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Published in:
Journal of the Peripheral Nervous System

DOI:
10.1111/j.1085-9489.2003.03021.x

2003

Citation for published version (APA):
Effects of FK506 on regeneration and macrophages in injured rat sciatic nerve

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Abstract Effects of FK506 [5.0 mg/kg body weight (BW), subcutaneous, daily] on nerve regeneration and presence of macrophages in lesioned rat sciatic nerves were studied. Models of autologous nerve graft or a nerve crush lesion were used and regeneration was evaluated by immunocytochemistry (also used to detect ED1/ED2 macrophages) and sensory pinch reflex test, respectively. Treatment with FK506 did not increase regeneration distance or regeneration rate in the autologous nerve grafts. However, regeneration distances after nerve crush were significantly longer following treatment with FK506. The number of macrophages (ED1/ED2) in nerve grafts increased over time, but treatment with FK506 had limited effects only in the presence of ED2 macrophages. Present and previously published studies may imply that there is a time-related and type-of-injury-related profile of FK506’s pro-regenerative effect.

Key words: FK506, macrophages, nerve crush, nerve graft, nerve regeneration

Introduction

Following severe nerve injuries with lacerations to both the proximal and the distal nerve segments, a gap may remain between the nerve segments after correction. Such a gap has to be bridged by one or several cables of autologous nerve grafts to support nerve regeneration. However, axonal outgrowth in nerve grafts is poor compared to that into a distal nerve segment after a direct nerve repair (Forman et al., 1979; Dahlin and Lundborg, 1998). It would be of advantage to use a drug that could improve axonal outgrowth in such nerve grafts. FK506 is used after various tissue transplantations to prevent graft rejection, and there are reports that nerve regeneration progresses more rapidly than anticipated in nerves of a hand transplant treated with FK506 (Jones et al., 2000; Piza-Katzer, 2001; Dubemard et al., 2002). Thus, FK506 may be a candidate drug to improve nerve regeneration also in autologous nerve grafts.

FK506 is a macrolide drug with immunosuppressive properties and has been reported to augment nerve regeneration in xenografts and allografts as compared with no immunosuppression or immunosuppression by cyclosporin A (Buttemeyer et al., 1995; Hebebrand et al., 1997). In a hand transplant, cutaneous innervation by ingrowing axons from the host occurs within dermal nerves in the allograft, which contains graft-derived Schwann cells detected in biopsy samples (Kanitakis et al., 2000). The suggested advantage of nerve regeneration capacity of FK506 in limb allografting has been attributed to a number of effects that FK506 may have on the neural system (Gold, 2000; Guo et al., 2001; Owen et al., 2001).

FK506 and FKBP (FK506-binding protein) ligands, e.g., GPI-1046, have experimentally been shown to improve nerve regeneration mainly after a nerve crush (Gold et al., 1995; Wang et al., 1997; Becker et al., 2000; Jost et al., 2000; Lee et al., 2000; Sulaiman et al., 2002; Udina et al., 2002). It has been suggested that
FK506 acts directly on the neuron (Gold et al., 1998; Guo et al., 2001; Sulaiman et al., 2002). Effects of treatment with FK506 on regeneration in an autologous nerve graft model have been studied (Fansa et al., 1999) and revealed morphological evidences of improved nerve regeneration when evaluated after 2 weeks of treatment, although no morphological differences were noted after 6 weeks. However, no one has studied the regeneration distance and rate and compared those with treatment with vehicle in autologous nerve grafts, not distal to the graft, thereby demonstrating how such a pro-regenerative action is executed. Theoretically, the regeneration rate and/or the calculated initial delay may be altered by treatment with FK506. These values are calculated from a regression line where the regeneration rate (mm/day) is equal to the slope of the line and initial delay (days) is the point where the regression line crosses the x-axis. Statistically, regression lines of different groups can be compared with an analysis of covariance (ANCOVA). However, calculation and comparison of the value of initial delay gives a higher statistical uncertainty because the values lie far outside of the studied time interval. Furthermore, initial delay is a mathematically derived parameter with no true biological significance, because the early period of regeneration must contain an acceleration period (Sjöberg and Kanje, 1990). Therefore, we have chosen not to calculate values of initial delay. Furthermore, we did not calculate regeneration rate when only two time points for evaluation were used.

