The effects of cold preservation and subimmunosuppressive doses of FK506 on axonal regeneration in murine peripheral nerve isografts

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BACKGROUND: FK506 is a frequently used immunosuppressant with neuroregenerative effects. The neuroregenerative and immunosuppressive mechanisms of FK506, however, are distinct, suggesting that FK506 may stimulate nerve regeneration at lower doses than are needed to induce immunosuppression. The effects of cold preservation, a technique known to improve axonal regeneration through nerve allografts, are not well studied in nerve isografts and are also reported here.

OBJECTIVES: To determine the effects of subimmunosuppressive doses of FK506 and cold preservation on nerve regeneration in isografts.

METHODS: The neuroregenerative properties of immunosuppressive and subimmunosuppressive doses of FK506 were compared in a murine model receiving either fresh or cold preserved nerve isografts. Sixty female BALB/cJ mice were randomized into six groups. Animals in groups I, III and V received fresh nerve isografts. Animals in groups II, IV and VI received cold-preserved nerve isografts. Mice in groups I and II received no medical therapy, while those in groups III and IV received subimmunosuppressive doses of FK506, and those in groups V and VI received immunosuppressive doses as confirmed by mixed lymphocyte reactivity assays. Nerve regeneration was evaluated with histomorphometry and functional recovery was evaluated with walking track analysis.

RESULTS: Pretreatment with cold preservation did not significantly affect neural regeneration. The potent neuroregenerative effect of immunosuppressive doses of FK506 was confirmed, and the ability of subimmunosuppressive doses of FK506 to stimulate axonal regeneration in murine nerve isografts is reported.

CONCLUSIONS: Less toxic subimmunosuppressive doses of FK506 retaining some neuroregenerative properties may have a clinical role in treating extensive nerve injuries.

Key Words: *Cold preservation; FK506; Isograft; Murine*

Effets de la conservation par le froid et de doses sous-immunodépresseuses de FK506 sur la régénération axonale dans des isogreffes de nerf périphérique chez des souris

CONTEXTE : Le FK506, un immunodépresseur souvent utilisé, produit des effets de neurorégénération. Toutefois, les mécanismes neurorégénératoires et immunodépresseurs du FK506 sont indépendants, ce qui donne à penser que le médicament peut susciter une régénération nerveuse à des doses plus faibles que celles nécessaires à l'immunodépresseur. Par ailleurs, les effets de la conservation par le froid, technique connue pour améliorer la régénération axonale dans les allogreffes de nerf, sont peu étudiés dans les isogreffes; nous nous pencherons donc sur leurs caractéristiques.

OBJECTIF : Déterminer les effets de doses sous-immunodépresseuses de FK506 et de la conservation par le froid sur la régénération nerveuse dans les isogreffes.

MÉTHODE : Nous avons comparé les propriétés neurorégénératrices du FK506 à des doses immunodépresseuses et sous-immunodépresseuses dans un modèle murin recevant soit des isogreffes fraîches, soit des isogreffes conservées par le froid. Soixante souris femelles BALB/cJ ont été divisées au hasard en six groupes. Les groupes I, III et V ont reçu des isogreffes nerveuses fraîches, tandis que les groupes II, IV et VI ont reçu des isogreffes nerveuses conservées par le froid. Les souris des groupes I et II n'ont été soumises à aucun traitement médical, celles des groupes III et IV ont reçu des doses sous-immunodépresseuses de FK506 et celles des groupes V et VI, des doses immunodépresseuses, confirmées par des épreuves de réaction lymphocytaire mixte. La régénération nerveuse a été évaluée à l'aide de l'histomorphométrie, et la réadaptation fonctionnelle, à l'aide de l'analyse des traces de marche.

RÉSULTATS : Le prétraitement à la conservation par le froid n'a pas amélioré sensiblement la régénération neurale. Le puissant effet neurorégénérateur des doses immunodépresseuses de FK506 a été confirmé; quant à la capacité des doses sous-immunodépresseuses de FK506 de stimuler la régénération axonale dans les isogreffes nerveuses de souris, elle est ici décrite.

CONCLUSION : Les doses sous-immunodépresseuses de FK506, moins toxiques que les doses immunodépresseuses mais possédant quand même des propriétés neurorégénératrices, pourraient jouer un rôle clinique dans le traitement de lésions nerveuses étendues.

