group, a segment of the peroneal nerve (to avoid mismatch between the nerve and the graft size) was obtained from a Sprague–Dawley rat. The wound was sutured and disinfected with povidone-iodine. To avoid autotomy after denervation, animals were pretreated with amitriptyline (Navarro et al., 1994). The experimental procedures were approved by the Ethics Committee of our university.

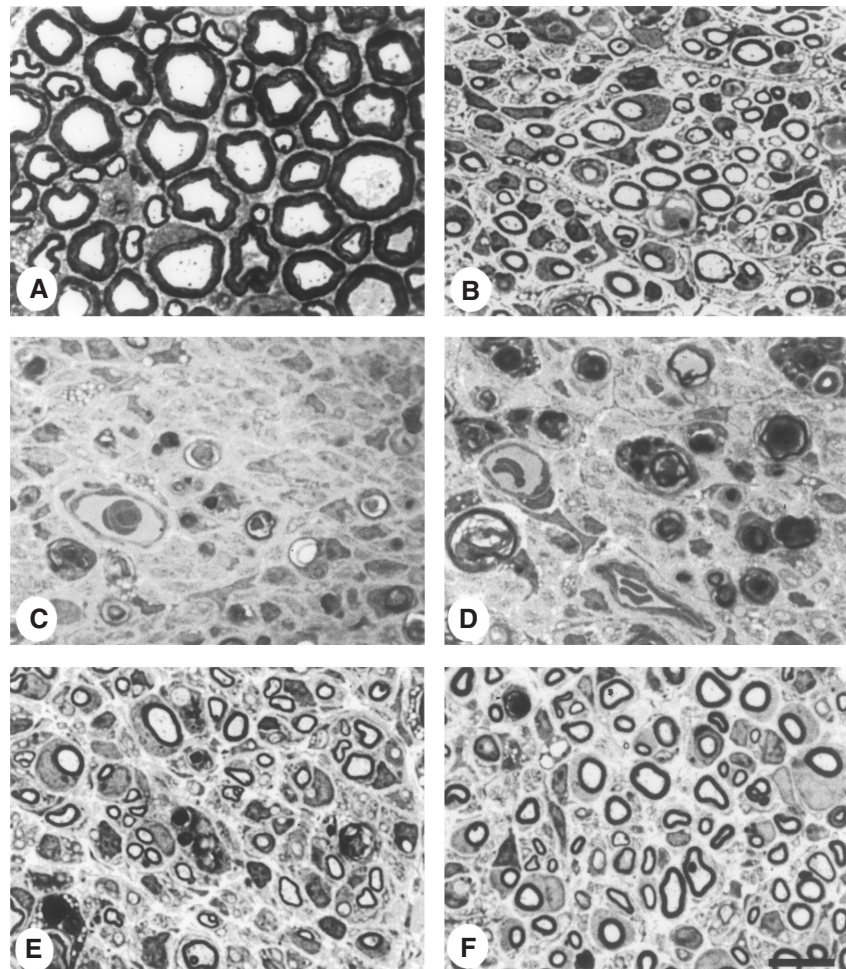
Different groups of mice repaired by allograft and xenograft were treated with a daily dose of 0.2, 2, or 5 mg/kg/day of FK506 (Fujisawa Pharmaceuticals, Osaka, Japan). The drug was diluted in saline solution and administered through subcutaneous injections in the back. The three doses were tested in allografts and only the highest dose (5 mg/kg/day) was tested in xenografts.