Our aim was to study how the expected enhanced regenerative effects of FK506 on neurons are executed in the clinically relevant autologous nerve graft model not only with respect to regeneration distance and rate of the outgrowing axons but also with respect to an inflammatory response (presence of subtypes or macrophages). Macrophages participate in the degradation of the myelin in a degenerating nerve, and a faster Wallerian degeneration process has been suggested to occur in subjects treated with FK506 (Owen et al., 2001) but also experimentally in rats (Fansa et al., 1999). We did not find any effects on nerve regeneration in autologous nerve grafts with FK506 treatment. Therefore, we also used the nerve crush model to confirm that FK506 had the previously reported effect on regeneration. The crush lesion was applied at the same level as in the autologous nerve graft experiments, i.e., more distally compared to previous studies (Gold et al., 1995; Wang et al., 1997).

Materials and Methods

Animals and anesthesia

Eighty-eight female Wistar rats [180 g body weight (BW)] were used in the study. The protocol was approved by the Animal Ethics Committee in Malmö/Lund, Sweden. The animals were anesthetized using a 1.8 ml intraperitoneal injection of a solution containing pentobarbital (60 mg/ml) and sterile saline at a 1:10 volume proportion. The experimental animals were divided into two groups according to surgical procedures, and these groups were further subdivided according to treatment and duration of nerve regeneration. All treatments were given as daily subcutaneous injections and consisted of FK506 (Lot Number 702385K; Fujisawa GmbH, Munich, Germany), vehicle (Lot Number 711059K; Fujisawa GmbH), or sterile saline (equal volume as that of FK506). FK506 (5 mg/kg/day, 1 mg/ml; Wang et al., 1997) and vehicle (correlating concentration as that of FK506) were dissolved in sterile saline according to the manufacturer’s instructions.

Autologous nerve graft

The left sciatic nerve was exposed in the rats \( n = 40 \) and a 10 mm graft was excised using proximal transection at hip level. The graft was orthotopically replaced and sutured to the proximal and distal nerve stumps using two to three epineurial sutures (9-0 Ethilon, Ethicon, Edinburgh, UK) at each anastomosis. The animals were treated with either FK506 \( n = 20 \) or vehicle \( n = 20 \). After 6 \( n = 13 \), 8 \( n = 13 \), or 10 \( n = 14 \) days, the animals were sacrificed with an overdose of pentobarbital and the sciatic nerve was re-exposed. The sciatic nerve was cut 3 mm proximal to the proximal suture and approximately 10 mm distal to the distal suture. After dissection, the nerve was fixed in Stefanini’s solution for 4 h and then transferred to phosphate-buffered saline with 20% sucrose. The autologous nerve grafts with their adjacent nerve segments were embedded in Tissue Tek® (Sakura, Netherlands) and frozen. Longitudinal sections, 10 mm thick, were cut in a cryostat and placed on slides. The sections were stained with monoclonal antibodies against 68 kDa neurofilaments (MO726, DAKO, Glostrup, Denmark), ED1 (MCA341, Batch Number 0796a, Serotec, Oxford, UK), or ED2 (MCA342, Batch Number G, Serotec), according to previous protocols (Arai et al., 2000), and evaluated by light microscopy. The regeneration distance, measured in the neurofilament stained slides, from the proximal suture line to the place where there was a front of neurofilament-positive axons (>5 continuously growing positive axons), was measured using a scale in the microscope (Fig. 1). The value obtained from the section (>7 sections from each rat evaluated) showing the longest regeneration distance from each animal was used to calculate the regeneration distance for the different groups.

The number of ED1- or ED2-positive cells (>7 sections from each rat evaluated) was counted using a
grid in the microscope and divided by area examined (at least 9 mm² for ED1 and 16 mm² for ED2 in each rat) to yield the cell density (n/mm²). The measured area was situated 4–6 mm from the proximal suture and within the endoneurium. Slides stained for ED1 and ED2 were not in consecutive cutting order, thereby preventing each cell from being counted more than once.