Nerve grafts are often required to affect a tension-free repair following peripheral nerve injury. Injured axons give rise to a regenerating unit comprised of a parent axon seeking motor or sensory targets by way of multiple terminal sprouts (1). Numerous agents have been tested for their ability to accelerate axonal regeneration in the peripheral nervous system and to hasten functional recovery. Such agents include

insulin-like growth factor (2-4), prosaposin (5,6), ciliary neurotrophic factor (7,8), acidic fibroblast growth factor (9), hyaluronic acid (10), leupeptin (11) and ganglioside GM1 (12). The immunosuppressive agent FK506, a neuro-immunophilin-binding ligand, has consistently demonstrated significant neuroregenerative properties both in vivo (13-17,18) and in vitro (19-22), while the neuroregenerative

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Figure 1) Schematic representation of experimental design. Every BALB/cJ mouse received an 8 mm long tibial nerve isograft from a syngeneic BALB/cJ donor. Animals in groups III and IV were treated with daily subimmunosuppressive doses of FK506 (0.5 mg/kg/day), while animals in groups V and VI received immunosuppressive doses of FK506 (2 mg/kg/day). Animals in groups II, IV and VI received tibial nerve grafts pretreated for one week with cold preservation. 0.5 FK = 0.5 mg/kg/day FK506; 2 FK = 2.0 mg/kg/day FK506; CP Cold preservation of tibial nerve graft at 4°C for one week

properties of other neuroimmunophilin-binding ligands such as GPI-1046, L-685,818, V-10,367 and rapamycin are less conclusive (21-27).

Cold preservation in University of Wisconsin solution decreases the antigenicity of a nerve allograft in part via the down-regulation of intercellular adhesion molecules (15,28-32). In fact, subimmunosuppressive doses of FK506 that are ineffective in stimulating nerve regeneration in a standard nerve allograft stimulate nerve regeneration in nerve allografts that have been pretreated with cold preservation (15). The ability of subimmunosuppressive doses of FK506 to stimulate nerve regeneration was previously attributed to the ability of cold preservation to render murine tibial nerve allografts immunologically inert. However, nerve graft explants pretreated with cold preservation may have promoted nerve regeneration by factors other than decreased immunogenicity. Recent studies have identified the presence of a series of inhibitory molecules, some of which are associated with myelin, that inhibit the regeneration of damaged neurons (33-37). While a majority of these molecules are found either exclusively (38,39) or predominantly in the central nervous system (40-43), myelin-associated glycoprotein (MAG) does occur in significant concentrations in the peripheral nervous system and inhibits axonal regeneration both in vivo and in vitro (44-46). MAG has demonstrated an inhibitory effect on the regeneration of unmyelinated murine axons that is negated in a dose-dependent manner by MAG-specific antibodies (36). These investigators have suggested that the release of MAG is

dependent on the duration of nerve segment predegeneration and the presence of myelin debris. Indeed, cold preservation of nerve grafts ensures a finite period of nerve graft predegeneration (30,31), and both FK506 and cold preservation significantly decrease myelin debris (15,16).

To determine whether cold-preserving nerve grafts for one week alone was sufficient to affect nerve regeneration, and to eliminate the confounding effects of nerve graft antigenicity, only nerve isografts were used in this study. Not previously reported, we postulated that, by modulating the duration of nerve graft predegeneration with one week of cold preservation, and the extent of myelin debris with cold preservation and either immunosuppressive or subimmunosuppressive doses of FK506, we would increase axonal regeneration in a reliable murine model.

METHODS

Experimental design

Sixty female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, Maine), each destined to receive a right tibial nerve isograft from a syngeneic BALB/cJ mouse donor, were randomly assigned to six groups (n=10). Mice in groups I, III and V received fresh tibial nerve isografts, while those in groups II, IV and VI received nerve isografts that had been pretreated for one week with cold preservation (Figure 1). Animals in groups I and II received no drug therapy, while those in groups III and IV were treated with subimmunosuppressive doses of FK506 (0.5 mg/kg/day) and those in groups V and VI were treated with immunosuppressive doses of FK506. Following nerve grafting, functional recovery was monitored on a weekly basis with walking track analysis. Three weeks postoperatively, animals were sacrificed, their tibial nerve grafts were harvested for histomorphometric analysis, their spleens were harvested to evaluate immune competence with mixed lymphocyte reactivity assays, and blood was drawn to determine serum FK506 levels. The three-week time point was specifically chosen based on previous work demonstrating that, in rodent models, even untreated short nerve allografts will accommodate nerve regeneration given sufficient time (15,16,47). Nerve regeneration has not been found to be significantly different even between positive and negative controls when evaluated at later time points. Therefore, a window of opportunity exists in this model at approximately three weeks, when control groups are clearly distinguishable and the effects of experimental interventions can be clearly evaluated (23). A central animal care facility was used to house all experimental animals in accordance with National Institutes of Health (Bethesda, Maryland) guidelines and our institutional animal studies committee.