### Morphological evaluation

After 21 days, the animals were reanesthetized, the operated nerve was dissected from the surrounding tissues, and a long segment (including several mm proximal and distal to the graft) was harvested. The regenerated nerves were fixed in glutaraldehyde–paraformaldehyde (3:3) in 0.1 M cacodylate buffer (pH 7.4, 4 h, 4 °C) and cut in three pieces (corresponding to the proximal, midgraft, and distal levels of the nerve). Tissue samples were postfixed in  $\text{OsO}_4$  (2%, 2 h), contrasted with uranyl acetate (2.5% in OH 70%), dehydrated through ethanol series, and embedded in Epon. Transverse semithin sections (0.5  $\mu\text{m}$ ) were stained with toluidine blue and examined by light microscopy. Images were acquired with an Olympus DP50 camera connected to a PowerMacintosh computer and printed at final magnifications of  $\times 200$  (for measuring the cross-sectional area of the whole nerve),  $\times 800$  (for measuring the number and size of endoneurial vessels), and  $\times 2000$  (for measuring perineurium thickness, for quantitation of regenerative myelinated axons and phagocytic cells).

For electron microscopy, ultrathin sections (90 nm) were made of the nerve at the midgraft and distal levels (only from samples demonstrating regenerating axons, as assessed by light microscopy). For each nerve, 10 fields were randomly photographed at a magnification of  $\times 1400$  for quantitation of the density of unmyelinated axons (number per  $\text{mm}^2$ ) and for measurement of cross-sectional axonal areas of myelinated fibers.

Data are expressed as mean  $\pm$  SE. Statistical comparisons between groups were made using Kruskal–Wallis test followed by the Mann–Whitney  $U$ -test. Differences were considered significant at  $p < 0.05$ .



**Figure 1.** Transverse semithin sections of a control sciatic nerve (A) and of regenerative nerves taken at the midpoint from an autograft (B), an allograft from an untreated animal (C), and allografts from animals treated with FK506 at a dose of 0.2 mg/kg/day (D), 2 mg/kg/day (E), or 5 mg/kg/day (F). Bar = 10  $\mu$ m.

## Results

### Autograft study

As expected, the autografts showed good regeneration and did not exhibit signs of rejection within or around the transplant (Fig. 1). The number of myelinated fibers 3 weeks following graft repair was  $1758 \pm 305$  at midgraft and  $1162 \pm 320$  in the distal nerve (Tables 1 and 2). The density of unmyelinated axons (number per  $\text{mm}^2$ ) was  $14400 \pm 1300$  at midgraft level and  $11200 \pm 2500$  in the distal nerve. Furthermore, abundant phagocytic cells and occasional myelinated fibers (mean  $314 \pm 33$ ) demonstrating features of degeneration were present at the level of the midgraft.

### Allograft study

Allografts from untreated animals showed poor axonal regeneration (Fig. 1C), although there was some degree of variability between animals. In two

animals, occasional regenerating myelinated fibers (about 90) and a preserved perineurium were found at midgraft, whereas in the other three animals, no regenerated fibers were seen and the perineurium was markedly thickened and infiltrated by immune cells (Table 3). In allografts treated with the lowest dose of FK506 (0.2 mg/kg/day), the grafts were similar to untreated allografts (Fig. 1D); only one animal in this group showed some regenerating myelinated fibers (174), but the others showed marked rejection, with thickened (Fig. 2A) and infiltrated perineurium. The density of unmyelinated axons at midgraft was significantly lower than in autografts (Table 1). There were no myelinated fibers distal to the graft in any mouse from these two groups.

The outcome of the allografts in the group treated with FK506 at a dose of 2 mg/kg/day was also variable. Two animals showed a low number of myelinated fibers and an enlarged perineurium. In the other three animals, however, the regenerated nerves were similar

**Table 1.** Morphological results of regenerated nerves at the midpoint of the graft in the different groups of mice with nerve autograft, allograft, or xenograft repair.