Nerve crush

Because the initial results in the autologous nerve graft experiments showed no difference between treatment with FK506 or vehicle, we decided to test the effect of FK506, vehicle, and saline in a nerve crush model since previous studies (Gold et al., 1995; Wang et al., 1997) have indicated that FK506 stimulates nerve regeneration after a crush injury. Furthermore, in these studies, the effect was demonstrated for sensory neurons alone, and therefore, we evaluated regeneration using a method that exclusively detects sensory axons, namely the sensory pinch reflex test. Because only two time points were used to evaluate axonal outgrowth, regeneration rate was not calculated. The left sciatic nerve in the rats (n = 48) was exposed at the proximal thigh and crushed twice (for a total of 30 s) at the level of the hip using forceps (number 5). The site of the crush was marked with an epineurial suture (9-0 Ethilon, Ethicon). The rats were treated with FK506 (n = 16), vehicle (n = 16), or saline (n = 16) as described above. Sensory pinch reflex test was performed after 3 (n = 24) or 6 (n = 24) days after crush in the anesthetized animals, as previously described (Kerns et al., 1993; Dahlin et al., 1996), to detect outgrowth of sensory axons and was expressed in mm.

Statistical analysis

All values are expressed as mean ± SE. A two-way ANOVA was used to test for differences in the material (factors: treatment and duration of treatment). A post-hoc study (Fisher’s protected least significant difference [PLSD]) was carried out to examine whether there were any significant differences between the groups for either the treatment or the duration. Regeneration rate was estimated using a linear regression line for each group in the nerve grafts. ANCOVA was used to compare regeneration rates of different treatments in the grafts. A p-value of <0.05 was considered significant (ANOVA/ANCOVA). Statview 5.0.1 (Abacus Concepts, Inc., Berkeley, CA, USA) was used to perform calculations. A power analysis was performed (Altman, 1994; see Discussion).

Results

Specimens from five rats were not possible to be stained due to problems with fixation. There was a front of neurofilament-positive axons (Fig. 1) in the autologous nerve grafts at the various evaluation time points.

Figure 1. Immunofluorescent micrographs of regenerating axons forming a front after treatment with FK506 (A) and vehicle (B). Scale bar = 100 μm.

Figure 2. Scattergram, with regression lines, of regeneration distances of axons in autologous nerve grafts showing regeneration rate (slope of line) after treatment with FK506 and vehicle. The calculated rates in the autologous nerve grafts were 1.8 and 2.0 mm/day in the rats treated with FK506 and vehicle, respectively (see also Table 1). Time was the only significant factor affecting the outgrowth of axons (p < 0.0001) in the autologous nerve grafts. There were no significant effects of FK506 treatment (p = 0.43), and the regeneration rate did not differ significantly (p = 0.54) in such grafts.
Regression analysis revealed a linear outgrowth of axons in the nerve grafts (Fig. 2) in relation to time after grafting, after both treatments (FK506 or vehicle). Time was the only significant factor affecting the outgrowth of axons ($p < 0.0001$, Table 1). There was no significant effect of treatment alone ($p = 0.43$), and furthermore, the two-way ANOVA identified no interaction between treatment and time ($p = 0.06$). Regeneration rate did not differ significantly ($p = 0.54$, ANCOVA) between treatment with FK506 (1.8 mm/day) or vehicle (2.0 mm/day, Table 1).

Macrophages in autologous nerve grafts

Morphologically, most of the ED1-positive macrophages in the autologous nerve grafts had a foamy appearance, indicating that the macrophages had ingested large amounts of myelin and the distribution of cells was even within the endoneurium (Figs 3-A and 3-B). The cell density in both groups of treatment increased with time (Fig. 4-A) and time was a significant factor ($p < 0.0001$, two-way ANOVA). Furthermore, there was a positive interaction (two-way ANOVA) between duration and treatment ($p = 0.0002$). Treatment alone did not affect the cell density of ED1-positive macrophages ($p = 0.72$).

In general, the cell density of ED2-positive macrophages was lower as compared with that of the ED1 in the autologous nerve grafts. The distribution within the endoneurium was even, and the density of ED2-positive macrophages in the vehicle group seemed to increase with time (Fig. 4-B), while the density in the FK506-treated group seemed to be stable at an initially higher level. Only occasionally ED2-positive cells had a foamy appearance, suggesting that these cells are not

Table 1. Nerve regeneration, evaluated by neurofilament staining, in autologous nerve grafts in rat sciatic nerves 6, 8, and 10 days after grafting following treatment with FK506 or vehicle

<table>
<thead>
<tr>
<th>Regeneration distance (mm)</th>
<th>FK506</th>
<th>Vehicle</th>
</tr>
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<tbody>
<tr>
<td>6 days</td>
<td>3.7 ± 0.4 (6)</td>
<td>2.1 ± 0.5 (6)</td>
</tr>
<tr>
<td>8 days</td>
<td>7.1 ± 0.6 (6)</td>
<td>8.0 ± 0.2 (6)</td>
</tr>
<tr>
<td>10 days</td>
<td>10.3 ± 0.5 (5)</td>
<td>10.1 ± 0.7 (6)</td>
</tr>
</tbody>
</table>

Regeneration rates (based on all three time points) are estimated using linear regression for each group. Neither regeneration distances (two-way ANOVA; factors: treatment and time) nor regeneration rate (ANCOVA) showed any significant differences between FK506 and vehicle, but time was a significant factor ($p < 0.0001$).

