Comparison of regeneration across nerve allografts with temporary or continuous cyclosporin A immunosuppression

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The efficacy of short-term immunosuppression in a nerve allograft model was examined by comparing regeneration across peripheral nerve allografts with either temporary (12 weeks) or continuous (30 weeks) cyclosporin A treatment. One-hundred fifty Lewis rats received 2-cm nerve grafts from allogeneic ACI or syngeneic Lewis rat donors and were allocated to the following groups: allogeneic grafts and continuous cyclosporin A, with 18 weeks (20 rats) or 30 weeks (20 rats) of survival after graft placement; allogeneic grafts and temporary cyclosporin A, with 12 weeks (10 rats), 18 weeks (20 rats), or 30 weeks (20 rats) of survival; and control rats with allogeneic and syngeneic grafts, no cyclosporin A, with 12, 18, or 30 weeks (10 rats each) of survival. Functional regeneration across the nerve grafts was serially assessed with walking-track analysis. Endpoint evaluations included electrophysiological, histological, and morphometric studies.

Both walking-track and electrophysiological function reached a plateau at a significantly worse level in nonimmunosuppressed allograft recipients than in syngeneic or treated allograft recipients. The group with temporary therapy experienced significant worsening in both motor and electrophysiological function at Week 18. 6 weeks after cyclosporin A withdrawal, compared to the group with continuous treatment. At Week 30, motor and electrophysiological function in the temporary-treatment group recovered to levels similar to those of the syngeneic and continuous cyclosporin A groups. Histological assessment of the graft segments from the temporary cyclosporin A group at 18 weeks showed evidence of retraction, with mononuclear cell infiltration and demyelination; morphometric evaluation demonstrated significantly decreased numbers of nerve fibers in the distal host segment. These histological and morphometric changes were no longer present in the nerves from the temporarily immunosuppressed rats at Week 30.

Withdrawal of immunosuppression after successful regeneration through nerve allografts results in short-term graft rejection. Eventual restoration of graft histological and function parameters is comparable to continuously immunosuppressed rats. Temporary immunosuppression of nerve allograft recipients is feasible.

KEY WORDS: nerve graft • nerve allograft • immunosuppression • cyclosporin A • nerve regeneration

INJURY to the peripheral nervous system results in significant patient morbidity with loss of sensory and motor function and frequently intractable chronic pain. The standard treatment for traumatic nerve deficits in reconstructive microsurgery remains the patient's own "expendable" sensory nerve autografts. An attractive alternative would be to use nerve allografts. This strategy would obviate the drawbacks associated with harvesting autografts, such as a scar, loss of sensation, and neuroma pain at the donor site, and would provide a potentially limitless supply of graft material for major reconstructive procedures.

Several recent experimental studies have demonstrated equivalent nerve regeneration across nerve allografts in rodents and primates immunosuppressed with continuous cyclosporin A therapy compared to nerve autografts. Although promising, these studies are controversial because of the use of potentially risky long-term immunosuppression for nonvital organ transplantation. It remains critical to address the feasibility of temporary host immunosuppression. We hypothesized that cyclosporin A immunosuppression is only necessary for the duration of time that the host axons are traversing the nerve allograft to reach host end-organs. The present study was designed to fully explore the possibility and assess the efficacy of short-term immunosuppression. This study compares temporary with continuous cyclosporin A immunosup-
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expression in a rat nerve allograft at multiple temporal endpoints, using histological, electrophysiological, and functional walking-track outcome measures.

Materials and Methods

Experimental Groups and Design

An overview of the experimental design and study groups is depicted in Fig. 1. A total of 150 Lewis rats received nerve grafts. Hosts received either temporary (12 weeks), continuous (until sacrifice up to 30 weeks), or no immunosuppression with cyclosporin A. Graft recipients were randomly assigned prospectively to the following experimental groups and sacrifice subgroups: allogeneic grafts and continuous cyclosporin A, with assessment at 18 weeks (20 rats) or 30 weeks (20 rats) of survival after graft placement; allogeneic and temporary cyclosporin A, with assessment at 12 weeks (10 rats), 18 weeks (20 rats), or 30 weeks (20 rats) of survival; and negative and positive control rats with syngeneic and syngeneic grafts, respectively, and no cyclosporin A, evaluated at 12, 18, or 30 weeks (10 rats each) postoperatively. Functional evaluation utilized a walking-track analysis of hind-footprint patterns, and was started preoperatively and performed serially over the entire course of the experiment. At sacrifice, each rat underwent electrophysiological assessment, after which the relevant nerves were removed for histological and morphometric studies.

Animal Model and Surgical Procedure

Lewis and ACI rats\(^*\) were acclimatized prior to surgical procedures, housed in flat-bottomed cages postoperatively, and allowed ad libitum access to standard rat chow and water. Inbred adult male Lewis (RT1\(^{b}\)) rats weighing 250 to 300 gm received nerve grafts from syngeneic Lewis (RT1\(^{b}\)) or allogeneic ACI (RT1\(^{b}\)) rat donors. These species differ at the major histocompatibility locus.\(^{16-18}\)

In all cases anesthesia was induced with inhalational ether followed by an intramuscular injection of 100 mg/kg ketamine hydrochloride (0.1 ml/100 gm Rogersee) and 10 mg/kg acepromazine maleate (0.1 ml/100 gm Atrave) into the lumbar paraspinal musculature.

