Comparative Dose-Dependence Study of FK506 and Cyclosporin A on the Rate of Axonal Regeneration in the Rat Sciatic Nerve

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ABSTRACT
The new immunosuppressant drug FK506 (Tacrolimus) increases the rate of nerve regeneration in vivo (Gold et al., 1994; Gold et al., 1995). In the present study, we have examined the dose-dependence of FK506’s ability to enhance nerve regeneration. In the first set of experiments, rats received daily s.c. injections of FK506 (2 mg/kg, 5 mg/kg or 10 mg/kg) for 18 days after a sciatic nerve crush injury. Signs of functional recovery in the hind feet appeared earlier than in saline-treated control rats at all three FK506 dosage; recovery was maximally accelerated in the 5-mg/kg group. Light microscopy at 18 days after nerve crush revealed more regenerating myelinated fibers in FK506-treated rats than in controls; this was most apparent in the 5-mg/kg group. Morphometric analysis of axonal areas in the soleus nerve confirmed that axonal calibers were maximally increased in the 5-mg/kg group. In the second set of experiments, the rate of axonal regeneration was determined by radiolabeling the L5 dorsal root ganglion. Regeneration rate for sensory axons was maximally increased (by 34%) in the 5-mg/kg group. In contrast, cyclosporin A (10 or 50 mg/kg; dosages were selected on the basis of the 1/10 lower potency of cyclosporin A) did not significantly alter the rate of axonal regeneration. Cyclosporin A (50 mg/kg) also failed to increase functional recovery or axonal calibers in the soleus nerve. Because the two drugs share a common mechanism for producing immunosuppression (i.e., calcineurin inhibition), these results indicate that FK506’s nerve regenerative property involves a distinct, calcineurin-independent mechanism.

The immunosuppressant drug FK506 (Tacrolimus) is a macrocyclic lactone that was isolated in 1984 from a soil sample obtained from Tsukuba, Japan, the Streptomyces strain of bacterium hence named Streptomyces tsukubaensis (Kino et al., 1987a; Kino et al., 1987b). FK506 is now being used as primary immunosuppressant therapy (Jain and Fung, 1996) and may replace CsA as the drug of choice (Harding et al., 1989; Siekierka et al., 1989) via the FK506-FKBP-12 complex, which inhibits the calcium and calmodulin-dependent protein phosphatase calcineurin (Liu et al., 1991; Clipstone and Crabtree, 1993; Wiederrecht et al., 1993). Because dephosphorylation of NF-AT is necessary for activation of IL-2 gene transcription, prevention of NF-AT dephosphorylation underlies FK506’s potent immunosuppressant action. Similarly, CsA produces its immunosuppressant effects by inhibiting the calcineurin-mediated dephosphorylation of NF-AT in T-cells after binding to cyclophilin (Mouzaki et al., 1992; for review, see Schreiber and Crabtree, 1992).

Our laboratory was the first to demonstrate that FK506 also increases nerve regeneration in vivo (Gold et al., 1994); concurrently, it was reported that FK506 increases neurite outgrowth in vitro (Lyons et al., 1994). In a subsequent study,
we showed that FK506 increases nerve regeneration by increasing the rate of axonal regeneration (Gold et al., 1995). The mechanism by which FK506 produces its axonal regenerative effect is unknown. However, on the basis of its mechanism of action in inhibiting T-cell proliferation (see above), the following hypothesis has been presented (Gold et al., 1995; Snyder and Sabatini, 1995). Besides being present in T-cells, the immunophilin FKBP-12 is also found in neurons, where it co-localizes with calcineurin (Steiner et al., 1992; Dawson et al., 1994), an enzyme that makes up 1% of brain protein (Klee, 1991). It is known that an important calcineurin substrate in neurons is GAP-43, which plays an important role in growth cone formation and axon elongation (Skene and Willard, 1981b; Skene and Willard, 1981a; Skene, 1989; Jap Tjoen San et al., 1995). Thus FK506 could increase nerve regeneration by increasing the phosphorylation of GAP-43 via its known ability to inhibit the activity of calcineurin. This proposal appears to be supported by a preliminary report (Steiner et al., 1991) showing that FK506 increases GAP-43 phosphorylation in vitro. Alternatively, FK506 may act via a different mechanism that does not involve calcineurin but is perhaps still mediated by FKBP-12. In this context, a role for FKBP-12 in nerve regeneration is supported, whether or not calcineurin plays a role, by the observation that axotomy increases FKBP-12 mRNA expression both in the motor (facial and lumbar spinal) and sensory (DRG) neurons (Snyder and Sabatini, 1995). A calcineurin-independent mechanism is particularly intriguing because it would indicate that the immunosuppressant and nerve regenerative properties of FK506 are separable.

