Peripheral nerve regeneration through allografts compared with autografts in FK506-treated monkeys

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Object. The clinical use of nerve allografts combined with immunosuppressant therapy has become a genuine possibility that could supersede the classic use of autografts. However, contradictory data have been reported on whether immunosuppressant therapy should be temporarily administered. The purpose of this study was to compare the nerve regeneration obtained using ulnar nerve allografts in nonhuman primates temporarily treated with FK506 (tacrolimus) with that obtained using nerve autografts.

Methods. Four-centimeter nerve autografts or allografts were placed in the distal ulnar motor nerve of eight monkeys. The FK506 was temporarily administered to the animals of the allograft group for 2 months. At periods of 3, 5, and 8 months postsurgery, quantitative electrophysiological recordings were obtained to estimate muscle response. A quantitative analysis of ulnar motor neurons in the spinal cord was performed and axons were counted stereologically.

No statistically significant differences were found in the neuronal and axonal counts between autograft and allograft groups at 8 months. The electrophysiological studies showed no differences relative to the amplitude, but the allograft group presented with a greater nerve conduction velocity (NCV). However, no statistically significant differences were found between the number of neurons and distal axonal counts in the two groups.

Conclusions. Nerve regeneration through cold-preserved allografts in a primate model temporarily treated with FK506 was similar to that obtained using nerve autografts, in terms of neuronal and axonal counts. Nevertheless, temporary immunosuppression produced lower NCV when allografts were used, with less maturation of the myelinated fibers, which indicated that a partial rejection had taken place.

Key Words • nerve allograft • FK506 • motor neuron • nerve regeneration • Macaca fascicularis

Nerve autografts are considered the gold standard technique for the repair of peripheral nerve lesions. However, because of the disadvantages related to autografts, interest in investigating other means of repairing peripheral nerve lesions has increased in recent years. Biomatereials such as veins, arteries, silicone, or polyglycolic acid allow relatively good nerve regeneration, especially in short nerve defects, such that the use of nerve allografts to repair nerve defects has become a real possibility in clinical practice. The main handicap in the use of nerve allografts is graft rejection. However, better understanding of alloimmunity has made it possible to apply several methods to reduce the antigenicity of allografts or to manipulate the recipient’s immune response by using immunosuppressive agents. In this context, protocols for cold-preserved nerve allografts are useful to decrease the dose of immunosuppressive drugs needed.

The new immunosuppressant FK506, also known as tacrolimus, has greater potential and fewer side effects than other immunosuppressants, and is now being used as primary immunosuppressive therapy. It has been established that FK506 has neuroregenerative and neuroprotective effects regardless of its immunosuppressive activity. These dual actions make FK506 ideal for situations requiring both immunosuppression and nerve regeneration, and the use of FK506 has been suggested to confer significant benefits in nerve transplants. Nevertheless, most of the research in peripheral nerve regeneration in which nerve allografts have been used with immunosuppression or cryopreservation has been performed in rats and mice. Lately, some experimental studies in cyclosporin A–treated nonhuman primates have demonstrated that peripheral nerve regeneration through nerve allografts is present. Moreover, the application of nerve allografts in humans has been practiced by Mackinnon, et al., and the groups involved in hand transplantation.

One of the most important questions in managing nerve allografts is to elucidate whether temporary administration of immunosuppressant drugs is sufficient to maintain the nerve activity once the axons have crossed the grafts, and to
avoid graft rejection. However, the findings from research into this phenomenon are controversial.\textsuperscript{24} Previous experimental studies in which the peripheral nerve regeneration in allografts was evaluated have focused on determining the number of axons and their correspondence with results of electrophysiological studies, whereas very few data on the number of motor neurons present when using nerve allografts in the spinal cord have been reported.\textsuperscript{17}

The main aim of this study was to examine the efficacy of temporary treatment with FK506 in nonhuman primates when using nerve allografts, compared with the classic use of autografts. We studied peripheral nerve regeneration by using the ulnar nerve as the experimental model. The number of motor neurons present in the spinal cord whose axons had regenerated across the grafts was determined. Moreover, the number of host axons was correlated with electrophysiological recordings of the regenerating process to compare nerve regeneration between the autograft and allograft groups.