Operative procedure

On the day of nerve grafting, recipient mice were anesthetized with a cocktail of 30 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa) and 0.2 mg/kg medetomidine hydrochloride (Pfizer Animal Health, Exton, Pennsylvania). A 1.5 cm skin incision was made on the right thigh and muscles were bluntly split using magnification (Wild M651 Leica Microsystems, Inc, Deerfield, Illinois). The right tibial nerve was identified and sharply transected 2 mm distal to the sciatic nerve trifurcation. An 8 mm long tibial nerve isograft (either freshly excised or pretreated with cold preservation) was reversed in orientation, interposed between the transected tibial nerve segments, and under 40 times

magnification was secured with four 11-0 microepineurial sutures. Muscle-splitting incisions were closed with 6-0 vicryl sutures and skin was closed with interrupted 6-0 nylon sutures. Animals were then recovered and housed until assessed for functional recovery. Tibial nerve harvests were performed with the identical anesthetic regimen and nerve exposure, except that both the left and right tibial nerves were taken from every donor mouse to limit the number of animals sacrificed. After 8 mm segments of tibial nerve were harvested, these donor animals were sacrificed with a lethal intracardiac dose of Euthasol (Delmarva Laboratories, Inc, Midlothian, Virginia).

Three weeks postoperatively, recipient mice were anesthetized as previously described, and the right tibial nerve graft was exposed through the initial operative incision. The nerve graft, along with a 0.5 cm cuff of native nerve on either side, was excised en bloc and taken for histological processing. The spleen was removed through a left subcostal incision using aseptic technique and was immediately taken for mixed lymphocyte reactivity assays. To test the immunocompetence of BALB/cJ mice, splenocytes from C57BL/6J mice were required for mixed lymphocyte reactivity assays and were harvested concomitantly through a subcostal incision under general anesthesia. To obtain FK506 levels, 0.5 mL of blood was drawn by cardiac puncture using an 18 gauge needle. These recipient animals were then sacrificed with a lethal intracardiac dose of Euthasol.

FK506 dosing regimen

Subcutaneous doses of FK506 were administered daily to animals in groups III to VI based on weight. FK506 stock solutions were made by dissolving 100 mg of FK506 powder (Fujisawa USA, Inc, Deerfield, Illinois) in 4 mL Cremaphor (Sigma Chemical Co, St Louis, Missouri) and 16 mL absolute ethanol (Quantum Chemical Co, Tuscola, Illinois). Stock solutions were then diluted to working solutions with a 3:1 mixture of propylene glycol (Sigma Chemical Co) and sterile water (Fujisawa USA, Inc). Both stock and working solutions were protected from light at all times, except when undergoing dilution or at the time of injection. Working solutions were changed at least every three days to prevent bacterial overgrowth and to minimize chemical breakdown (16,23). FK506 was administered at immunosuppressive (2.0 mg/kg) or subimmunosuppressive (0.5 mg/kg) doses.

Cold preservation

Cold preservation solution was made fresh for each nerve harvest. Aliquots of Belzer-University of Wisconsin solution (DuPont Pharma, Research Triangle Park, North Carolina) were stored at 4°C and were treated with 200,000 U/L penicillin G-sodium (Sigma Chemical Co), 40 U/L Humulin R (Lilly, Indianapolis, Indiana) and 16 mg/L of dexamethasone (Elkins-Sinn, Inc, Cherry Hill, New Jersey) at the time of nerve harvest. Once harvested, tibial nerves were transferred into the cold preservation solution and vials were sealed, labelled and stored at 4°C (48,49). Seven days later, mice in groups II, IV or VI received these tibial nerve isografts pretreated with cold preservation (15,31).