In the present study, we have extended our initial observations by using behavioral, morphological and radiolabeling techniques to examine the dose-dependence of FK506's effect on nerve regeneration. In addition, as an initial step toward determining the mechanism by which FK506 increases nerve regeneration, we examined, using radiolabeling techniques, whether CsA shares FK506's nerve regenerative property. A preliminary report of these findings has been presented (Gold et al., 1996b).

Materials and Methods

Animals and drug administration. A total of 69 male Sprague-Dawley rats 6 weeks old were used for morphological and transport studies. FK506 was obtained from Fujisawa Pharmaceuticals Inc. (Osaka, Japan) as a new formulation that readily dissolves in saline. Because our initial studies showed that a 1-mg/kg dose of FK506 produces a modest increase in regeneration (Gold et al., 1995), rats were given three higher doses of FK506 (2, 5 or 10 mg/kg) in the present study. CsA (Sigma Chemical Co., St. Louis, MO) is approximately 1/10 as potent as FK506 in terms of its ability to inhibit T-cell proliferation (Kino et al., 1987a; Kino et al., 1987b; Toci et al., 1989) and as an immunosuppressant in humans (Starzl et al., 1989; Hoffman et al., 1990). Thus, in view of our finding that a significant effect of FK506 on regeneration was found using a 1-mg/kg dose (Gold et al., 1994; Gold et al., 1995), and because the maximal response was obtained using a 5-mg/kg dose of FK506 (see "Results"), rats were given CsA at a dose of either 10 or 50 mg/kg. Both drugs were given as a s.c. injection in the back of the neck beginning on the day of nerve crush (see below); controls received an equivalent volume of saline (5 ml/kg).

Surgical procedures. All surgical procedures were conducted under sterile conditions using protocols approved by the University Animal Care and Use Committee (AAALAC accreditation). As previously described (Gold et al., 1994; Gold et al., 1995), rats were anesthetized with 2% halothane, and the sciatic nerves were exposed bilaterally and crushed twice (for a total of 30 s using a No. 7 Dumont jeweler's forceps) either at the level of the hip (for morphological studies) or at the junction of spinal nerves L4 and L5 (for measurement of axonal regeneration rate). A sterile 9-0 suture was tied through the epineurial sheath to mark the crush site.

Clinical assessment. Functional recovery was assessed in FK506-treated (2, 5 and 10 mg/kg; n = 3 per dosage group) and CsA-treated (50 mg/kg; n = 3) animals used for morphological study only. Because the three dosage groups were studied separately, 1 to 2 saline-treated axotomized control rats were used for each group (total n = 5). As previously described (Gold et al., 1994), animals were examined blindly by two investigators each day until perfusion (18 days). The number of days after nerve crush until the animal demonstrated onset of an ability to right its foot and move its toes (termed "onset") and the number of days until the animal demonstrated an ability to walk on its hind feet and toes (termed "walking") were recorded for each animal. To obtain records during walking, we marked the hind feet with tempora paint and allowed the animals to freely walk across a sheet of paper between days 14 and 18. Toe spread during walking was defined as the distance between the first and fifth, and between the second and fourth, digits (measured to the nearest 0.5 mm); this represents a reliable and reproducible index of functional recovery that, unlike the sciatic nerve function index, is not influenced by how fast the animal walks (Walker et al., 1994). At least three footprints were analyzed for each animal, and the resultant average value for each animal was used to calculate mean values and standard errors for each group (i.e., FK506, CsA and saline). Mean values were compared using one-way ANOVA followed by FSLD for comparison of individual values (STATVIEW, Abacus Concepts, Inc., Berkeley, CA). The distances between the first and fifth digits and those between the second and fourth digits gave similar results, so only the data from the former analysis are presented in this report.