Materials and Methods

Experimental Animals

The study was performed in eight male nonhuman primates (Macaca fascicularis), each weighing 3 to 4 kg. To determine if the primates had the same or different human leukocyte antigen identities, it was necessary to perform a prescreening DNA test for the Class II DRB genes and type them for DQA and DQB. The primate DNA in the allogenic group was quantified using a spectrophotometer (Pharmacy-Biotech, Kit QIAdnap Mini Kit; Qiagen, Venlo, The Netherlands). The DNA samples were sent to the Biomedical Primate Research Center (Rijswijk, The Netherlands). The results of the DRB denaturing gradient gel electrophoresis test\textsuperscript{19,27} showed that the banding patterns of the monkeys were different from each other, and that they therefore did not share DRB alleles and haplotypes, indicating that the animals were not genetically related.

The animals were divided into two groups of four: Group I received nerve autografts, whereas Group II received nerve allografts. For surgical procedures and electrophysiological studies, the monkeys underwent induction of profound anesthesia with a mixture of ketamine (12 mg/kg) and midazolam (1 mg/kg) given by intramuscular injection, and repeated as needed. The animals underwent surgical procedures and electrophysiological recordings of the regenerating process to examine the histological features of the nerve grafts, respectively.

Surgical Procedures

In three monkeys in each group, a 3- to 4-cm segment of the distal motor ulnar nerve was removed bilaterally. In the remaining monkey in each group, this procedure was only done unilaterally, and these animals served as controls. The defect was immediately repaired with a 4-cm nerve to ensure a lack of tension at the level of the anastomoses. The repairs were made with autografts obtained from the superficial fibular nerve (Group I) or allografts obtained from the same donor site in another animal (Group II). The grafts were secured to the proximal and distal stumps of the nerve branch with five 10-0 nylon sutures (Fig. 1).

Group II had special characteristics. The nerve autografts were stored in University of Wisconsin Cold Storage Solution at 4°C for 3 weeks to reduce antigenicity before insertion of the graft into the ulnar nerve. In addition, the primates in this group received immunosuppressive therapy with FK506 (Fujisawa, Vienna, Austria); an oral dose of 9 mg/kg/day was administered by gavage for 2 months, beginning the morning before surgery, to ensure immunosuppression with minimal side effects in the monkeys.\textsuperscript{20,30} The FK506 administration was then stopped. Two months proved to be long enough for the axons to cross the 30- to 40-mm grafts at a growth rate of 2 to 3 mm/day.\textsuperscript{22}

Electrophysiological Studies

To evaluate nerve regeneration, we performed electrophysiological studies at various time points during the experiment. A control study was performed in all animals before the ulnar nerve was sectioned. Repeated studies were performed immediately after nerve sectioning and at 3, 5, and 8 months after surgery in both groups. The NCV of nerve grafts and the maximum amplitude of CMAPs of hypothermic muscles were measured with the aid of an electrophysiological recorder (MS 92a; Marconi Instruments Medical, Old Woking, United Kingdom). The proximal ulnar nerve was stimulated with a supramaximal stimulus by using monopolar needle electrodes. The hypothermic muscle response was recorded by needle electrodes placed in the abductor digiti minimi muscles. The distance between the site of stimulation and the muscle was 10 cm and this distance was kept constant in repeated studies.

Application of the Retrograde Neuronal Tracer

Four days before the monkeys were killed, they were again profoundly anesthetized according to the same anesthetic protocol mentioned earlier. A small silicone chamber was sutured into the distal anastomosis of the nerve grafts. With the aid of a Hamilton syringe, we placed 1 μl of the neuronal retrograde tracer FluoroGold (Sigma Chemical Co., Inc., San Francisco, CA) in the chamber so that the entire distal stump was embedded with the tracer (Fig. 2).