Functional recovery

Walking track analysis was used to confirm tibial nerve function preoperatively and to monitor functional recovery after nerve grafting. Hindpaws of experimental mice were dipped in develop-

er and these animals were then walked along a corridor lined with exposed, undeveloped x-ray film preoperatively and at one, two and three weeks postoperatively. Measurements of print length were then made on the operated (experimental) side (EPL) and the normal side (NPL), using a SummaSketch II Plus digitizing tablet (Summagraphics, GTO CalComp, Inc, Scottsdale, Arizona). These measurements were then related to each other by calculating the print length factor (PLF) (15,23,50-52):

$$PLF = (EPL - NPL) / NPL$$

Mixed lymphocyte reactivity assays

To measure the degree to which experimental animals were immunosuppressed, their harvested spleens were placed in 5 cm³ of cold RPMI-1640 medium (Washington University Medical School Tissue Culture Center, St Louis, Missouri), fractured with a 30 µm mesh and spatula, and centrifuged three times. The supernatant was discarded and the residual 2.5×10⁵ responder cells were spun down into a pellet and suspended in complete Iscove's tissue culture media supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, Utah), 10 nM Hepes buffer, 1% sodium pyruvate, 1% penicillin-streptomycin (Washington University Medical School Tissue Culture Center) and 1×10⁻⁵ M beta-mercaptoethanol (Sigma Chemical Co). These suspended responder cells were divided into 100 µL aliquots and were cultured in four separate environments. Responder cells were cultured alone in media, with 100 µL of Concanavalin A (Sigma Chemical Co), or were cocultured with equally concentrated 100 µL aliquots of either dysgenic C57BL/6J or syngenic BALB/cJ mouse splenocytes treated with Mitomycin C (Sigma Chemical Co). Using 96-well flat bottom microtitre plates (Costar, Cambridge, Massachusetts), eight separate wells were allocated to each culture environment for every experimental animal. Cultured splenocytes were incubated for five days at 37°C in 95% humidified air mixed with 5% carbon dioxide. On day five, 2 µCi of ³H-thymidine was added to each well, and 16 h later the cells were harvested and the incorporation of ³H-thymidine was quantified in a liquid scintillation counter (1450 Microbeta Plus, Wallac-Gaithersburg, Maryland). Greater thymidine uptake and increased lymphocyte proliferation were characterized by higher scintillation counts.

Data from every experimental animal were evaluated. Cultured splenocytes that failed to proliferate in the presence of the known stimulator Concanavalin A were deemed nonviable and were eliminated from the study. Mean values of thymidine uptake for responder cells cultured alone in media, and with C57BL/6J or BALB/cJ stimulator cells were independently calculated. A stimulation index (SI) relating mean thymidine uptake in responder cells stimulated by a dysgenic stimulator with mean thymidine uptake in responder cells cultured in media alone was then calculated for every animal. The highest and lowest SI values from every experimental group were then discarded, and a mean SI was calculated from the remaining data for every experimental group (16,23,53). These data were then combined into three groups consisting of the experimental animals receiving no immunosuppression (groups I and II), 0.5 mg/kg/day of FK506 (groups III and IV), and those receiving 2 mg/kg/day of FK506 (groups V and VI).

Figure 2) The print length factor was used to quantify the degree of functional recovery. Although no significant differences were delineated between groups, animals receiving higher doses of FK506 trended toward more rapid functional recovery than did those in the untreated groups. 0.5 FK = 0.5 mg/kg/day FK506; 2 FK = 2.0 mg/kg/day FK506; CP Cold preservation of tibial nerve graft at 4° C for one week

Histomorphometry

Following harvest three weeks postoperatively, tibial nerves were placed in cold, buffered 3% glutaraldehyde solution (Sigma Chemical Co). Nerves were then postfixed in osmium tetroxide and embedded in Araldite 502 (Polysciences Inc, Warrington, Pennsylvania). Cross sections of 1 μ m were cut with an LKB III Ultramicrotome (LKB-Produkter AB, Broma, Sweden), stained with 1% toluidine blue and examined under light microscopy. Preservation of nerve architecture, quality and quantity of regenerated nerve fibres, extent of myelination, and the presence of ongoing Wallerian degeneration were thus qualitatively evaluated. Quantitative data were obtained with the aid of a video monitor calibrated to 0.125 μ m/pixel and an automated digital image-analysis system linked to morphometry software (Leco Instruments, St Joseph, Michigan). In every animal, cross-sections from the exact midpoint of the tibial nerve graft were digitized, converted to grey and white scales, and assessed for total fascicular area and total fibre number. Six fields, magnified 1000 times, were randomly selected for every nerve (or a minimum of 500 myelinated fibres) and evaluated for axon width, myelin width and fibre diameter. These primary values were used to calculate nerve fibre density (fibres/mm²), total number of myelinated fibres, percentage of neural tissue (100% $\times$ neural area/intrafascicular area), and percentage of neural debris (100% $\times$ area of debris/intrafascicular area).