Tissue fixation and preparation for morphological studies. At 18 days after nerve crush, the rats were deeply anesthetized with 4% halothane, heparinized and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 s followed by 1 liter of 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and fixed at 4°C for 24 h. The following tissues were sampled at known distances from the crush site: sciatic nerve at (5, 10 and 15 mm from the crush site); peroneal nerve, sural nerve and tibial nerves (30 mm from the crush site); distal tibial nerve (at 40 mm from the crush site). Samples were also taken from the following tissues: tibial branches supplying the medial and lateral gastrocnemius muscles and the soleus muscle; interosseus muscles. Tissues were placed in 0.1 M sodium phosphate buffer (pH 7.4), postfixied with 1% osmium tetroxide (in 0.1 M phosphate buffer) for 2.5 h, dehydrated in ethanol and embedded in plastic. Semithin sections (0.5 µm) were stained with toluidine blue; thin sections were stained with uranyl acetate and lead citrate and examined in a JOEL 100X electron microscope.

Morphometric analysis. Analysis of axonal calibers was performed in the branch of the posterior tibial nerve supplying the soleus muscle; tissues were mounted onto film-supported 75-mesh grids. The entire nerve cross-sections were photographed and printed at a final magnification of ×10,000. Axonal areas of both myelinated and unmyelinated fibers were determined by tracing the axolemma using a Houston Instrument HI-PAD digitizing tablet connected to an IBM XT computer with appropriate software (Bioquant IV, R&M Biometrica, Nashville, TN). Cumulative histograms were constructed from these data, and mean values and standard errors were calculated. Because axonal areas did not demonstrate a normal distribution, the largest 30% of axons were selected from each animal for the purpose of statistic analysis. From this population, mean axonal areas were determined for each nerve. For each group (i.e., FK506, CsA and saline), mean values and standard errors were calculated on the basis of these individual values. Mean values
for axonal areas were compared using ANOVA followed by FLSD for comparison of individual values (STATVIEW).

Measurement of axonal regeneration rate. For the measurement of nerve outgrowth distances, FK506-treated (5- and 10-mg/kg groups only; *n* = 6 per dosage group), CsA-treated (10 and 50 mg/kg; *n* = 4 per dosage group), and saline-treated (*n* = 6) axotomized rats were anesthetized with 2% halothane, a laminectomy performed and both L5 DRG injected with 50 µCi/DRG of [3H]-leucine (Amersham, Arlington Heights, IL; specific activity 150 Ci/mm mol) on day 11 or 14. Body temperature was thermostatically maintained at 37°C (Harvard Apparatus, South Natick, MA) during the period of anesthesia. The maximal extent of transported protein-incorporated radioactivity was determined as previously described (Gold et al., 1995). Briefly, 24 h later (days 12 and 15, respectively), the rats were killed by euthanasia solution, and the nerves (L5 spinal roots to the distal sciatic nerve branches) were harvested intact (Droz and Warshawsky, 1963), cut into 2-mm segments and each segment solubilized in 0.5 ml Solvable (NEN Research Products, Boston, MA) at 37°C for 24 h. Radioactivity was determined in a liquid scintillation spectrometer (Packard, Downers Grove, IL). Then dpm were normalized to the amount of radioactivity at the crush site (100%) and plotted against the distance from the crush site for each nerve. The maximal extent of outgrowth was determined from the point of intercept between a line drawn through the front of radioactivity collected in the distal portion of the nerve and background radioactivity (Ochs and Ranish, 1970). Mean values for the maximal outgrowth distances were calculated and the values were plotted against the number of days since axotomy. Next a regression line was generated (STATVIEW), and the slope of the line used to estimate the rate of axonal regeneration between the two time-points studied (days 12 and 15).

All values are mean ± S.E.M.

Results
FK506 dose-dependently speeds functional recovery. The time until the first signs of toe movement and return of ability to walk on the toes was reduced in all three FK506-treated groups (table 1). Overall, the 5 mg/kg FK506-treated group demonstrated the earliest recovery of function in the hind feet. For example, the number of days until the onset of an ability to right the foot and move the toes (“onset”) and the number of days until the animal demonstrated an ability to walk on its hind foot and toes (“walking”) were reduced from 16.8 ± 0.2 days to 14 ± 0 days and from 17.8 ± 0.2 days to 15 ± 0 days, respectively, in the saline-treated animals (*n* = 5) and in the 5-mg/kg group (*n* = 3), respectively. Representative footprints are shown in figure 1.