Perfusion of Animals and Processing of the Neural Tissue

Four days after the tracer was injected, the animals were again anesthetized and transcardially perfused with 1 L of saline solution and 2 L of a solution of 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) to fix the neural tissue. The spinal cord and ulnar nerves were then removed and stored in a 30% sucrose solution overnight at 4°C. The middle and lower thoracic spinal cord was cut using a freezing microtome in 40-μm-thick slices; the sections were then mounted onto subbed slides and dried, and coverslips were affixed. A section of nerve that contained the entire graft and extended 4 mm beyond either end was removed and embedded in paraffin. It was then cut into 2-μm-thick transverse sections and processed using the Bodian and hematoxylin techniques to observe axons located proximal and distal to both anastomoses to examine the histological features of the nerve grafts, respectively.

Stereological Counting of Motor Neurons and Axons

Neuronal density in the spinal cord was studied using stereological methods. Representative sections were systematically selected at random to avoid double counting of the same motor neuron. To achieve a 33% sample fraction, 40 sections in each case were counted. To estimate the density of the labeled motor neurons in the spinal cord, we used the optical dissector technique, an unbiased stereological method\textsuperscript{23} performed with the aid of an Olympus fluorescent microscope (Olympus Optical Co. Europe GmbH, Hamburg, Germany) at 40 magnification (Fig. 3A–C). The microscope was connected to a video camera and the motorized stage was connected to a Commodore AMIGA 2000 computer (AMIGA, Inc., Snoqualmie, WA). The dissector volume was calculated by taking into account the area of the dissector grid (29.284 μm²) and multiplied by the distance between the two focal planes. Meander sampling was done, keeping the same fraction in every section, and a maximum of 20 dissectors in the widest sectors were analyzed. Neurons were counted in each dissector, and then totaled in each sector to obtain the SqD- parameter. Neuronal density was calculated using the following formula: Nv = Sqd-SVdis (cells/mm²), where Nv is neuronal density, Sqd= means summatory of all neurons counted in each dissector of a sector, and SVdis is the summatory of all dissector volumes in a sector.

Axons were counted stereologically with the aid of an image analyzer (CAST-Grid V1.10; Olympus Corp., Copenhagen, Denmark) coupled to an Axioscope microscope. The total number of axons (N)
can be estimated directly by using the fractionator technique\textsuperscript{16,24} and the following formula: $N = Q/sf$, in which the number of axons counted using the unbiased counting frames ($Q$) is multiplied by the inverse of the sampling fraction ($sf$). The sampling fraction is determined using the following formula: 

\[ sf = \left( \frac{a_{\text{frame}}}{a_{\text{sample}}} \right)^{1/4} \]

(four frames are used) in which $a_{\text{frame}} = 501.96 \, \text{mm}^2$ and $a_{\text{sample}} = 1633.9 \, \text{mm}^2$. Axons located outside the four frames and any in contact with the left and lower borders of the frames were not counted (Fig. 3D).

**Statistical Analysis**

Statistical Analysis was performed using nonparametric tests. The number of neurons and axons in autografts and allografts were compared using the Mann–Whitney test. The results obtained in the electrophysiological studies performed at different times during the experiment were compared using the Mann–Whitney U-test. The Wilcoxon test was performed in each group to compare the mean values of NCV and CMAP at 3, 5, and 8 months to observe the change in the variables during the follow-up time. Significance was considered at the level of 0.05.