$n =$ number of rats. The regeneration distances were analyzed using a two-way ANOVA (factors: treatment and time) with post-hoc Fisher’s PLSD. Regeneration rates (based on all three time points) are estimated using linear regression for each group.

Figure 3. Light micrographs showing macrophages stained for ED1 at 6 days treated with either FK506 (A) or vehicle (B). Note the foamy appearance of the macrophages (inset in A, scale bar = 50 μm). At 6 days, there is a visible difference in the number of ED2-stained macrophages after FK506 treatment (C) as compared with vehicle treatment (D). Scale bar = 100 μm.
actively ingesting myelin in vivo but may be active in cultures (in vitro; Leonhard et al., 2002) (Figs 3-C and 3-D). Both time (p = 0.02) and treatment (p < 0.0001) had significant effects on the cell density of ED2-positive macrophages, but the two-way ANOVA identified no positive interaction between time and treatment (p = 0.21). The post-hoc analysis showed a significantly higher cell density in the FK506-treated group than in the vehicle-treated group (p < 0.0001, Fisher’s PLSD).

Regeneration after nerve crush

In three rats, it was not possible to evaluate the regeneration distance after the nerve crush due to problems with anesthesia. The two-way ANOVA identified a positive interaction between treatment and time (p = 0.003, Table 2). Time was a significant factor (p = 0.0001, ANOVA). In contrast to the autologous nerve grafts, FK506 treatment had a significant effect (p = 0.004, ANOVA) on regeneration distance after nerve crush. The post-hoc analysis of regeneration distance demonstrated significant (p < 0.0001, Fisher’s PLSD) differences between FK506 and the other two groups, but no difference was shown between treatment with saline and vehicle (p = 0.56, Table 2).

Discussion

The present study showed that there was a linear outgrowth of axons in autologous nerve grafts, but there was no significant short-term (up to 10 days) effect of treatment with FK506 neither on the regeneration distance nor on the regeneration rate compared to treatment with vehicle. Other reports examining long-term effects (weeks and months) of FK506 on regeneration in autologous and predegenerated nerve grafts and in isografts and allografts (Buttemeyer et al., 1995; Doolabh and Mackinnon, 1999; Fansa et al., 1999; Wang and Gold, 1999; Navarro et al., 2001) have shown varying efficiency of FK506 in the regeneration process in such grafts. FK506 has been reported to augment and induce Schwann cell proliferation, as well as to induce a higher axonal sprouting in the grafts initially, an effect which may subside after some time (Fansa et al., 1999; 2000). In contrast, we did not observe any effects on axonal outgrowth of FK506 in autologous nerve grafts. A higher cell density of ED2 macrophages after FK506 treatment, however, was found in our short-term experiments. The effect on ED2 macrophages indicates that FK506 was biologically active in the nerve grafts. We have no biological explanation as to why no effect on nerve regeneration was observed in our nerve graft model, while other authors have reported long-term effects of FK506 (Doolabh and Mackinnon, 1999; Navarro et al., 2001).

To rule out the possibility that our failure to detect a
difference was due to the design of the study, in this particular case the number of animals in the different experimental groups, we performed a power analysis. Statistically, a power analysis can be performed to calculate the number of animals required in each group to be able to detect a significant difference, where the power is set at 80%, at probability of 0.05. Such a calculation was performed using a nomogram (Altman, 1994), and we supposed that a clinically, or rather an experimental, relevant difference in regeneration distance is considered significant up to at least 1.7 mm (Haapaniemi et al., 1998; 2001; Nishiura et al., 2001; Widerberg et al., 2002). With a standard deviation of 1.0, the sample size in each group will be five animals (Altman, 1994). Based on the present sample size in each group, regarding the autologous nerve grafts, we had been able to detect a difference of around 1.6 mm at 8 days, between the FK506 and vehicle groups, using a formula with the same power and probability as above. In accordance, using the same formula to detect a difference of less than 1.7 mm in 8-day group will require five animals in each group. An experimentally observed difference set at 1.7 mm during the first 10 days seems to be relevant in this context, even if such a difference is probably not enough to improve the functional outcome, such as recovery of muscle force when analyzed at later time points (3 months) as seen after other types of treatments (Haapaniemi, et al., 2001; 2002). We conclude, based on the power analysis and the discussion above, that the lack of effect of FK506 on nerve regeneration in the nerve graft model is most likely due to some still unknown biological factor and not related to the design of this study.