Footprints were analyzed by determining toe spread distances between the first and fifth digits (see “Materials and Methods”). The greatest improvement was observed in the 5 mg/kg FK506-treated group. These distances were significantly (*P* < .05) greater in the 5 mg/kg FK506-treated (*n* = 3) compared with the saline-treated (*n* = 5) animals; mean values for the distance between the first and fifth digits were 12.2 ± 0.40 mm and 10.7 ± 0.30 mm, respectively; in normal, uninjured animals, values ranged from 19 to 21 mm.

TABLE 1

| Functional recovery after sciatic nerve crush in saline-treated and FK506-treated rats |
|---------------------------------|---------------------------------|----------------|----------------|----------------|
|                                 | Saline                         | 2 mg/kg | 5 mg/kg | 10 mg/kg |
| Days to onset                   | 16.8 ± 0.2^a^                 | 15 ± 0  | 14 ± 0^†^ | 14.3 ± 0.3^†^ |
| Days to walking                 | 17.8 ± 0.2^a^                 | 16 ± 0  | 15 ± 0^††^ | 15.3 ± 0.3^††^ |

^a^ Number of days until initial signs of return of toe movement.

^b^ Number of days until initial return of ability to walk on hind feet and toes.

^c^ Values are mean ± S.E.M.

^†^ *P* < .0001, compared with saline group (one-way ANOVA followed by FLSD).

^††^ *P* < .01, compared with 2-mg/kg group (one-way ANOVA followed by FLSD).

^†††^ *P* < .001, compared with 2-mg/kg group (one-way ANOVA followed by FLSD).

Fig. 1. FK506 speeds functional recovery. Representative footprints 18 days after axotomy from animals given daily s.c. injections of saline (panel A) or FK506 at a dose of 2 mg/kg (panel B), 5 mg/kg (panel C), or 10 mg/kg (panel D). Each image was generated by scanning the original footprint using MacImage (Xerox Imaging Systems, Inc.). All animals exhibit signs of recovery from axotomy as shown by an ability to walk on the foot. Note the near normal appearance of the footprint from the rat given the 5-mg/kg dose of FK506; all five toes are clearly discernible, there is good toe spread, and there is no imprint made by the heel, which reflects the ability of this animal to walk on the ball of its foot and toes. Prints from all other animals show less toe spread and walking ability.

FK506 dose-dependently accelerates the maturation and elongation of regenerating axons. As previously reported (Gold et al., 1994; Gold et al., 1995), the regenerated axons in rats treated with FK506 are in a more advanced stage of maturation. Light microscopy (fig. 2) revealed the presence of more regenerative myelinated axons in all three groups treated with FK506 (i.e., 2 mg/kg, 5 mg/kg and 10 mg/kg; *n* = 3 per group) compared with the group treated with saline for 18 days after axotomy, the most numerous being observed in the group treated with 5 mg/kg FK506 (fig. 2C). By electron microscopy (fig. 3), the regenerated axons in the FK506-treated group were larger in size and contained more myelinated axons than those in the saline-treated group. In agreement with the clinical appearance of the animals (see above), the most pronounced increase in axonal sizes was apparent in the 5 mg/kg FK506-treated group (fig. 3C).

These morphological impressions were confirmed by morphometric analysis of axonal areas in soleus nerve at 18 days after axotomy. First, the numbers of myelinated axons were found to increase from control values by 60%, 217% and 150%, in the 2 mg/kg, 5 mg/kg and 10 mg/kg FK506-treated.
groups, respectively (table 2). Second, axonal calibers demonstrated a shift to larger axonal areas, compared with saline-treated rats, in all three groups of FK506-treated rats (fig. 4). Mean axonal areas in the soleus nerve at 18 days after axotomy were significantly ($P < .01$) increased from control values in all three FK506 dosage groups (table 2); values were $0.6 \pm 0.02 \mu m^2$ in saline-treated rats and, in FK506-treated rats, $1.1 \pm 0.03 \mu m^2$ (2-mg/kg group), $1.3 \pm 0.04 \mu m^2$ (5-mg/kg group) and $1.2 \pm 0.05 \mu m^2$ (10-mg/kg group) which represents an increase of 83%, 120% and 100%, respectively. The mean axonal area for the 5-mg/kg group was also significantly ($P < .05$) greater than that obtained for the 2-mg/kg group (table 2). For comparison with our previous study (Gold et al., 1995), a 1-mg/kg dose elicited a 67% increase (the mean value being $1.0 \pm 0.08 \mu m^2$) in mean axonal area.