All surgical procedures were performed aseptically and employed standard microsurgical techniques using an operating microscope. The sciatic nerves from donors were exposed through dorsal glutal-splitting incisions and externally neurolysed to isolate and resect a 2-cm measured segment of posterior tibial nerve as the nerve graft. An identical exposure of the host sciatic nerve was performed. The host tibial nerve was sharply transected at a standard position, 2 to 3 mm distal to its natural branching from the non-neurolysed sciatic nerve in the popliteal fossa. The nerve graft was then interposed and sutured to the transected posterior tibial nerve of the recipient Lewis animal with 10-0 nylon epineurial suture. The gluteal muscles and skin were approximated with interrupted 4-0 and continuous 3-0 nylon sutures, respectively. The ipsilateral peroneal and contralateral left sciatic nerves were unchanged to serve as normal intra-animal controls for electrophysiological and functional walking-track evaluation, respectively.

For endpoint electrophysiological studies, the animals were deeply anesthetized, tracheotomized, paralyzed with tubocurarine (0.5 ml of a 3-mg/ml intraperitoneal solution), and ventilated. After electrophysiological assessment, the nerves were removed for histological study. Ventilation was discontinued, following which the anesthesized animals were sacrificed with high doses of inhalational ether.

Immunosuppression and Cyclosporin A Levels

Cyclosporin A oral solution at a dose of 5 mg/kg was administered subcutaneously once a day starting 1 day preoperatively.\(^{6,34}\) The cyclosporin A dosage was adjusted weekly according to weight changes. Toxicity was monitored by daily observation, by weekly weighing, by determination of blood cyclosporin A levels (see below), and in selected cases by measurement of serum creatinine and hepatic enzyme (serum glutamic-oxalocetic transaminase, serum glutamine-pyruvic transaminase, and alkaline phosphatase) levels and examination of renal and hepatic histology at autopsy.

Whole-blood trough cyclosporin A levels were measured in cohorts of rats to ensure adequate dosing and to monitor for toxicity. In addition, four temporarily immunosuppressed rats underwent repeated cyclosporin A level determinations over a 15-day period after drug cessation to ascertain the pharmacokinetics of cyclosporin A clearance. The methods of measuring cyclosporin A levels in rats have recently been described.\(^{34}\) Briefly, determination of whole-blood cyclosporin A levels was performed by radioimmunoassay.

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\(^*\) Lewis and ACI rats were supplied by Harlan Sprague-Dawley, Indianapolis, Indiana.

Fig. 1. Study design: 150 Lewis rats received nerve grafts. The hosts received either temporary, continuous, or no cyclosporin A (CsA) immunosuppression. Within each therapy group, rats were randomly assigned to 12-, 18-, or 30-week endpoints for nerve conduction studies and histomorphometry. Motor function was assessed by walking-track performance serially over the course of the study, as indicated in the experiment scheme.
using a commercially available kit.† This radioimmunoassay, measuring mainly parent cyclosporin A rather than its metabolites, is based on a competitive procedure using double-antibody separation and an ¹²⁵I-labeled tracer.¶

Functional Assessment

A previously described walking-track analysis technique was used to evaluate rat hind-footprint patterns.⁰ Novak briefly, the walking track consisted of a track, 8.2 cm × 42 cm, darkened at one end with an appropriately cut length of exposed film placed on the bottom of the track. The hind feet of the rat were placed in a Petri dish containing x-ray film developer and the rat was then allowed to ambulate down the track, making hind-footprints. Measurements of walking tracks included the footprint length (PL). These measurements were taken for both the left normal control side (NPL) and the right experimental side (EPL) by means of a computer-linked digital pen and morphometry software.† The print length factor was obtained as follows: EPL – NPL/NPL, and was utilized to evaluate the walking-track patterns of each rat.

All rats underwent walking-track assessment preoperatively for baseline values, and then at 4, 8, 10, and 12 weeks postoperatively. Additional evaluations at 14, 16, 18, 20, 24, and 30 weeks were performed where appropriate. An interpretable walking track was always obtained, although on occasion an individual rat had to be walked more than once for this. All the tracks were measured in a blinded fashion for print length parameters.

Electrophysiological Studies

At the time of sacrifice, electrophysiological recordings across the grafts were made using a computer-assisted electromyographic machine.§ The animals were deeply anesthetized with ketamine hydrochloride and acepromazine maleate. The right sciatic nerve was exposed above the sciatic notch 2 cm proximal to the proximal repair site, and the tibial nerve was exposed distally 0.5 cm beyond the distal repair site. A tracheotomy was then performed, following which the animal was paralyzed with tubocurare and ventilated using a small-animal ventilator. An electrocardiographic monitor was used to follow the heart rate throughout the procedure. Body temperature was maintained at approximately 37°C using a heating lamp and a water-heated plastic blanket. Muscle temperature was monitored using a needle probe.