**Results**

**General Observations**

The primates displayed no side effects from the bilateral ulnar nerve lesions during the study. After transection of the motor branch of the ulnar nerve, all monkeys exhibited an ulnar paralysis that was confirmed by the electrophysiological recording and in terms of function. After 8 months, the monkeys exhibited apparently normal movement with a normal contraction of the abductor digiti minimi muscle when stimulated. No accessory nerves arising from the proximal and distal nerve graft anastomoses were detected. No side effects of the administration of FK506, such as diarrhea, intussusception, vomiting, or other effects described in the literature were observed in the monkeys in the allograft group. However, as has been noted previously in the literature,\textsuperscript{33} these animals did display a lower rate of weight gain than those in Group I.

**Neuronal and Axonal Counting**

The extent of the distribution of labeled ulnar neurons along the rostrocaudal axis of the thoracic spinal cord was 2860 $\mu$m in Group I and 3400 $\mu$m in Group II. The statistical analysis of the mean labeled neuronal population showed no statistically significant differences between Groups I (1272 ± 339 [mean ± standard deviation throughout]) and II (1589 ± 416) ($p = 0.1$). There were no statistically significant differences between autograft and allograft groups in the number of axons counted proximally and distally ($p = 0.442$ and 0.694, respectively; Fig. 4). Moreover, there were no statistically significant differences between the number of proximal axons in the autograft group (1258 ± 468) compared with the number of axons counted distally (1505 ± 628; $p = 0.15$), nor were there statistical differences between the number of axons proximally (1377 ± 590) and distally (1476 ± 918) in the allograft group ($p = 0.95$; Fig. 4).

**Histological Findings**

All animals displayed enlargement of the nerve graft, which was more evident in the allograft group. Few inflammatory cells were observed in the autograft cases (Fig. 5A and C). In contrast, more connective tissue, fibrin, and inflammatory cells around the vessels, with some giant cells, were detected in the allograft cases (Fig. 6A and C) than in the autograft group. All the nerves in both groups were

![Intraoperative photograph showing application of the neuronal retrograde tracer (FluoroGold) in a small silicone chamber sutured in the distal anastomosis of the nerve graft. Bar = 1 cm.](image)
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Fig. 3. Photomicrographs of sections of nerves obtained in experimental monkeys. A and B: Distribution of the motor neurons of the motor branch of the ulnar nerve in the spinal cord of the autograft and allograft groups, respectively, is shown. C: Motor neurons are depicted. D: Frames used for axonal counting are shown. Bar = 100 μm (A and B), 40 μm (C), or 15 μm (D).

Fig. 4. Bar graph showing neuronal and distal axonal quantification in autograft and allograft groups at the end of the study. No statistically significant differences are observed in the neuronal and axonal quantifications.
structurally intact, with Schwann cells showing no signs of atrophy. Vacuolization was visible in Schwann cells in the allograft group.

**Electrophysiological Studies**

Animals in both groups exhibited reinnervation through the ulnar nerve grafts. The mean values for reinnervation are shown in Figs. 7 and 8. After sectioning of the ulnar nerve, no NCV and CMAP were recorded in any case. After 3 months, the autograft group demonstrated a greater NCV than the allograft group, with statistically significant differences (p = 0.029) that were maintained throughout the study. In the autograft group, an NCV increment was observed that was mostly apparent during the first 3 months after grafting, and it remained at similar values during the rest of the study, whereas in the allograft group the NCV increased slightly during the study (p = 0.029). On the other hand, the CMAP increased gradually in both groups without statistically significant differences at 3 (p = 0.486), 5 (p = 0.231), or 8 months (p = 0.146).

**Discussion**

In this study, in which we used a nonhuman primate model temporarily treated with FK506, cold-preserved nerve allografts exhibited regeneration levels similar to nerve autografts in terms of neuronal and axonal counts. However, withdrawal of immunosuppressive therapy led to lower NCV in the allografts, with no changes in the CMAP.