Because there are numerous very small regenerating nerve fibers, we also examined the top 30% of axonal areas (see Gold et al., 1995), as shown in figure 5. Mean axonal areas of the largest 30% of axons (table 2) increased significantly from $1.6 \pm 0.05 \mu m^2$ in saline-treated rats to $2.5 \pm 0.07 \mu m^2$ (2 mg/kg), $3.0 \pm 0.09 \mu m^2$ (5 mg/kg) and $2.9 \pm 0.10 \mu m^2$ (10 mg/kg) in FK506-treated rats ($P < .001$). Significant increases in the largest 30% of axonal areas were also found in rats treated with 5 mg/kg FK506 and in those treated with 10 mg/kg, compared with that in rats treated with 2 mg/kg FK506 ($P < .001$). No significant difference was found between rats treated with 5 mg/kg and 10 mg/kg.

FK506 dose-dependently increases the maximal rate of sensory axonal regeneration. We measured axonal regeneration rate for sensory neurons in the 5 and 10 mg/kg FK506-treated groups ($n = 6$ per group) only because we had previously (Gold et al., 1995) determined the regeneration rate for the 1-mg/kg dose, and the present morphological data revealed only a slight difference in axonal areas between the 1-mg/kg and 2-mg/kg groups. Maximal nerve outgrowth was determined by measuring the distance traveled by the fast axonally transported radiolabeled proteins in sensory axons at 12 and 15 days after axotomy (see "Material and Methods"). Radiolabeled profiles showed that the maximal extent of outgrowth was more advanced along the sciatic nerve of FK506-treated rats both at 12 and at 15 days (fig. 6). The axon regeneration rate (fig. 7) was significantly ($P < .05$) increased from 3.8 mm/day in the saline-treated rats to 5.1 mm/day (5-mm/kg group) and 4.9 mm/day (10-mm/kg group) in the FK506-treated groups; the difference between the 5-mg/kg and 10-mg/kg groups was not statistically significant (fig. 6). These data represent a 34% and a 29% increase in regeneration rate for the 5-mg/kg and 10-mg/kg groups, respectively.
CsA fails to increase the maximal rate of sensory axonal regeneration. In marked contrast to FK506, CsA did not significantly (P > .05) alter the rate of axonal regeneration for sensory neurons (figs. 8 and 9). Maximal regeneration distances were slightly more advanced along the nerve, and to a similar extent, in both the 10-mg/kg and 50-mg/kg groups (n = 4 per group) compared with controls (fig. 8); this difference, though not significant, was somewhat greater at the earlier (i.e., day 12) time-point. Accordingly, both the 10-mg/kg and 50-mg/kg dosages resulted in a slight, nonsignificant, reduction in overall regeneration rate between days 12 and 15 (fig. 9); regeneration rates decreased from 3.8 mm/day in the saline-treated rats to 3.7 mm/day (10-mg/kg group) and 3.6 mm/day (50-mg/kg group), which represents a 3% and a 5% decrease, respectively. Thus a dose-dependent alteration in axonal regeneration was not observed using dosages an order of magnitude greater than for FK506; dosages were selected on the basis of CsA being 1/10 as potent as FK506 in other systems (Kino et al., 1987a; Kino et al., 1987b; Tocci et al., 1989).

CsA fails to increase functional recovery and elongation of regenerating axons. Functional and morphological studies confirmed the failure of CsA to speed nerve regeneration. CsA (50 mg/kg) did not significantly alter functional recovery. The number of days until “onset” of functional recovery (see “Materials and Methods”) in the CsA-treated rats (n = 3) was slightly but not significantly (P > .05) increased from the control values (16.8 ± 0.20 days) to 17.6 ± 0.33 days; only one of the three CsA-treated rats reached the “walking” stage by the termination of the experiment (at 18 days).