Bipolar hooked platinum electroencephalographic stimulating and recording electrodes¶ were placed under the sciatic nerve proximally and the tibial nerve distally, respectively. A ground was placed in muscle midway between the stimulating and recording electrodes. A stimulus duration of 0.05 msec was used and stimulus intensities ranging incrementally from 1 to 10 mA were delivered. A total of three to seven averaged direct compound nerve action potentials were obtained for each nerve. An identical setup, but with the distal recording electrode around the peroneal nerve, was used to obtain peroneal nerve compound action potentials from each animal. In addition, eight age- and weight-matched normal (previously unoperated) Lewis rats underwent similar electrophysiological evaluation.

From the supramaximal response of the recordings from each rat's tibial and peroneal nerves, the latency, amplitude, and negative area under the curve was obtained. Nerve conduction velocities were calculated based on derived latencies and measured distances. Conduction velocities were automatically corrected for any deviations in body temperature by an equation incorporated in the computer software.

Histological and Morphometric Studies

Following electrophysiological recordings, the entire sciatic nerve, including the proximal segment, nerve graft, and distal segment, was removed and fixed by immersion in a 3% (wt/vol) glutaraldehyde solution. The tissue was postfixed with osmium tetroxide and embedded in Araldite 502. Toluidine blue was used to stain cross sections 1 μm thick for light microscopy. Slides of the nerve allograft and distal host segment were evaluated by an observer blinded to treatment groups to assess the overall nerve architecture, quality and quantity of regenerated nerve fibers, degree of myelination, and the presence or absence of Wallerian degeneration and inflammatory cell infiltration.

Quantitative histological examination was performed on cross sections from host nerve segments 5 mm distal to the suture line. At 1000 magnification, six representative fields, chosen by an observer blinded to treatment groups, were evaluated per nerve, using a digital image-analysis system linked to morphometry software.* This system digitalizes the microscopic image and displays it on a video monitor with a calibration of 0.125 μ/ pixel. Analysis of the digitalized information, based on gray and white scales, is performed by the computer software. From each nerve segment, the fascicular area was measured, and 300 to 700 myelinated axons were evaluated for the area and diameter (smallest sieve diameter) of the axon, myelin, and fiber. Calculated from these primary measurements were the following additional morphometric indices: total number of myelinated fibers, percent neural tissue, nerve fiber density (no. of fibers/sq mm of tissue), axon/myelin ratio, and G ratio (axon/fiber diameter).

Statistical Analysis

An overall analysis of the differences in group means was calculated by a one-way analysis of variance (AN-

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† Kit manufactured by CycloTrac SP, INCSTAR, Stillwater, Maine.
‡ Software manufactured by Bioquant, R & M Biometrics, Nashville, Tennessee.
¶ Electodes manufactured by Nicolet Biomedical Instruments, Madison, Wisconsin.

* Digital image-analysis system manufactured by Leco Instruments, Montreal, Quebec, Canada.
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OVA) for the three electrophysiological variables: mean nerve conduction velocity, mean amplitude, and mean area. If the ANOVA demonstrated significant (p < 0.05) overall group effects for any of the three, then specific group mean comparisons were performed for that variable using post hoc independent sample t-tests, with a level of significance set at 0.05. In analyzing the print length factor parameter from the walking-track data, an initial ANOVA ascertained if significant inhomogeneity in the group means at any of the 11 assessment times was present. If this condition was fulfilled, then individual group means underwent Tukey's pairwise comparisons for the appropriate time points to correct for multiple comparisons and to maintain an overall alpha level of 0.05. Individual morphometric indices were similarly analyzed by ANOVA and Tukey's pairwise comparisons. All statistical tests were two-tailed. Statistics were calculated using SAS (version 6.03) and CRUNCH (version 3.0) software programs for personal computers.

Results

General Observations

During the 30-week course of the study, the majority of the 150 rats remained healthy and gained weight (average 175 gm) with age. Twelve (8%) rats died prematurely; deaths ranged from 11 to 29 weeks postoperatively. In nine of these 12 cases, rats exhibited a profound weight loss (range 50 to 100 gm) over several weeks before succumbing to death, consistent with cyclosporin A toxicity. In these nine rats, mortality was predominantly in the groups receiving continuous cyclosporin A treatment, with five deaths in the 30-week subgroup and two in the 18-week subgroup. Among the two deaths in the temporary cyclosporin A 18-week subgroup, both animals died within 2 weeks after drug cessation. A detectable cyclosporin A blood level at 197 ng/ml was found in the one rat that was measured. The three other deaths occurred in a temporary cyclosporin A 18-week subgroup rat at Week 17 secondary to an acute paraplegic syndrome, in a continuous cyclosporin A 30-week subgroup rat at Week 14 because of urinary tract sepsis, and in an autograft 30-week subgroup rat at Week 28 for unknown reasons. General autopsy, histological examination of the liver and kidneys, and serum levels of creatinine and hepatic enzymes were all within normal limits in these animals. The 12 rats that died were included for walking-track analysis, but were excluded for electrophysiological and histological analysis.