Statistical analysis

Walking track analyses were evaluated with SigmaStat, version 2.0 (SPSS Science, Chicago, Illinois), while all other analyses required Statistica Version 5.5 (StatSoft, Tulsa, Oklahoma). Significant differences in animal weight and FK506 levels between experimental groups were compared with an independent

Figure 3) Stimulation indices were calculated to demonstrate the data from the mixed lymphocyte reactivity assays. The stimulation index (SI) = (³H-thymidine uptake from experimental splenocytes)/(³H-thymidine uptake from dysgenic control splenocytes). The amount of ³H-thymidine uptake served as a marker for T cell proliferation in response to a foreign antigen. Experimental animals treated with 2 mg/kg/day FK506 (2 FK) were characterized by T cell anergy and had a significantly lower SI ($P < 0.005$) than did untreated experimental animals (No FK) or those treated with 0.5 mg/kg/day FK506 (0.5 FK)

t test. The presence of statistically significant differences in functional recovery between groups was evaluated with a one-way repeated measures analysis of variance (ANOVA) and pair-wise multiple comparisons were then made post hoc using the Student-Newman-Keuls method. A one-way ANOVA detected the presence of significant differences between mean histomorphometric data. When two groups with equal sample sizes were directly compared, a student's *t* test was used, while the Dunnetts test identified significant differences between the untreated isograft and all other experimental groups. Comparisons of stimulation indices between groups were made post hoc using the least significant difference test. Statistical significance was set at $P < 0.05$ for all evaluations.

RESULTS

General observations

All 60 experimental animals survived and gained weight during the study. Preoperatively, mice weighed 19.4 ± 1.6 g, and postoperatively they weighed 20.3 ± 1.7 g. There were no significant weight differences between groups with respect to nerve graft pretreatment with cold preservation, or the administration of subimmunosuppressive or immunosuppressive doses of FK506. As expected, animals receiving no form of FK506 therapy had no detectable serum levels of FK506. FK506 levels in group III and IV animals receiving 0.5 mg/kg/day were 1.6 ± 0.9 ng/mL, and in group V and VI animals receiving 2 mg/kg/day, FK506 levels were 21.0 ± 3.1 ng/mL.

Functional recovery

The PLF approached zero in all groups before surgery, thus confirming that tibial nerve function was symmetrical between the normal and experimental limbs before nerve injury (Figure 2). Following tibial nerve injury, all of the experimental groups demonstrated gradual functional recovery as the PLF

Figure 4) Histomorphometric values for **A** – Total fibre number; **B** – Nerve fibre density (fibres/mm²); and **C** – Per cent nerve (100% × nerve area/intrafascicular area) are displayed. 2 mg/kg/day of FK506 significantly improved nerve regeneration, while cold preservation did not significantly affect nerve regeneration in this model. * $P < 0.05$; ** $P < 0.01$. 2 FK = 2 mg/kg/day FK506; 0.5 FK = 0.5 mg/kg/day FK506; CP Cold preserved

approached zero. Although no significant differences were identified, animals receiving higher doses of FK506 demonstrated a trend toward more rapid tibial nerve recovery.

Mixed lymphocyte reactivity assays

Demonstrated in Figure 3, splenocytes derived from experimental BALB/cJ mice receiving no immunosuppression and those receiving 0.5 mg/kg/day FK506 had significantly higher stimulation indices when challenged with dysgenic splenocytes relative to the experimental animals treated with 2 mg/kg/day of FK506 ($P < 0.005$). These data confirm that animals in groups V and VI receiving 2 mg/kg/day of FK506 were immunosup-

Figure 5) Histomorphometric values for **A** – Fibre width (μm); **B** – Per cent neural debris (100% × area of debris/intrafascicular area). 2 mg/kg/day of FK506 significantly decreased the amount of neural debris in fresh nerve grafts relative to untreated mice, and those receiving 0.5 mg/kg/day FK506. * $P < 0.05$. 2 FK = 2 mg/kg/day FK506; 0.5 FK = 0.5 mg/kg/day FK506; CP Cold preserved

pressed, while those in groups III and IV received a subimmunosuppressive (0.5 mg/kg/day) dose of FK506.