We did not use predegenerated autologous nerve grafts (Wang and Gold, 1999) because the outgrowth in such grafts is already markedly improved due to a shorter initial delay or, more precisely, a faster acceleration. An initial delay is calculated from a regression analysis, i.e., calculated time at which axons are considered to start growing (interception of the regression line with the x-axis). The initial delay value, however, has a high uncertainty due to the calculation, and therefore, such a calculation was not performed in the present experiment. Furthermore, the regeneration rate is not improved in such predegenerated nerve grafts. In contrast, FK506 had a positive effect on the regeneration distance of sensory neurons after nerve crush compared to treatment with vehicle or saline (Table 2). This is supported by previous findings that FK506 enhanced regeneration in nerve crush model (Gold et al., 1995; Wang et al., 1997; Becker et al., 2000; Lee et al., 2000; Udina et al., 2002), and it also indicates that we had an active substance.

We used neurofilament staining and the sensory pinch reflex test to determine the regeneration distance in our models. It has been previously demonstrated that there is a strong correlation between those methods (Dahlin et al., 1996). Because the purpose of our study was to evaluate the effects of FK506 on autologous nerve grafting, a more clinically relevant model than a nerve crush, we deliberately chose neurofilament staining to detect both motor and sensory axon regeneration distances. In a clinical setting, both motor regeneration and sensory regeneration are of importance to the final clinical outcome. Surprisingly, we did not detect any positive effect of FK506 on the autologous nerve grafts. Therefore, regeneration distances were specifically measured using the pinch reflex method during sensory axon regeneration in an experimental model in which such a positive effect has previously been found—the nerve crush model. High regeneration rates (up to 5.1 mm/day; FK506 5.0 mg/kg/day; 3.8 mm/day for salinetreated rats) have been calculated in rats subjected to nerve crush after treatment with FK506 (Wang et al., 1997). In contrast to our study, a different evaluation method (axonal transport) and longer regeneration time (12 and 15 days as end points) were used in such studies. The axonal transport method is prone to criticism due to the problem of defining significant labeling above background (Danielsen et al., 1986).

The pinch reflex test measures only the outgrowth of sensory fibers and does not detect the regeneration of motor fibers, which the immunocytochemical methods used for the autologous nerve graft do. FK506 probably only enhances sensory regeneration because radioactive labeling of dorsal root ganglia (DRG) and

<table>
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<tr>
<th>Days after injury</th>
<th>Regeneration distances (mm) [mean ± SE (n)]</th>
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<tr>
<td></td>
<td>FK506*</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
</tr>
<tr>
<td>3</td>
<td>6.3 ± 0.2 (6)</td>
</tr>
<tr>
<td>6</td>
<td>18.6 ± 0.6 (8)</td>
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</table>

n = number of rats. The regeneration distances were analyzed using a two-way ANOVA (factors: treatment and time) with post-hoc Fisher’s PLSD. *Statistical difference (p = 0.004; ANOVA) of FK506 compared as a group with the other two groups (vehicle and saline)
the pinch reflex test, as used in other and our studies, (Wang et al.; Udina et al., 2002) only detects sensory regeneration. It has been suggested that the effects of FK506 may have a time profile after nerve crush injury, and axonal outgrowth may be dependent on the dose or a delayed or discontinuous administration of FK506 (Wang et al., 1997; Gold et al., 1999; Udina et al., 2002). The dose and treatment regimens used in our study were based on previous studies indicating that the most efficient dose of FK506 was 5 mg/kg, administered subcutaneously during the whole study period (Wang et al., 1997; Fansa et al., 1999; Gold et al., 1999; Udina et al., 2002). Recently, however, Udina et al. (2002) reported that there is a bimodal dose dependence of FK506 on the rate of axonal regeneration after a nerve crush in mice. That study indicates that our selection of a FK506 dose of 5 mg/kg/day subcutaneously is valid and appropriate.