Cyclosporin A Levels

Figure 2 displays the changes in blood cyclosporin A levels over 15 days following drug cessation in four rats receiving temporary treatment. Drug levels fell from greater than 2000 ng/ml (upper limit of assay) on the day of the last drug treatment to a mean of 138 ng/ml by Day 15 postadministration. Thus, substantial amounts of circulating cyclosporin A were detectable for approximately 2 weeks after drug withdrawal in the temporary-treatment groups in this study.

Fig. 2. Graph showing blood cyclosporin A (CsA) levels in temporarily immunosuppressed rats at various times after drug cessation. Plots show individual levels for four rats on the day of withdrawal (Day 0) and on Days 4, 8, and 15 after the last dose. Levels on Day 0 were > 2000 ng/ml (upper limit of assay) and declined in an exponential manner over 2 weeks.

With an injection dose of 5 mg/kg/day, trough cyclosporin A blood levels were greatly elevated (> 1600 ng/ml and often > 2000 ng/ml). Because of the apparent chronic toxicity and mortality (seven deaths) in some of the drug recipients, we changed the dose schedule half-way into the study to 5 mg/kg/48 hours. Drug toxicity was reduced with this regimen (two further deaths), yet the blood levels remained high (mean 1311 ng/ml) and well above the previously reported therapeutic range for immunosuppression for nerve allografts.2

Walking-Track Analysis

Rats underwent walking-track assessment preoperatively to establish baseline values, and then again at 4, 8, 10, 12, 14, 16, 18, 20, 24, and 30 weeks postoperatively. Figure 3 summarizes the mean print length factor results at each of the 11 assessment times for the four study groups in this experiment. At baseline, the print length factor was close to 0 for all animals as the EPL and NPL for each rat were virtually identical, and therefore the numerator (EPL - NPL) for the print length factor equation was 0. With a tibial nerve injury and resulting denervation of the ankle extensors, the right footprint pattern length increased as did EPL and hence the print length factor. At Week 4, the mean print length factor in the four groups ranged from 0.2 to 0.3, with no significant differences between groups; the mean print length factor recovered to some degree in all of the groups. By Week 10, the recovery was similar in the autograft and both immunosuppressed allograft groups, but the mean print length factor remained significantly elevated (worse) in the untreated allografted rats (p = 0.002, ANOVA; p < 0.05). Tukey's). This statistical relationship between an increased mean print length factor in the nonimmunosuppressed allograft recipients was maintained at the 12-week (p = 0.02) and 14-week (p = 0.012) assessment times. By Week 16, 4 weeks after cyclosporin A withdrawal, mean (± standard error of the mean) print length factor in the temporary-treatment group had
risen to 0.21 ± 0.02. This was similar to the untreated allograft group (0.20 ± 0.03), but significantly higher compared to the mean print length factor of both the autograft (0.061 ± 0.03) and the continuous-treatment allograft groups (0.066 ± 0.02; p = 0.0001). The mean Week 18 print length factor for the temporary-treatment group remained similarly increased (p = 0.0001). There was a progressive decrease in the mean print length factor of the temporary-treatment group from Week 20 (0.11 ± 0.03) through Weeks 24 (0.09 ± 0.02) and 30 (0.08 ± 0.02). Indeed, for these three assessment times the mean print length factor in the temporary-treatment group was not significantly different from the autograft and the continuous-treatment allograft groups. At both Weeks 24 and 30, the mean print length factor of the untreated allograft groups remained significantly higher than the other three groups (p < 0.005).

In summary, as measured by the print length factor, the temporary-treatment rats demonstrated a marked worsening and then steady recovery of motor function after cessation of cyclosporin A treatment. The eventual level of function recovery was similar to that achieved by nerve autograft and continuous treatment allograft recipients. In contrast, untreated allograft recipients recovered only partial function, which reached a plateau at a significantly lower level than the other groups.

**Electrophysiological Results**

The mean amplitude, the negative area under the curve, and the nerve conduction velocity data from tibial nerve recordings are presented in summary form in Table 1. As illustrated by the large standard errors, the amplitude and area data demonstrated great variability even within groups, precluding meaningful statistical comparisons between groups. In contrast, the latencies of the waveforms and the conduction velocities were much less variable. Additionally, to correct for any possible variability in recording techniques from one rat study to the next, tibial nerve conduction velocities were divided by peroneal nerve conduction velocities for each rat and the group means of the resulting ratio data are displayed in Fig. 4. The overall ANOVA indicated significant group effects for the nerve conduction velocity variable (F = 6.22, p < 0.001).