Histomorphometry

As an independent variable in this model, cold preservation failed to significantly affect any histomorphometric parameter of axonal regeneration, while the dose-dependent neuroregenerative effect of FK506 was confirmed (Figure 4). At 2 mg/kg/day, FK506 significantly increased total fibre number in cold preserved nerves ($P < 0.05$), per cent neural tissue in cold preserved nerves ($P < 0.05$), and nerve fibre density in both fresh ($P < 0.05$) and cold preserved nerves ($P < 0.01$). Fresh nerve grafts in mice receiving 2 mg/kg/day FK506 also demonstrated significantly less neural debris than fresh nerve grafts in FK506-untreated mice ($P < 0.05$) or those treated with FK506 0.5 mg/kg/day ($P < 0.05$). This finding was less dramatic in cold preserved nerve grafts (Figure 5).

The 0.5 mg/kg/day dose of FK506 significantly increased both the per cent of neural tissue ($P < 0.05$) and nerve fibre

density ($P < 0.05$) in cold preserved nerves. Where significant differences in the absolute and relative degrees of neural regeneration were not found, there was a trend whereby mice treated with FK506 at 0.5 mg/kg/day had more neural regeneration compared with untreated mice, but less than mice treated with FK506 at 2 mg/kg/day. Fibre width was not significantly different between any of the experimental groups and no trends were noted (Figure 5).

DISCUSSION

The present study confirmed the neuroregenerative effects of immunosuppressive doses of FK506 in the murine model, but also suggested that subimmunosuppressive doses of FK506 increase neural regeneration. Mixed lymphocyte reactivity assay data demonstrated that the splenocytes of mice treated with 2 mg/kg of FK506 on a daily basis were unable to mount a significant cell-mediated immune response to genetically disparate antigen, while animals treated with 0.5 mg/kg/day of FK506 mounted a significant immune response that was indistinguishable from untreated controls. Yet, relative to untreated controls, mice receiving subimmunosuppressive doses of FK506 consistently manifested increased nerve fibre numbers reaching statistical significance in the histomorphometric parameters of neural density and per cent of neural tissue in the cold-preserved nerve isografts. While subimmunosuppressive doses of FK506 did stimulate neural regeneration in this model, the effect is likely dose-dependent because immunosuppressive doses of FK506 improved neural regeneration to a more significant extent (54). While a trend toward improved functional recovery was noted in animals receiving immunosuppressive doses of FK506, PLF was not significantly different between any of the groups. This finding may have been due to the insufficient sensitivity of walking track analysis as an instrument for measuring functional recovery, but may have also corroborated the findings of previous studies noting only moderate correlation between histomorphometric data, nerve conduction studies and functional recovery (55).

Recent studies of structural analogues of FK506 and other neuroimmunophilin-binding ligands have enabled researchers to differentiate the putative neuroregenerative mechanism of FK506 from its immunosuppressive mechanism (21,22,56). As such, it is not surprising that the doses of FK506 required to induce immunosuppression differ from those required to stimulate nerve regeneration. To induce immunosuppression, FK506 forms a complex with one of its binding ligands, FKBP-12, to inhibit the phosphatase activity of calcineurin and ultimately inhibit T cell proliferation (57,58). The neuroregenerative effects of FK506 are now thought to be independent of calcineurin, but to still involve binding to FKBP-12, or perhaps another binding ligand such as FKBP-52 (19-21,59). The administration of other nonimmunosuppressive FKBP-12-binding ligands has demonstrated a neuroregenerative effect in some *in vivo* (25,26,60,61) and *in vitro* studies (21,22,62), but not in others (23,24,63,64). By demonstrating a neuroregenerative effect in the absence of immunosuppression, the present study provides further *in vivo* evidence that the neuroregenerative and immunosuppressive effects of FK506 are distinct from one another.