Morphometric analysis demonstrated that axonal areas in the soleus nerve at 18 days after axotomy from rats given CsA (50 mg/kg) were not significantly (P > .05) different from saline-treated rats (see above). The number of myelinated axons was slightly but not significantly (P > .05) reduced from control values (table 2). Mean axonal areas for the entire population and the largest 30% of axons were 0.7 ± 0.03 μm² (n = 1524 axons) and 1.8 ± 0.06 μm² (n = 458 axons), respectively, which were similar to control values (table 2).

TABLE 2

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<th>10 mg/kg</th>
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<td>6</td>
<td>5</td>
<td>6</td>
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<tr>
<td>Number of myelinated axons</td>
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<td>10 ± 1.1</td>
<td>19 ± 3.5*†</td>
<td>15 ± 3.7*</td>
<td>2 ± 1.1</td>
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<tr>
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<td>All axons</td>
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<td>1.1 ± 0.03**</td>
<td>1.3 ± 0.04**†</td>
<td>1.2 ± 0.05**</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>Largest 30%</td>
<td>1.6 ± 0.05</td>
<td>2.5 ± 0.07*</td>
<td>3.0 ± 0.09*†</td>
<td>2.9 ± 0.10*</td>
<td>1.8 ± 0.06</td>
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</table>

* Values are mean ± S.E.M.
** P < .01, compared with saline group (one-way ANOVA followed by FLSD).
† P < .05, compared to 2-mg/kg group (one-way ANOVA followed by FLSD).

Fig. 4. Axons in the soleus nerve are larger in FK506-treated animals. Cumulative histograms showing the distribution of axonal areas 18 days after nerve crush. Note the shift to the right for axonal areas (which indicates larger axons) from FK506-treated rats (constructed from 1285, 1209 and 933 axons from the 2 mg/kg, 5 mg/kg and 10 mg/kg FK506-treated rats, respectively) compared with the saline-treated rats (constructed from 1251 axons). The greatest shift is present in the 5-mg/kg group.

Fig. 5. The largest 30% of axons in the soleus nerve from FK506-treated animals show an even greater increase in size. Cumulative histograms of the top 30% of axonal areas (presented as a percentage of the total numbers of axons in each subpopulation) showing the distribution of axonal areas 18 days after nerve crush. Each histogram was constructed from 377, 386, 365 and 279 axons from saline-treated and 2 mg/kg, 5 mg/kg and 10 mg/kg FK506-treated groups, respectively. Just as for the entire population (see fig. 4), the greatest shift is present in the 5-mg/kg group.
Discussion

**FK506 and peripheral nerve regeneration.** The present study supports and extends our initial findings (Gold et al., 1994; Gold et al., 1995) by showing that FK506 increases nerve regeneration in a dose-dependent fashion. Our data thereby establish FK506’s *in vivo* nerve regenerative property. FK506 dose-dependently increases the rate of axonal regeneration, the morphological equivalent being the presence of large, more myelinated regenerating axons. From a functional standpoint, the animal demonstrates a faster return of function in the affected hindlimb. Moreover, these dose-dependence studies reveal that the most effective dose for promoting axonal regeneration for sensory fibers in the rat is 5 mg/kg as a s.c. daily injection. This dosage was consistently found to be the most effective, regardless of the method used (i.e., functional recovery, morphometric analysis or axonal regeneration rate) to assess nerve regeneration. Interestingly, the 10-mg/kg dose of FK506 was less effective in promoting nerve regeneration in all three analyses. Although the reason for this is presently unclear (see below), we have also noted that whereas low (pM to nM) concentrations markedly promote neuritic outgrowth *in vitro* (using SH-SY5Y cells), high (µM) concentrations of FK506 actually inhibit neuritic elongation (B.G. Gold and M. Zeleny-Pooley, unpublished observation).