The location of the injury is important for a treatment effect because an injury far proximal at the L4–L5 junction (Wang et al., 1997) may increase the risk for post-traumatic apoptosis of the cell bodies. This has been observed after proximal lesions in rats and mice (Ygge, 1989; Shi et al., 2001). FK506 has been reported to be protective against apoptosis (Bavetta et al., 1999) and to reduce the size of the infarcted area in the brain after vascular occlusion (Bochelen et al., 1999). The suggestion that FK506 may act directly on the neuron, e.g., by increasing the synthesis of GAP43 (Gold et al., 1998), may be more relevant when there is a chronic axotomy of motor neurons (Sulaiman et al., 2002). This is also supported by the fact that there might be a chronic axotomized situation for a long-term period in hand-transplanted patient where FK506 was attributed to the improved nerve regeneration (Owen et al., 2001). The present findings of a lack of effect of FK506 on axonal outgrowth in autologous nerve graft do not justify the fact that such a drug is used to treat patients with severe nerve injuries reconstructed by nerve grafting, unless the immunosuppressive properties of FK506 are needed (Buttemeyer et al., 1995).

We could find neither any difference regarding change in the general cell density nor any morphological differences in ED1-positive macrophages between the FK506-treated group and the vehicle-treated group. In both groups, there was an even distribution of macrophages, most of them with a foamy appearance. Thus, we could not find any indication of enhanced Wallerian degeneration, as measured by calculated density of ED1-positive macrophages in the autologous nerve grafts, as described previously (Fansa et al., 1999). However, a significantly higher density of ED2-positive macrophages was observed after treatment with FK506 compared to vehicle, but the difference seems to decrease gradually up to 10 days (Fig. 4B), analogous to the stimulating effect found by Fansa et al. (1999; 2000) on the number of Schwann cells. No morphological differences between the FK506-treated and vehicle groups were detected regarding ED2-positive macrophages. We conclude that these cells perform other tasks than myelin removal in vivo (Hirata et al., 1999), like stimulation of other cell types in the Wallerian degeneration, in contrast to a recent report using cell cultures (Leonhard et al., 2002). ED2-positive cells in the central nervous system (Angelov et al., 1998) and ED1-positive cells in peripheral nerves (Lindholm et al., 1987) produce interleukin-1 (IL-1), which stimulates nerve growth factor synthesis in Schwann cells, with subsequent stimulation of regeneration of sensory axons (Lindholm et al., 1987). The invasion of macrophages in nerve segments with preserved circulation is also more efficient than in avascular nerve segments (Dahlén, 1995). These factors could also be an explanation as to why we see improved axonal outgrowth after a nerve crush but not in the autologous nerve grafts. We, however, have no explanation for the discrepancy between the effects of FK506 on ED1 and ED2 macrophages.

In conclusion, the present study showed that FK506 has a pro-regenerative effect on sensory neurons after a nerve crush lesion, which is in accordance with previous studies using other evaluation methods (Gold et al., 1995; Wang et al., 1997; Lee et al., 2000; Udina et al., 2002). Such an effect, however, could not be demonstrated in autologous nerve grafts, in contrast to recently published studies using a lower dose of FK506 and a discontinuous treatment regime (Navarro et al., 2001). We also observed a limited effect of FK506 concerning the presence of ED2 macrophages in the autologous nerve grafts and no effect on ED1 macrophages. Present and previously published studies imply that there might be a type of injury-related profile of the pro-regenerative effect of FK506, indicating that only nerve crush lesions and chronic axotomized neurons may benefit from treatment with FK506. The present study does not justify the use of FK506 after autologous nerve grafting procedures but may have a dual task in human hand transplantation, such as immunosuppression and stimulation of chronic axotomized neurons (Jones et al., 2000; Owen et al., 2001; Sulaiman et al., 2002).

Acknowledgements

The study was supported by grants from the Swedish Research Council (5188), Lundgren’s Foundation, Segerfalks Foundation, and the University Hospital in Malmö. We thank Fujisawa GmbH for FK506 and vehicle. We express our sincere gratitude to Prof B. Holmquist, Department of Statistics, Lund University,
Lund and to Associate Professor J.-Å. Nilsson, Department of Medicine, University Hospital, Malmö, Sweden. We also thank I. Hallberg for technical assistance and M.K. Reimer for scientific advice.

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