Normal unoperated rats had a mean nerve conduction velocity of 74.2 m/sec. This was similar to the 12-week mean conduction velocity of the rats receiving autografts (68.7 m/sec) and the immuno-suppressed rats receiving allografts (71.1 m/sec). These three conduction velocities were significantly superior to the mean conduction velocity of the untreated allograft recipients (40.3 m/sec; p < 0.001, t-tests). The mean nerve conduction velocity of untreated allograft recipients at Weeks 18 and 30 remained significantly slower than all other groups (p < 0.05). At Week 18, the temporary-treatment group demonstrated a decreased level of nerve conduction velocity; this group's mean conduction velocity was 49.9 m/sec, which was significantly slower than the mean nerve conduction velocity of the continuous-treatment group (62.6 m/sec; p = 0.0064). For those animals assessed at 30 weeks, the mean conduction velocity of the temporary-treatment group (53.8 m/sec) and the continuous-treatment group (49.9 m/sec) were similar (p = 0.48).

**Histological Results**

The sections from the tibial nerve proximal to the nerve grafts had a similar and normal histological appearance in all rats and were indistinguishable between
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**Fig. 5.** Photomicrographs of allograft segments from temporarily (a and c) and continuously (b and d) immunosuppressed rats at Weeks 18 (a and b) and 30 (b and d). a: Wallerian degeneration (w) and mononuclear inflammatory cell infiltration within the endoneurium are observed. Nerve fiber density is somewhat decreased. Most striking, however, is the presence of many large but thinly myelinated nerve fibers (arrows). b: In contrast, a diverse population of well-myelinated regenerated axons is present with no inflammatory infiltrate or evidence of Wallerian degeneration. c: A dense population of well-myelinated nerve fibers is observed in a normal-appearing endoneurial environment. d: An endoneurial inflammatory cell infiltrate, especially disposed perivascularly, is observed among a dense population of well-myelinated axons. Calibration bar = 20 μm. Toluidine blue, × 280.

treatment groups. In contrast, the histological appearance of the donor graft and distal host segments was discernibly different among groups.

**Untreated Syngeneic Graft Group.** The syngeneic graft segments at all time assessments had a normal appearing fascicular architecture. There was evidence of excellent regeneration with a dense population of small and large well-myelinated fibers. Wallerian degeneration and inflammatory cells were absent. The host nerves distal to the grafts had normal fascicular patterns, and were well populated with a diverse size distribution of normally myelinated nerve fibers.

**Untreated Allogeneic Graft Group.** The allografts were occasionally atrophic and frequently demonstrated disruption of nerve architecture. Although there was some evidence of regeneration into most allografts, degeneration and lymphocytic infiltration were also common. The distal host segments appeared to have a decreased fiber population.

**Temporary-Treatment Allogeneic Graft Group.** These groups demonstrated marked differences in histological findings depending on the assessment times. For rats assessed at 12 weeks, the histological appearance of the allograft and distal host segments was indistinguishable from the syngeneic graft group. At Week 18, a dramatic change was evident. The allograft was now infiltrated by lymphocytes, which were observed in the perineurium and endoneurium, and often disposed perivascularly (Fig. 5a). Wallerian degeneration and a decrease in the fiber population was seen, but more striking was the presence of many large, morphologically normal axons with a thin myelin sheath (Figs. 5a and 6). The nerves distal to these grafts contained sharply reduced numbers of nerve fibers but a paucity of Wallerian degeneration. At Week 30, both graft and distal host segments exhibited normal overall appearances and nerve fiber populations (Fig. 5c) similar to the 30-week syngeneic graft and continuous-
treatment allogeneic graft groups. Inflammatory cell infiltration was absent.

Continuous-Treatment Allogeneic Graft Group. In contrast to the temporary-treatment group, at Week 18 the nerves from the rats in the continuous-treatment group had histological characteristics similar to normal rat regenerated nerves. Specifically, a diverse distribution of small and large fibers with an appropriate caliber of myelination was seen (Figs. 5b and 7). At 30 weeks, significant numbers of infiltrating inflammatory cells were observed in approximately 50% of the allografts (Fig. 5d). Despite their presence, the graft and distal nerve had normal architecture and contained a dense population of well-myelinated nerve fibers (Fig. 5d).

Morphometric Results

At Week 12, nerve fiber density in the distal host nerve segment was similar in the autogeneic graft, immunosuppressed, and untreated allogeneic graft groups (Fig. 8). However, the untreated allograft group exhibited a significantly decreased mean myelinated fiber diameter in the distal nerve segment (p < 0.05, ANOVA and Tukey’s comparisons; Fig. 9). Quantitative histological examination of the distal nerve segments confirmed the qualitative impression that at Week 18 the temporary-treated group demonstrated a considerable decrease in the number of myelinated nerve fibers (p < 0.01). This was reflected by the nerve fiber density data (Fig. 8) as well as the percent of neural tissue and total number of fiber data (not shown). Despite an overall decrease in the number of nerve fibers in the temporary-treatment group at Week 18, the morphometric qualities of the remaining fibers were similar to the other groups. For instance, the mean nerve fiber diameter (Fig. 9) was unaltered, as were axon area, myelin area, and G ratio (axon/fiber diameter) (data not shown). At Week 30, all morphometric parameters were similar in the four experimental groups.