In comparing the present morphological and axonal regeneration data with our previous study (Gold et al., 1995), it is important to note that the 1-mg/kg dose reported in our initial studies (Gold et al., 1994; Gold et al., 1995) was an estimate. This was due to the nature of the FK506 material (pharmaceutical capsules for human use) available at the time. After extraction of the drug, the final concentration in our dosage solution was estimated by high-performance liquid chromatography (Gold et al., 1994). The present data, by showing that a 2-mg/kg dose of FK506 increases mean axonal area for regenerating axons slightly, though

**Fig. 6.** FK506 increases the axonal regeneration distance at 12 (panel A) and 15 (panel B) days after nerve crush in both the 5-mg/kg (top) and 10-mg/kg (bottom) groups. Shown is average percent distribution of disintegrations per minute of radiolabeled proteins 24 h after radiolabeling the L5 DRG (three animals per group) from saline-treated (●) and FK506-treated (○) animals. Transport profiles from the saline-treated animals at 12 and 15 days are reproduced in both the top and bottom figures. The front of each transport profile (the average position being marked by arrows) indicates the maximal extent of the growing axonal sprouts, which are further advanced along the nerves from the FK506-treated rats. The increase is more dramatic in the 5-mg/kg group than in the 10-mg/kg group. Bars are S.E.M. Lack of error bar indicates that S.E.M. is smaller than symbol.
Fig. 7. FK506 increases the axonal regeneration rate after sciatic nerve crush. The plots show mean transport distance measured at 12 and 15 days. By regression analysis, regenerative rates (slope of the line) between the time-points studied (i.e., 12 and 15 days) were significantly ($P < .05$) increased in both the 5-mg/kg and 10-mg/kg groups compared with the saline-treated rats; regeneration rates were increased from controls by 34% and 29% in the 5-mg/kg and 10-mg/kg groups, respectively. Bars are S.E.M. Lack of error bar indicates that S.E.M. is smaller than symbol.

Fig. 8. CsA does not increase the axonal regeneration distance at 12 (panel A) or 15 (panel B) days after nerve crush in either the 10-mg/kg (top) and or the 50-mg/kg (bottom) group. Shown is average percent distribution of disintegrations per minute of radiolabeled proteins 24 h after radiolabeling the L5 DRG (three animals per group) from saline-treated (●) and CsA-treated (○) animals. Transport profiles from the saline-treated animals at 12 and 15 days are reproduced in both the top and bottom figures. The front of each transport profile (the average position being marked by arrows) indicates the maximal extent of the growing axonal sprouts, which are slightly advanced along the nerves from the CsA-treated rats. Bars are S.E.M. Lack of error bar indicates that S.E.M. is smaller than symbol.
not significantly, more than the value previously reported for a 1-mg/kg dose (see fig. 4), provide confirmation of our original dosage estimate (Gold et al., 1994).

Separation of FK506’s nerve regenerative and immunosuppressant properties. The major new finding of the present study is that the immunosuppressant drug CsA does not share FK506’s ability to alter nerve regeneration. Although CsA produced a slight but not significant increase in regeneration distance at the earlier (i.e., day 12) time-point of study, this effect was not dose-dependent, and regeneration rate was not altered. In fact, a slightly greater reduction in rate was noted at the higher dose (50 mg/kg), which indicates that our failure to observe a significant effect on regeneration rate was not altered. In fact, a slightly greater reduction in rate was noted at the higher dose (50 mg/kg), which indicates that our failure to observe a significant effect on regeneration by CsA is not due to examination of too low a dose. In agreement with these in vivo findings, CsA is ineffective in promoting neuritic outgrowth in SH-SY5Y cells (as determined by failure of the drug to alter the overall distribution of neuritic lengths), although (interestingly, considering our in vivo findings) some rare cells with long processes are observed (B.G. Gold and M. Zeleny-Pooley, unpublished observation). This is a surprising and unexpected finding, because both agents produce immunosuppression via similar mechanisms (see below). Thus the present results strongly suggest that the ability of FK506 to enhance nerve regeneration is separable from its immunosuppressant properties (for further discussion, see Gold et al., 1994).