Group	n	Nerve area (mm <sup>2</sup> )	Number of myelinated fibers	Density of unmyelinated fibers (number/mm <sup>2</sup> )	Average axonal area (μm <sup>2</sup> ) (myelinated axons)
Autograft	5	0.15 ± 0.09	1758 ± 305	14400 ± 1300	1.23 ± 0.07
Allograft	5	0.19 ± 0.01	35 ± 21*	3100 ± 700*	
Allograft + FK506 0.2	5	0.18 ± 0.02	35 ± 35*	4400 ± 1600*	
Allograft + FK506 2	5	0.22 ± 0.02*	1101 ± 444‡	6400 ± 2600*	1.00 ± 0.02*
Allograft + FK506 5	5	0.20 ± 0.01*	1669 ± 293‡‡	9300 ± 2200‡	1.18 ± 0.06
Xenograft	5	0.30 ± 0.05*†‡¶	5 ± 5*§¶	1900 ± 600*¶	
Xenograft + FK506 5	5	0.19 ± 0.03**	559 ± 82*†‡¶**	4900 ± 500*†**	0.97 ± 0.05*

Values are expressed as mean ± SE. p < 0.05 versus \*autograft, †allograft, ‡allograft + FK506 0.2 mg/kg/day, §allograft + FK506 2 mg/kg/day, ¶allograft + FK506 5 mg/kg/day, and \*\*xenograft.

to those repaired with autografts (Fig. 1E). The mean number of myelinated fibers was 1101 ± 444 at midgraft. In the distal nerve, only three of the five mice showed presence of regenerative fibers (the mean for the group being 323 ± 200). The density of unmyelinated axons at both levels was significantly lower than in the autograft group.

The best results were obtained in the allograft group treated with the higher dose (5 mg/kg/day) of FK506 where all the grafts appeared similar to autografts (Fig. 1F). The numbers of regenerating myelinated fibers were similar in both groups at midgraft and distally, being also significantly higher in the 5 mg/kg/day allograft group than in the untreated allograft group at midgraft (Table 1) and in the 2 mg/kg/day allograft group at the distal level (Table 2). The density of unmyelinated axons in the 5 mg/kg/day group was lower than in autografts (Fig. 3) but significantly higher than in untreated allografts and xenografts at midgraft (Table 1) and higher than, in the distal nerve, xenografts treated with 5 mg/kg/day and allografts treated with 2 mg/kg/day of FK506 (Table 2). The perineurium of the graft was thinner (Fig. 2B), and the number of phagocytic cells was reduced in comparison with the other groups repaired with allografts (Table 3).

**Xenograft study**

In the group that received a xenograft without treatment, one sample had about 30 myelinated fibers at midgraft, although they did not reach the distal seg-

ment of the nerve. The other xenografts showed disorganization of the fascicular architecture, markedly thick perineurium, and infiltration of enlarged, foamy macrophages (Fig. 4).

In animals given the 5 mg/kg/day dose of FK506 (the only one used in this group, because the other two dosages were insufficient for allografts), none of the xenografts showed evidence of rejection. The vascularization, the perineurium thickness (Fig. 2C), and the number and size (data not shown) of macrophages were considerably decreased with respect to untreated xenografts (Table 3). Administration of FK506 allowed regeneration of nerve fibers through the graft, although the number of myelinated fibers was lower (approximately one-third) than in autografts or allografts treated with the same dose of FK506 (Table 1). Furthermore, only three of the animals exhibited regenerative fibers distal to the graft (Table 2). The numbers of myelinated and unmyelinated axons, however, were significantly higher in this group than in untreated allografts or xenografts and the number of for myelinated fibers was also higher than in allografts treated with 0.2 mg/kg/day of FK506 (Table 1).

**Discussion**

In severe nerve transection injuries, where there is loss of nerve tissue, the consequent gap between the stumps can be bridged using a nerve graft. The origin of this graft has a profound effect on functional

**Table 2.** Morphological results of regenerated nerves distal to the graft in the different groups of mice with nerve autograft, allograft, or xenograft repair.