Discussion

History of Nerve Allografts

Nerve grafting utilizing autologous tissue has become the treatment of choice for the management of a significant peripheral nerve gap. The first nerve graft was reported by Phillippeaux and Vulpian in 1870. The first human allogeneic nerve graft was attempted by Albert in 1878. The early results of nerve allografts were consistently more disappointing than autografts. Functional regeneration was poor and allografts were deemed useless. The allografts, as opposed to autografts, elicited a severe inflammatory response with lymphocytic infiltration, Schwann cell necrosis, and disruption of neurilemmal tubes. With advances in immunology and solid organ transplantation, it became clear that rejection of foreign tissue was due to the disparity of major histocompatibility antigens between the donor and recipient tissue as well as any tissue-specific or minor histocompatibility antigens.

More recent studies in peripheral nerve allografting have used various strategies to prevent nerve allograft rejection, including histocompatibility matching, pretreatment of the nerve grafts, or host immunosuppression. Nerve allografts from minor, as compared to major, histocompatibility antigen-disparate donors elicited decreased immunological responsiveness and lesser nerve allograft rejection and resulted in greater regeneration across the allogeneic nerve graft. Several methods of pretreating the donor nerve to de-
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| TABLE 1 |
|-------------------|-------------------|-------------------|
| Study Group       | Conduction Velocity (m/sec) | Area (μV·msec) | Amplitude (μV) |
| Week 12           | 40.3 ± 7.35*       | 35.6 ± 15.8      | 68.6 ± 24.9    |
| allograft, no cyclosporin A |                       |                   |                 |
| allograft & cyclosporin A | 71.1 ± 3.90       | 44.3 ± 11.1      | 95.3 ± 18.8    |
| autograft         | 68.7 ± 3.31       | 49 ± 18.3        | 96.4 ± 25.4    |
| Week 18           | 37.7 ± 7.46*      | 27.3 ± 11.3      | 53.1 ± 19.3    |
| allograft, no cyclosporin A |                       |                   |                 |
| allograft & temporary cyclosporin A | 49.9 ± 3.26+     | 23 ± 3.3         | 46.2 ± 6.3     |
| allograft & continuous cyclosporin A | 62.6 ± 3.72      | 81.4 ± 26.6      | 127.4 ± 34.6   |
| autograft         | 66.0 ± 3.07       | 66.6 ± 28.3      | 131 ± 37.9     |
| Week 30           | 43.7 ± 6.3†       | 48 ± 17.6        | 108.5 ± 24.3   |
| allograft, no cyclosporin A |                       |                   |                 |
| allograft & temporary cyclosporin A | 53.8 ± 2.25       | 53 ± 13.4        | 121.7 ± 19.3   |
| allograft & continuous cyclosporin A | 42.9 ± 2.46       | 67 ± 14.5        | 128.3 ± 13.9   |
| autograft         | 52.8 ± 3.5        | 25.7 ± 11.7      | 79.5 ± 13.6    |
| normal, unoperated rats | 74.2 ± 2.4        | 2077 ± 473       | 3482 ± 707     |

* Values are mean ± standard error of the mean for each group.
† Statistically significant different (p < 0.05) from other groups at similar assessment times.

crease its antigenicity have been employed, including sectioning the nerve prior to transfer to allow for Wallerian degeneration, freezing, boiling, irritating, and lyophilizing either individually or in combination.36,33,45 Pretreatment with irradiation alone, lyophilization alone, or combinations of irradiation plus lyophilization or irradiation plus freezing were effective, but remained inferior to autografts.20,28 Various nonspecific immunosuppressive agents including azathioprine, actinomycin D, methotrexate, corticosteroids, antilymphocytic preparations, and combinations thereof have been used to try to decrease the nerve allograft rejection response, but they have enjoyed only limited and varied success.27,36,40

With the isolation of cyclosporin A from the fungi *Tolypospadium inflatum* in 1970 and the discovery of its immunosuppressive activity by Borel,10 organ and tissue transplantation has advanced greatly. Many types of solid organ and tissue transplantation have become increasingly successful in the experimental and clinical setting.44,45

Peripheral nerve transplantation with cyclosporin A immunosuppression was first attempted by Zalewski, *et al.*54 in rats with major histocompatibility complex disparities. In this and further studies, Zalewski and coworkers3,54 showed histological evidence of regeneration into the allograft following a proximal nerve repair, but no quantitative assessments were performed. In a rat model, Bain, *et al.*7 established a dose-response curve and determined the minimum effective dose (5 mg/kg/day) required to prevent rejection of nerve allografts with major histocompatibility complex disparities. By histological, morphological, electrophysio-

![Fig. 8. Bar graph showing nerve fiber densities (no. of myelinated nerve fibers/sq mm of tissue) from distal host nerve segments. At Week 18, cyclosporin A (CSA) temporary-treatment rats (***) demonstrated a sharp decline (p < 0.01, ANOVA and Tukey's comparisons) in axon density (mean ± standard error of the mean) compared to autografted and continuously immunosuppressed rats. Untreated allograft recipients (*) showed a similar decrease at Week 18 (p < 0.05). In contrast, fiber density at Week 30 was not significantly different between the four groups.]