**Neuronal and Axonal Counts: Correlation With Electrophysiological Results**

Most investigations of nerve regeneration achieved using immunosuppressive therapy are performed in rats and mice, and few data are described in nonhuman primates. Thus, Mackinnon’s group described a similar regeneration through 3-cm ulnar nerve auto- and allografts in cyclosporin A–treated and –nontreated primates. They provided the background data necessary to permit investigators to embark on clinical peripheral nerve allograft implantation protocols. Investigation in this area has been based particularly on electrophysiological, histological, and morphometric assessment, but little is known about the number of neurons present in the spinal cord of the primates when allografts are used in the nerve regeneration process.

In our study we observed that the number of labeled ulnar motor neurons in the spinal cord was similar in the autograft and allograft groups, with no statistical difference, as was the number of axons present in the proximal and distal stumps of the grafts. It appears that nerve regeneration through cold-preserved allografts and those temporarily treated with FK506 was similar to that observed through autografts, at least in terms of neuronal and axonal counts. Nevertheless, electrophysiological recordings obtained at 3,
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5, and 8 months showed that although CMAP is similar in both groups, there were significant differences in NCV between them (see Figs. 4 and 7). The differences relative to the NCV could be explained by the loss of Schwann cells after cessation of immunosuppression, resulting in a demyelination of regenerated axons. The number of axons stays constant but the maturity of the regenerated fibers decreases, which is translated into a lower NCV.

Cold Preservation of Nerves Combined With Temporary FK506 Treatment in Allografts

As is widely described in the literature, the use of non-treated nerve allografts transplanted in a nonimmunosuppressed host leads to an absence of axonal growth into the nerve allografts. This has been demonstrated in swine, sheep, and monkeys (Macaca fascicularis) that have exhibited no functional outcome. In mice and rats, axonal regeneration is detected only at the first 2 cm of the nerve allografts. We have used previous experimental data published in the same species (Macaca fascicularis), even studies in which the ulnar nerve was used as the experimental model, in which no axonal regeneration through the nontreated nerve allografts was observed. We decided that avoiding the killing of four more monkeys is an important consideration when this phenomenon is well known and is described in the literature.

The use of cold-preserved nerve allografts remains controversial. Some authors assert that cold preservation of nerve allografts, which reduces immunogenicity and thus decreases the dose of immunosuppressive agents needed, would not be an ideal alternative, because pretreated allografts have a reduced number of vital Schwann cells and therefore a decreased capacity to sustain nerve regeneration. Nevertheless, the viability of Schwann cells in cold-preserved nerves has been demonstrated, and the additive beneficial effect of the combination of cold preservation of nerve allografts and administration of FK506 has been described in rats and mice. This protocol leads to nerve regeneration parameters significantly superior to the positive con-

Fig. 6. Photomicrographs showing cross-sections of the motor branch of the ulnar nerve in the allograft group. A and B: Cross-sections of the nerve proximal to the graft. C and D: Cross-sections of the ulnar nerve distal to the graft. Hematoxylin (A and C), Bodian (B and D). Bar = 150 μm.
trol isografts, which previously were considered the gold standard of nerve regeneration.\textsuperscript{13}

Other investigations have focused on nerve regeneration through allografts temporarily treated with FK506, but the results remain controversial, varying from successful regeneration to complete allograft rejection.\textsuperscript{3,24,31} However, more recent reports point to a partial more than to a total rejection of nerve grafts, with a partial loss of function.\textsuperscript{31} In our study, the presence of more inflammatory cells, connective tissue, and fibrin in the allograft than in the autograft group could indicate a partial rejection in allograft cases after withdrawal of the immunosuppressant drug. Nevertheless, the autograft and allograft groups had similar values in the neuronal and axonal counts, which suggests similar activation of the target muscles.

**Conclusions**

Although good results have been found in rats and mice, withdrawal of FK506 has the effect of causing a partial graft rejection in monkeys. This rejection does not affect the neuronal and axonal counts, however, and in the functional assessment we observed recovery even to the extent of completed activation of hypothenar muscles in both groups.

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