Group	n	Nerve area (mm <sup>2</sup> )	Number of myelinated fibers	Density of unmyelinated fibers (number/mm <sup>2</sup> )	Average axonal area (μm <sup>2</sup> ) (myelinated axons)
Autograft	5	0.18 ± 0.01	1162 ± 320	11200 ± 2500	1.13 ± 0.07
Allograft + FK506 2	5	0.17 ± 0.01	323 ± 200*	1800 ± 1100*	0.94 ± 0.06
Allograft + FK506 5	5	0.19 ± 0.02	1106 ± 370‡	7600 ± 2200‡	1.11 ± 0.08
Xenograft + FK506 5	5	0.09 ± 0.02	125 ± 81*‡	2500 ± 1400*‡	1.02 ± 0.15

Values are expressed as mean ± SE. p < 0.05 versus \*autograft, †allograft + FK506 2 mg/kg/day, and ‡allograft + FK506 5 mg/kg/day.

**Table 3.** Results of vascular area, perineurium thickness, and number of phagocytic cells in semithin transverse sections of the graft in the groups of mice studied.

Group	n	Total vascular area ( $\mu\text{m}^2$ )	Perineurium thickness ( $\mu\text{m}$ )	Number of phagocytic cells
Autograft	5	5158 $\pm$ 1085	2.92 $\pm$ 0.15	111 $\pm$ 19
Allograft	5	6926 $\pm$ 1251	17.03 $\pm$ 5.91*	121 $\pm$ 23
Allograft + FK506 0.2	5	7893 $\pm$ 2866	19.47 $\pm$ 1.96*	126 $\pm$ 41
Allograft + FK506 2	5	5739 $\pm$ 2175	12.35 $\pm$ 5.58*	82 $\pm$ 13
Allograft + FK506 5	5	4684 $\pm$ 1822	6.92 $\pm$ 2.92‡	65 $\pm$ 8‡
Xenograft	5	5694 $\pm$ 2399	37.42 $\pm$ 8.09*‡§¶	183 $\pm$ 56
Xenograft + FK506 5	5	2192 $\pm$ 1181	7.46 $\pm$ 1.38*‡**	72 $\pm$ 10

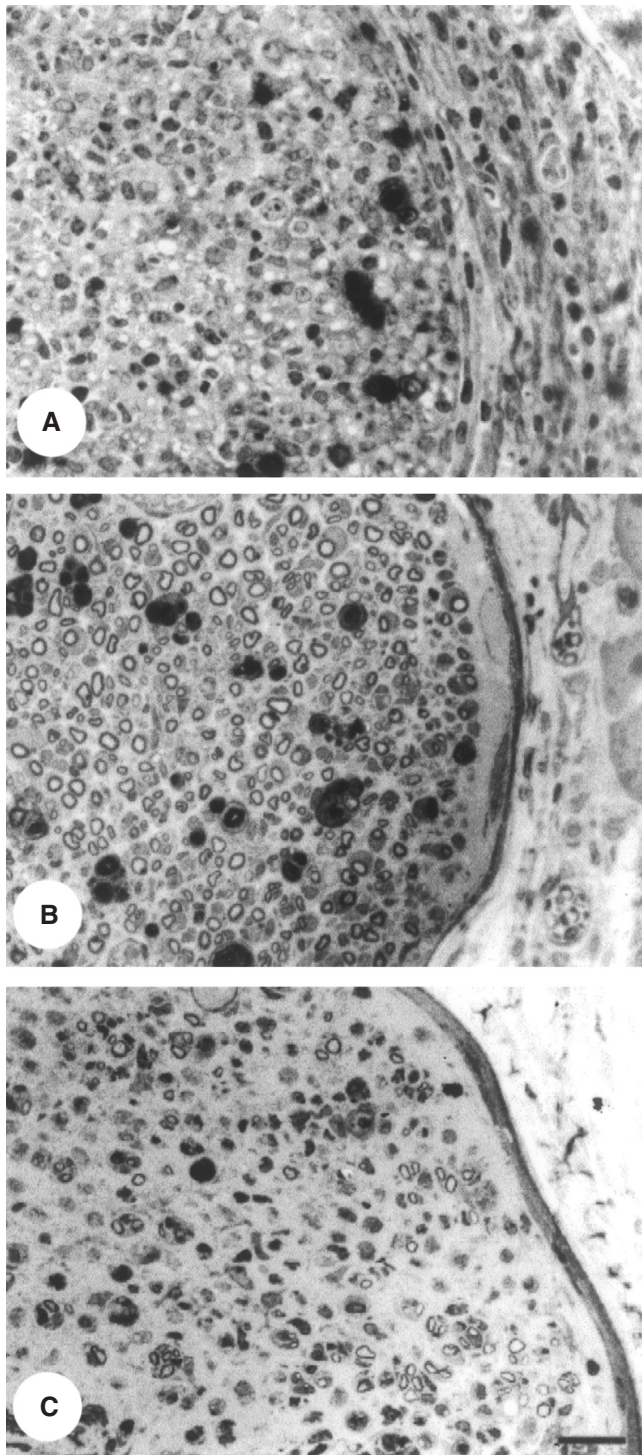
Values are expressed as mean  $\pm$  SE.  $p < 0.05$  versus \*autograft, †allograft, ‡allograft + FK506 0.2 mg/kg/day, §allograft + FK506 2 mg/kg/day, ¶allograft + FK506 5 mg/kg, and \*\*xenograft.