![Fig. 9. Bar graph showing mean nerve fiber diameters of myelinated axons from the distal host nerve. All groups had similar mean diameters (± standard error of the mean) diameters, except at Week 12 where the untreated allograft group (*) had a significantly lower diameter in comparison to the cyclosporin A (CSA) immunosuppressed allograft group (p < 0.05. ANOVA and Tukey's comparisons).]

logical, and functional assessments, Bain, *et al.*7 showed that regeneration across nerve allografts in rats immunosuppressed with cyclosporin A was comparable to that across control syngeneic grafts, and significantly superior to that across allogeneic nerve grafts in untreated recipients. Regeneration equivalent to that produced by autografts was similarly demonstrated across nerve allografts in cyclosporin A immunosuppressed primates as measured by clinical, histological, and electrophysiological parameters and by recovery of muscle contractile function.5,15 Successful suppression of the immune response and regeneration across a nerve allograft using cyclosporin A treatment was also recently documented in a murine model.23

**Temporary Immunosuppression**

A peripheral nerve transplant may restore lost function or enhance recovery, but is not essential for patient

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survival; thus, the benefits of restored function must clearly outweigh the risks of immunosuppression. Is host immunosuppression of short duration sufficient? Unique to peripheral nerve grafts is that, unlike other organ transplants, the foreign graft provides only a structural conduit, namely Schwann cells, neurilemmal sheaths, and neurotrophic factors, through which autogenous axons must regenerate to reinnervate distal end-organs of autogenous origin. Hence, immunosuppressive therapy may only be required temporarily to prevent allograft rejection during the time that regeneration is actively proceeding. Once host axons have traversed the graft, cessation of immunosuppressive therapy becomes a possibility. The ensuing rejection of non-neuronal support cells may not result in axonal loss or in a permanent loss of function.

The present study was designed to test the hypothesis that a temporary course of cyclosporin A immunosuppression would suffice to permit functional regeneration across peripheral nerve allografts, comparable to continuous cyclosporin A immunosuppression. We used an inbred rat nerve allograft model of proven antigenicity in which nerve allografts without immunosuppression failed, but which allowed regeneration comparable to autografts when continuous cyclosporin A immunosuppression was provided. Although the rat nerve injury model has been criticized on the basis of allowing good histological regeneration, but poor to moderate long-term functional recovery, this is not dissimilar to patients who undergo repair of complete nerve injuries. A tibial rather than a sciatic nerve injury model was chosen to optimize recovery from which deterioration could be more readily documented by print length determinations from walking-track analysis. A 12-week course of cyclosporin A therapy was chosen as the minimum necessary for all immunosuppressed rats in this study to allow for regeneration across the nerve grafts and reinnervation of end-organs, based on known rates of rat nerve regeneration, and to attain a plateau of walking-track recovery after tibial nerve transection and repair. The 18-week sacrifice endpoint (6 weeks after cyclosporin A cessation) was chosen to demonstrate a rejection episode in the temporary-treatment group and was based on a pilot study. A final evaluation at 30 weeks was considered to be of sufficient time after cyclosporin A cessation to allow for any possible recovery to be demonstrated. Finally, at each of the three assessment times, an autograft group, a nonimmunosuppressed allograft group, and a continuous-treatment allograft control group were included.

The walking-track print length factor data, nerve conduction velocity results, and histological evaluation at 12 weeks all verified that immunosuppressed allograft recipients had comparable functional regeneration to autograft recipients; both groups were superior to the nonimmunosuppressed allograft group. Moreover, the attainment of the print length factor plateau as early as Week 10 (Fig. 3) indicated that reinnervation of the ankle extensors (gastrocnemius and soleus muscles) with resulting muscle function had occurred. Upon cessation of cyclosporin A immunosuppression after 12 weeks, blood levels declined over a 2-week period (Fig. 2). In this temporary-treatment group, unambiguous evidence of graft rejection was demonstrated after cyclosporin A withdrawal. First, both mean nerve conduction velocity and the mean print length factor were significantly inferior to the continuous-treatment rat group at Week 18 (Figs. 3 and 4). Second, the histological sections demonstrated inflammatory cell infiltration into the allograft with attendant axonal damage, Wallerian degeneration, and striking hypomyelination (Figs. 5a and 6). The latter suggests early remyelination of spared but demyelinated axons. Distal to these allografts, the nerve fiber population was significantly diminished (Fig. 8). In contrast, at Week 18 the control continuous-treatment subjects had normal histology. By Week 30, mean print length factor, mean nerve conduction velocity, and histological characteristics of the temporary-treatment group had recovered and were not significantly different as compared to the continuous-treatment and autograft control groups. In summary, cessation of immunosuppression after successful regeneration through nerve allografts resulted in short-term graft rejection. However, eventual recovery of graft histological and functional parameters was observed to be comparable to continuously immunosuppressed rats.

A limitation of the present study was in the use of a rat nerve allograft model and a relatively short (2-cm) unifascicular graft segment. Future studies are necessary to assess the efficacy of temporary immunosuppression for longer nerve allografts and in other species, including nonhuman primates.