In addition to confirming the neuroregenerative properties of FK506 on nerve isografts, we also studied the effects of pretreating nerve isografts with cold preservation. It is well established that cold-preserved nerve allografts are less antigenic and express lower surface concentrations of intercellular adhesion molecules and the major histocompatibility complex (MHC) (32,48,65). Cold preservation clearly facilitates axonal regeneration through the nerve allograft (15). However, a number of studies have demonstrated the presence of inhibitory factors in myelin that prevent axonal regeneration (36,38,39,66-74), and may be affected by cold preservation or FK506. In fact, these myelin inhibitory factors are now believed to be key factors in explaining why significant neural regeneration fails to occur in the central nervous system (74). One such protein, Nogo, localizes exclusively to central nervous system myelin and contributes to an environment that is inhibitory to neural regeneration (38,39,71). While 10 times more concentrated in central nervous system myelin, MAG is also found in significant concentrations in the myelin of peripheral nerves (44-46).

MAG has demonstrated a significant inhibitory effect on peripheral nerve regeneration (36,37,45,46). In one study, two-day-old peripheral nerves, thought to be devoid of Schwann cells, were inhibited by a transected nerve allowed to degenerate for seven days (36). Conversely, more mature regenerating nerve sprouts that were at least three days old and already ensheathed with Schwann cells were not susceptible to the predegenerated nerve end containing MAG. These investigators suggested that MAG was released from the distal end of a cut nerve at a specific time after injury, and that the inhibition of axonal sprouts in close proximity to this distal cut end depended on nerve sprout maturity (36,37). When transected and allowed to degenerate for six days first, the distal nerve stump had no influence on the regeneration of naked two-day-old axons. However, if allowed to degenerate for at least seven days before exposure to immature, regenerating axons, the distal nerve stump inhibited the regeneration of these immature axons. To confirm that the inhibition of axonal regeneration was related to MAG, the administration of a monoclonal antibody to MAG in this environment reversed the inhibitory effects of the seven-day-old distal stump in a dose-dependent manner.

In the present study, nerve grafts treated with cold preservation underwent a seven-day period of predegeneration. Based on previous work, predegeneration of this duration should have correlated with higher MAG concentrations in the vicinity of regenerating immature host axons, and less neural regeneration (36,37). That pretreatment of nerve grafts with a seven-day period of cold preservation had no significant effect on axonal regeneration was likely related to a number of factors.

Cold preservation may have down regulated the expression of MAG and other myelin-associated inhibitors of neural regeneration. Cold preservation decreases nerve graft antigenicity and is associated with the decreased expression of intracellular adhesion molecule (ICAM-1) and MHC-II (65). It is conceivable, then, that cold preservation would alter the expression of myelin-based proteins such as MAG. The administration of FK506 may have also affected the expression or efficacy of MAG. Neural debris, of which fragmented, degen-

erated myelin is a constituent, was significantly reduced in the fresh nerve grafts of mice treated with immunosuppressive doses of FK506. The ability of FK506 to reduce this myelin debris has been previously reported (16) and may have counteracted the inhibitory effects of MAG, which is thought to be expressed in degenerating myelin (75,76). Also, one may have speculated that animals treated with FK506 were characterized by more rapidly maturing, wider, myelinated nerve fibres less susceptible to the inhibitory effects of MAG. However, FK506 did not significantly increase mean fibre width in this study, thus suggesting that it did not significantly alter fibre maturity. Unfortunately, there are no reports in the literature studying the effects of any form of nerve preservation or FK506 on MAG expression.

Subimmunosuppressive doses of FK506 stimulated nerve regeneration in the murine isograft model. The neuroregenerative effect of FK506 was also shown to be dose dependent, because higher immunosuppressive doses of FK506 had a more powerful neuroregenerative effect. We have also shown that,

when evaluated as an independent variable, cold preservation failed to significantly affect any parameter of neural regeneration. To further delineate the relationship between MAG, cold preservation and FK506, quantitative molecular techniques to measure levels of MAG expression will be required. It will then be possible to evaluate the effects of FK506 therapy, cold preservation or other forms of nerve pretreatment on MAG expression and subsequent neural regeneration. The ability to modulate the expression of proteins that are inhibitory to neural regeneration and to use less toxic, subimmunosuppressive doses of FK506 have exciting clinical applications that must first be tested on larger animal models.

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