outcome. The best results are obtained using an autologous graft, which elicits the greatest degree of nerve regeneration and recovery of function (Lundborg, 2000). As the supply of autologous material is insufficient, allogenic transplants are a good alternative. Nevertheless, allografts require systemic immunosuppression to avoid rejection (Pollard and Fitzpatrick, 1973a; 1973b; Zalewski and Gulati, 1984b; Mackinnon et al., 1987; Bain et al., 1988; Yu et al., 1990). Regardless of the graft used, the rate of recovery is slow. Thus, the availability of a drug to prevent rejection of the graft and speed regeneration would be great advantages in therapy. FK506, a drug that has both immunosuppressive and nerve regenerative properties, meets both of these criteria. Previous studies have shown that maximal acceleration of the rate of axonal regeneration by FK506 following a sciatic nerve crush is obtained with a dose of 5 mg/kg/day in both rats (Wang et al., 1997) and mice (Udina et al., 2002). The present study confirms and extends these findings by demonstrating that the 5 mg/kg/day dose of FK506 is the most efficacious to avoid rejection of nerve allografts and xenografts. This dose (the maximal dose employed in our study), however, did not elicit the same degree of regeneration in xenografts as compared with treated allografts or untreated autografts.

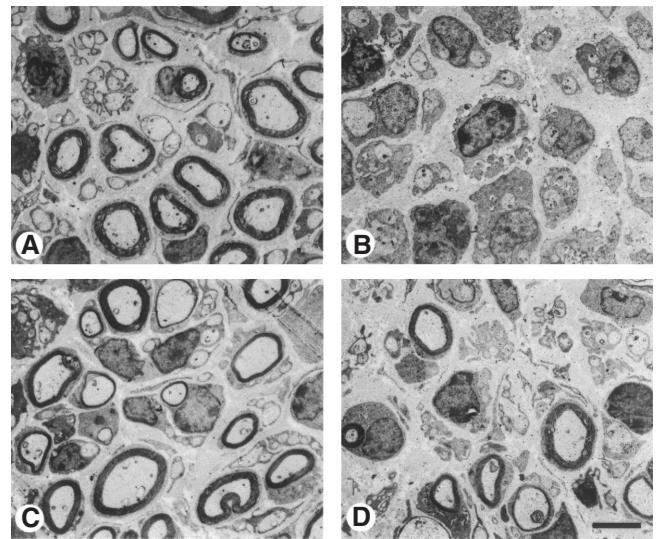
An extensive dose–response analysis (Udina et al., 2002) revealed that FK506 exerts a bimodal dose–response for acceleration in the rate of nerve regeneration following sciatic nerve crush in the mouse. Although the best regeneration rate was achieved with 5 mg/kg/day, 0.2 and 2 mg/kg/day dosages elicited a very similar regeneration course through the first week following injury (Udina et al., 2002). Accordingly, in the present study, we also examined the two lower dosages. Although the lowest dose (0.2 mg/kg/day) increases regeneration in the crush model, it was not found to be as effective as the immunosuppressive dose (5 mg/kg/day) for nerve allografts. This finding is in accordance with another report (Sakai et al., 1991) showing that, to avoid rejection of xenogenic