It has been suggested that nerve allograft segments in animals immunosuppressed with cyclosporin A will be rejected with loss of axons, cellular elements, and graft function upon cessation of immunosuppression. However, these conclusions were based on studies that suffered from an inadequate duration of cyclosporin A treatment, small sample size, short survival times after cyclosporin A withdrawal, and the performance of a proximal but not a distal nerve repair. Two recent studies have investigated the nerve allograft response after cessation of cyclosporin A immunosuppression. Yu, et al., reported long-term functional survival of rat nerve allografts as measured by maintained electrophysiological function in three of seven rats 4 months after the termination of a long period of immunosuppressive therapy. In a small study (14 rats), Mackinon, et al., observed that following withdrawal of cyclosporin A immunosuppression at 8 weeks following allogeneic nerve graft placement, sciotic nerve function index decreased significantly, reaching a nadir at 14 weeks, but then recovered by 20 weeks after graft placement. Histological analysis revealed evidence of graft rejection and denervation at 14 weeks and remyelination at 20 weeks. Although supporting our findings, both of the previous studies can be criticized for their small size and lack of appropriate control groups.

Pathogenesis of Allograft Rejection Following Temporary Immunosuppression

The finding of axon preservation in a thinly myel-
nated state and the evidence of axonal damage in the 18-week temporarily treated allograft segments (Fig. 8) may suggest the pathogenesis of allograft rejection in this model. Schwann cells are regarded as the primary immunogenic components of the peripheral nerve, capable of up-regulating major histocompatibility complex expression and presenting antigen to elicit immunological responses from T lymphocytes. Donor-derived Schwann cells in the naive allograft would therefore be the primary targets of the host immunological response. In an immunosuppressed allograft recipient, regenerated host axons become ensheathed by donor Schwann cells. Hence, withdrawal of immunosuppression may have differential effects on the graft constituents, with rejection of donor-derived Schwann cells, but survival of demyelinated host axons. Precisely such a disparity was observed in our study. The additional presence of axonal injury observed in the Week 18 temporary-treatment group may be partially explained by the bystander injury phenomenon which occurs to axons in several other demyelinating conditions where the immunological response is targeted against axon-ensheathing glial cells. Other axons may have been injured because of a vascular ischemic insult secondary to the rejection of allogeneic graft endothelial cells and microvascular thrombosis.

If donor Schwann cells were destroyed, then what series of events is responsible for the eventual recovery of the temporary-treatment allograft group? We hypothesize that, following a rejection response after cessation of immunosuppression, host Schwann cells repopulated the donor nerve graft to account for remyelination. Indeed, differences in Schwann cell origin and thus immunogenicity may have accounted for the fact that one-half of the Week 30 continuous-treatment allografts were infiltrated by lymphocytes, which were absent in the Week 30 temporary-treatment allograft segments. Replacement by host-derived Schwann cells could underlie eventual restoration of function in a nerve allograft whose foreign cellular elements have been rejected. Aguyao, et al., found the migration of morphologically identifiable Schwann cells, presumably of host origin, into rejecting human to mouse xenografts following discontinuation of immunosuppression. In an immunohistochemical study using species-specific rat monoclonal antibody markers, Lassner, et al., studied the cellular rejection mechanisms in peripheral nerve allografts. They discovered that while donor-specific major histocompatibility complex markers were no longer detectable 6 weeks postoperatively, an acellular nerve graft architecture was maintained and recipient-specific Schwann cells were present in the nerve allograft. Since no specific markers for host Lewis versus donor ACI rat Schwann cells were employed in the present study, the replacement hypothesis remains speculative. Future studies are planned to test this hypothesis.

Conclusions

Following demonstrable nerve regeneration across rat nerve allografts with cyclosporin A treatment, withdrawal of immunosuppression results in nerve allograft rejection, demyelination, and axonal loss. Over time, recovery of graft electrophysiological and related motor function occurs, accompanied by remyelination of surviving graft axons and regeneration of injured axons. Replacement of rejected foreign donor Schwann cells by host-derived Schwann cells may partially underlie the recovery process. This study supports the use of temporary cyclosporin A immunosuppression for nerve allografts. Further studies in nonhuman primates and humans are required to assess the potential clinical application of temporary immunosuppression for nerve allografts.

Acknowledgments

The authors thank Janos Patkai and Dr. Tomoo Maeda for technical assistance, Marco Katic for assistance with statistical analysis, Dr. Gerard Murphy of Sandoz for supplying cyclosporin A oral solution, and Drs. Larry Becker, Reg Gorczynski, and Alan Hudson for research advice.

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Manuscript received December 19, 1991. Accepted in final form May 27, 1992. This work was funded by the Medical Research Council of Canada and Sandoz, Canada.

Research fellowships for Drs. Midha and Evans were supported by the Medical Research Council of Canada.

This paper received the 1992 Mayfield Award given by the Joint Section on Disorders of the Spine and Peripheral Nerves. This work was presented in part at the Society for Neuroscience Meeting, New Orleans, Louisiana, November, 1991, and at the Annual Meeting of the American Association of Neurological Surgeons. San Francisco, California. April 11–16, 1992.

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