The discordant effect of FK506 and CsA on nerve regeneration stands in marked contrast to their action in T-cells. The present findings, therefore, provide new insight into the mechanism underlying FK506’s nerve regenerative property. FK506 and CsA produce their immunosuppressant effect by inhibiting calcineurin activity, though via binding to distinct immunophilins, FKBP-12 and cyclophilin, respectively. Calcineurin plays a key role in regulating T-cell proliferation by dephosphorylating NF-AT, thereby enabling it to enter the nucleus where it increases IL-2 secretion, which stimulates T-cell proliferation. In our previous paper (Gold et al., 1995), we proposed that FK506 may accelerate axonal regeneration by increasing the phosphorylation state of GAP-43 as a consequence of calcineurin inhibition. This hypothesis is untenable in light of our demonstration that CsA does not alter nerve regeneration. However, the slight inhibitory effect of CsA on axonal regeneration suggests that overstimulation of this pathway, perhaps leading to loss of the dynamics of GAP-43 phosphorylation (Meiri et al., 1991), may explain why high dosages of FK506 are less effective in promoting nerve regeneration (present study) and neuritic outgrowth in vitro (B.G. Gold and M. Zeleny-Pooley, unpublished observation). Recent studies in other systems reveal that calcineurin inhibition is also not responsible for mediation of FK506’s ability to overcome multiple drug resistance in yeast (Hemenway and Heitman, 1996) and aldosterone-stimulated sodium transport in kidney cells (Rokaw et al., 1996).

Immunophilins in nerve regeneration. Although the present findings rule out a role for calcineurin in FK506’s ability to increase nerve regeneration, FKBP-12 may still mediate FK506’s regenerative effect. Apart from its interaction with calcineurin, FKBP-12 has also been shown to be associated with two calcium release channels: the ryanodine receptor (Jayaraman et al., 1992) and the IP3 receptor (Cameron et al., 1995b). Interestingly, a very recent preliminary report (Takei et al., 1996) shows that inactivation of the type 1 IP3 receptor in chick DRG growth cones inhibits neuritic growth. Whether FK506’s ability to stabilize these channels and alter calcium release (Brillantes et al., 1994; Cameron et al., 1995a) is involved in its regenerative effects is an important area for future investigation (see Snyder and Sabatini, 1995). However, support for this possibility is diminished by the apparently higher concentrations of FK506 (10–100 nM) necessary to disrupt association of FKBP-12 with the IP3 receptor (Cameron et al., 1995b), compared with the concentrations that produce neurite outgrowth in vitro (B.G. Gold and M. Zeleny-Pooley, unpublished observation).

An alternative mechanism by which FK506 could alter nerve regeneration via FKBP-12 is suggested by the discovery that FKBP-12 functions as an inhibitor of TGF-β1 receptors (Wang et al., 1994; Wang et al., 1996). Thus FK506, by dissociating FKBP-12 from TGF-β1 receptors, could activate the TGF-β1 pathway that is known to stimulate NGF synthesis in glial cells (Lindholm et al., 1990). In view of the
propose that this might be achieved by infecting or neuromodulating brain neurons that have also been shown to be protected by FK506 against experimental brain ischemia (Sharkey and Butcher, 1994; Tokime et al., 1996; Ide et al., 1996). This finding suggests that FKBP-52-glucocorticoid receptors play a role in the ability of FK506 to reduce cerebral infarction. Whether this pathway plays a role in FK506’s ability to increase nerve regenerative is unknown. An important issue for future research is whether FK506’s various in vivo neuronal properties, including nerve regeneration (Gold et al., 1994; Gold et al., 1995), prevention of kindling-induced sprouting of mossy fibers (Zhuang et al., 1996) and neurotoxicity (Lopez et al., 1991; Mueller et al., 1994; Wijdicks et al., 1994; Bronster et al., 1995; Vincenti et al., 1996), are mediated by different FKBP.

Prospective new class of drugs for nerve regeneration. In summary, the present study confirms the nerve regenerative properties of FK506. Furthermore, our results indicate that distinct mechanisms underlie the immunosuppressant (calcineurin-dependent) and nerve regenerative (calcineurin-independent) properties of FK506. Thus, on the basis of structural analysis of FK506-FKBP interactions (Griffith et al., 1995; Itoh et al., 1995; Itoh and Navia, 1995; Shuker et al., 1996), it should be possible to separate these properties and design new FKBP ligands (Batchelor et al., 1994; Armistead et al., 1995) that do not inhibit calcineurin. These compounds could then be developed (Navia and Chaturvedi, 1996) for in vivo testing of their ability to enhance nerve regeneration. The development of such compounds may lead to the generation of new drugs for the treatment of human peripheral nerve injuries.

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