neural transplants in mice, it is necessary to use a high dose of FK506. The intermediate dose (2 mg/kg/day) is not sufficient to guarantee effective immunosuppression in all the allografts in mice, although it has been reported to prevent rejection of nerve allografts (Büttemeyer et al., 1995; Feng et al., 2001; Okajima et al., 2002) and xenografts (Hebebrand et al., 1997) in rats. Nevertheless, the 2 mg/kg/day dose reportedly promotes regeneration of myelinated axons (at the midpoint of tibial allografts) in mice, close to (Myckatyn et al., 2002) or even higher (Grand et al., 2002) than in untreated isografts. Our study, which examined the nerves not only in the graft but also in the distal regenerative stump, further shows that this dose is indeed able to increase nerve regeneration, most likely different from the immunosuppressive action of the drug.

While experimental studies have shown some degree of regeneration in allografts without immunosuppressive treatment (Bain et al., 1992), the success of functional recovery depends on the length of the graft, because short allografts enable the regenerating axons to cross the gap before the immune reaction exerts its negative consequences on regeneration (Pollard and Fitzpatrick, 1973b). A 3 cm allograft of the cubital nerve at the distal forearm showed similar functional and morphological recovery in immunosuppressed non-human primates compared to placebo-treated non-human primates (Bain et al., 1992). In contrast, regeneration through longer allografts (8 cm in the peripheral nerve of sheep) was not successful (Strasberg et al., 1996). In humans, where the severity of their injury did not allow autograft reconstruction, nerve regeneration was achieved through long peripheral nerve transplants. In these cases, patients were immunosuppressed by either FK506 or cyclosporin A (along with prednisolone) and graft acceptance with some degree of functional recovery was observed, although a few cases exhibited rejection (Bain, 1998; 2000). In our 6 mm allograft model in the mouse, regeneration was very poor, with few regenerating myelinated fibers and a low number of regenerating



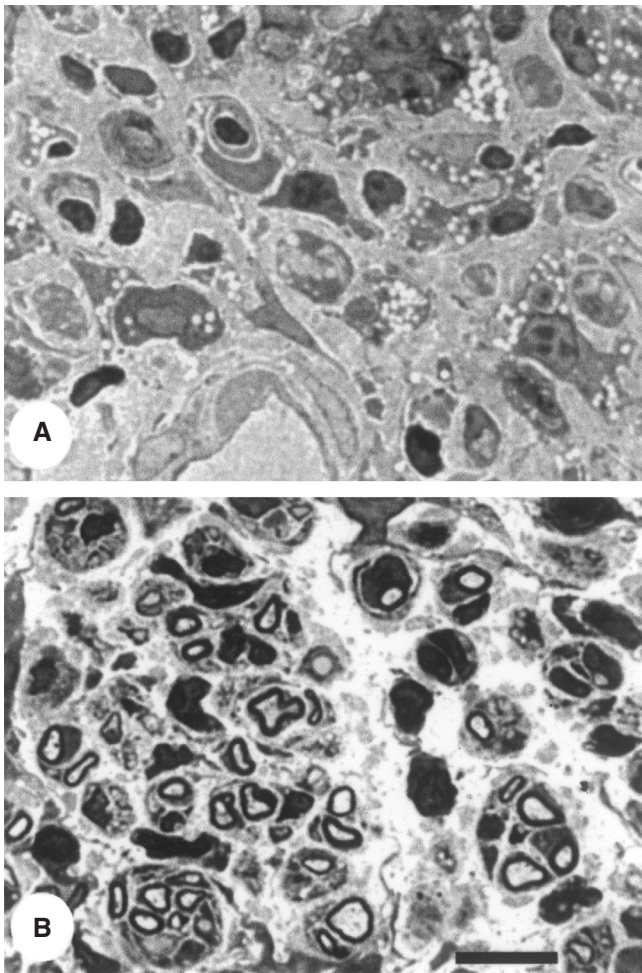
**Figure 2.** Transverse semithin sections of regenerated nerves at midpoint from allografts treated with 0.2 mg/kg/day (A) and 5 mg/kg/day (B) of FK506 and from a xenograft treated with 5 mg/kg/day (C) of FK506. The perineurium thickness is increased in the allograft treated with a low dose of FK506, whereas it is close to normal in grafts treated with 5 mg/kg/day of FK506. Bar = 20  $\mu$ m.



**Figure 3.** Ultrathin sections of regenerated nerves at midpoint from an autograft (A) and an untreated allograft (B), and from an allograft (C) and a xenograft (D) from animals treated with the highest dose (5 mg/kg/day) of FK506. Note the numerous myelinated and unmyelinated axons in the autograft (A) and the treated allograft (C), whereas the untreated allograft (B) demonstrates only nonmyelinated axons. The nerve from the treated xenograft contains fewer and smaller myelinated axons compared to the treated allograft. Bar = 3  $\mu$ m.

unmyelinated axons in the midgraft; no regeneration was found in the distal nerve. On the other hand, treatment with 2 mg/kg/day of FK506 elicited robust regeneration in three of the five allografts (being more limited in the other two allografts); even in the three robust cases, axonal regeneration was decreased (one-third) in the distal nerve. In contrast, regeneration was effective in the distal nerve level in all autografts and allografts treated with 5 mg/kg/day of FK506, with a reduction of 33% for myelinated fibers and 20% for unmyelinated fibers, compared to values in the midgraft. All these results are indicative of nerve regeneration being a length- and time-dependent process.

While immunosuppressive treatment seems compulsory for repair using allografts, prolonged immunosuppression in humans is associated with serious complications, some life threatening (*Shapiro et al., 1990*). One of the most immunogenic components of allografts are the Schwann cells (*Aguayo et al., 1979*), which play a crucial role in axonal regeneration (*Fu and Gordon, 1997; Verdú and Navarro, 1998*). Unless effective immunosuppression is provided, donor Schwann cells in allografts and xenografts are destroyed by the immunological reaction during the first weeks after transplantation (*Pollard and Fitzpatrick, 1973a; Osawa et al., 1987*). Similarly, withdrawal of the immunosuppressive



**Figure 4.** Transverse semithin sections of regenerated nerves taken at the midpoint from xenografts obtained from an untreated animal (A) and an animal treated with the 5 mg/kg/day dose of FK506 (B). Bar = 10  $\mu$ m.

treatment leads to rejection of the graft, largely due to the presence of allogenic Schwann cells because the axons are of host origin. Loss of Schwann cells renders that graft acellular, providing a poor environment for nerve regeneration (*Ide et al., 1983; Lassner et al., 1989*). In such instances, the rate of axonal regeneration is slower, axonal maturation and myelination being delayed compared to autografts and immunosuppressed grafts. The normal ratio of unmyelinated to myelinated axons in the mouse sciatic nerve (average being 1.95) was increased in grafts demonstrating good regeneration (i.e., autografts and allografts treated with 2 and 5 mg/kg/day of FK506 and xenografts treated with 5 mg/kg/day of FK506), with values ranging from 2.2 to 2.8. This increase in the proportion of unmyelinated axons can be attributed to the fact that the leading front of growing axons in a regenerating nerve consists of nonmyelinated axons (*Fu and Gordon, 1997*) that may become myelinated with time

or remain unmyelinated. In the grafts undergoing rejection, a number of unmyelinated axons growing in scattered minifascicles were found but only a few or no small myelinated fibers, indicating a slow and limited degree of regeneration. Although some reports (*Mackinnon et al., 1992; Ishida et al., 1993a; Midha et al., 1993a; 1993b*) indicate a limited degree of recovery over time after withdrawal of immunosuppression, probably due to repopulation of the graft by host Schwann cells (*Ishida et al., 1993b; Midha et al., 1994*), the success of temporary immunosuppression is controversial (*Zalewski and Gulati, 1981; 1984a; Yu et al., 1989*).

The morphological features of the nerves varied considerably between our experimental groups. In the allografts and xenografts, the area of the nerves was increased (due to larger amounts of collagen in the endoneurium) and the perineurium was thickened when compared to autografts. Both these features are commonly observed in injured nerves exhibiting deficient regeneration and in rejected grafts (*Pollard and Fitzpatrick, 1973a; Gómez et al., 1996*). It has been shown that fibroblasts migrate and proliferate readily within acellular nerve grafts, their differentiation into flat perineurial-like cells being dependent on axonal regeneration (*Popovic et al., 1994*). Therefore, the limited axonal regeneration found in untreated allografts and xenografts and in allografts treated with low doses of FK506 was accompanied by expanded connective tissue and increased, although not significantly, area occupied by blood vessels. The regenerating axons in such cases were grouped in small minifascicles, each surrounded by a loose perineurial-like structure (Figs. 1E and 4B), a pattern usually seen in rejected allografts (*Pollard and Fitzpatrick, 1973a; Zalewski and Gulati, 1984b*). Regenerating myelinated axons demonstrated thinner myelin sheaths compared to normal nerve (Fig. 1), which was expected given the short time of our study. Previous studies, however, have shown that axons regenerating through a graft or across a gap remain smaller than normal even at longer times (*Gutman and Sanders, 1943; Giannini et al., 1989; Gómez et al., 1996*).

Another limitation of immunosuppressant therapy is that macrophages also play a positive role in Wallerian degeneration and nerve regeneration (*Beuche and Friede, 1984; Perry and Brown, 1992*), demonstrating the important link between the immune system and nerve regeneration. The slow leukocyte infiltration in C57BL/6/Ola mouse strain in an injured peripheral nerve results in a slow myelin removal and a slow Wallerian degeneration (*Perry et al., 1990*) that leads to the impairment of axonal regeneration (*Bisby and Chen, 1990*). Both cyclosporin A and FK506, however, suppress cellular immunity by blocking T-lymphocyte proliferation (*Schreiber, 1991*), and a higher dose is

needed to alter non-specific immunity (Keicho et al., 1991; Andersson et al., 1992). Our results indicate a reduced number of phagocytic cells within the endoneurium in allografts and xenografts treated with the high dose of FK506 (5 mg/kg/day) compared to untreated grafts or grafts treated with the 0.2 mg/kg/day dose. Moreover, since the initial studies of FK506 showing neuroprotective (Dawson et al., 1993; Sharkey and Butcher, 1994) and neuroregenerative properties (Gold et al., 1994; Lyons et al., 1994), it is now clear that the mechanism underlying nerve regeneration is distinct from that involved in immunosuppression. While the availability of nonimmunosuppressive FK506 analogs (Gold et al., 1997; Steiner et al., 1997a; 1997b) is important for the treatment of neurological situations not requiring immunosuppression, we suggest that FK506 may be beneficial to repair by nerve allografts where both its immunosuppressive and neuroregenerative activities would be advantageous.

## Acknowledgements

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