JOURNAL OF NEUROTRAUMA
Volume 22, Number 11, 2005

Pp. 1267–1281

Immunosuppression with Either Cyclosporine A or FK506 Supports Survival of Transplanted Fibroblasts and Promotes Growth of Host Axons into the Transplant after Spinal Cord Injury

YOSHIKAZU HAYASHI,[1,2] JED S. SHUMSKY,[1] THERESA CONNORS,[1] TAKANOBU OTSUKA,[2] ITZHAK FISCHER,[1] ALAN TESSLER,[1,3] and MARION MURRAY[1]

ABSTRACT

Fibroblasts that have been genetically modified to secrete neurotrophins can stimulate axonal regeneration, rescue injured neurons, and improve function when grafted into a spinal cord injury site. These grafts are usually allografts that require immunosuppression to prevent rejection. In this study, we compared the effects of two immunophilin-ligands (cyclosporine A [CsA] and FK506) that are used clinically to prevent transplant rejection on protection of grafted fibroblasts. As there are risks associated with prolonged immunosuppression, we compared the effects of 2 or 8 weeks of administration of these drugs, in combination with our standard methylprednisolone protocol, in animals that survived for 8 weeks, to determine whether a shorter course of immunosuppression would be effective. Outcome measures included fibroblast survival, infiltration of activated macrophages and microglia into the graft, final lesion size, and growth of host axons into the graft. The graft consisted of a Vitrogen matrix into which fibroblasts were suspended; the graft was placed into a C3/C4 lateral funiculus lesion. The fibroblasts were isolated from a transgenic strain of Fischer rats that produce the marker alkaline phosphatase (Fb/AP). This enabled us to track the grafted fibroblasts and to evaluate the extent of their survival. The grafted matrix filled the lesion cavity. The density of fibroblasts within the matrix differed according to treatment. Fibroblast survival was most robust in animals that received 8 weeks of immunophilin-ligand treatment. FK506 supported greater Fb/AP survival than CsA. ED-1 immunostaining for activated microglia and macrophages showed an inverse correlation between AP immunoreactivity and the density of immune cells within the graft. Thus, prolonged administration of either FK506 or CsA was necessary for maximal fibroblast survival and for limiting the macrophage invasion of the graft. None of the FK506 or CsA protocols modified the size of the lesion, indicating that these immunophilin-ligands had little effect on secondary enlargement of the lesion and therefore little neuroprotective effect. Because immunophilin-ligands have been shown to be neurotrophic, we used RT-97 immunostaining for neurofilaments and calcitonin gene related protein (CGRP) staining for dorsal root axons to visualize axons that grew

[1]Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, Pennsylvania.
[2]Department of Orthopedic Surgery, Nagoya City University Medical School, Nagoya, Japan.
[3]Department of Veterans Affairs Medical Center, Philadelphia, Pennsylvania.

into the graft. Some axons grew into the matrix even in the absence of immunophilin-ligand treatment, suggesting that the Vitrogen matrix itself is permissive, but all of the immunophilin-ligand protocols were much more effective in eliciting axonal growth. Growth of axons into the transplants was equally increased by drug treatment for 2 or 8 weeks. Thus, both treatments improved fibroblast survival, diminished immune cell invasion, and promoted axonal growth, and a 2-week course of treatment with either immunophilin-ligand was as effective as 8 weeks in stimulating axonal growth.

Key words: allograft survival; axonal growth; cyclosporine A; FK506; immunophilin ligands; spinal cord injury; transplantation

INTRODUCTION

THE USE OF CELLULAR TRANSPLANTS as a treatment to repair injured spinal cord is now quite common in animal models. Most of these transplants are allografts and thus, in the absence of immune suppression, subject to rejection mediated by the host immune system (Tobias et al., 2001, 2005). The use of host cells as a source of transplant material could obviate this problem but this approach may not be widely applicable clinically. Courses of immune suppression are therefore likely to continue to be required in transplant paradigms. There are, however, risks associated with long-term immune suppression, including increased rates of infection and malignancy (Finberg and Fingeroth, 2004). It is possible that the need for immune suppression may be more limited in the case of spinal cord repair mediated by cellular transplants if the function of the donor cells is primarily to protect neurons from the immediate toxic environment induced by the injury, to protect axotomized neurons from retrograde degeneration and to encourage axonal growth during the early post-transplantation period. Cessation of immune suppression when axonal growth is complete may lead to a gradual rejection of the implanted cells without loss of function recovered as a result of the transplant. Successful regeneration has been observed, for example, after temporary immunosuppression in rats with peripheral nerve allografts (Katsube et al., 1998). Persistence of regenerated axons has also been observed in experiments in which transplanted hybridomas that secrete the myelin inhibitor IN-1 are protected for several weeks by treatment with cyclosporin A (CsA), then rejected when CsA is discontinued (Schnell and Schwab, 1990).

The only drug approved for use in humans with spinal cord injury (SCI) is the glucocorticoid methylprednisolone, which has physiological effects that might preserve damaged spinal cord, including decreasing the number of infiltrating inflammatory cells (Bartholdi and Schwab, 1995), decreasing gray matter damage (Takami et al., 2002), suppressing lipid peroxidation and opposing the effects of free radicals (Anderson et al., 1988; Hadley, 2002). Its beneficial effects, however, appear to be modest in animal studies (Rabshevsky et al., 2002), and its clinical utility has been questioned (Hadley, 2002). Because it is still used clinically, however, we have incorporated administration of methylprednisolone into our standard protocol (Liu et al., 1999, 2002; Tobias et al., 2003). Methylprednisolone administration alone does not prevent rejection of non-autologous cells (Tobias et al., 2005). Administration of CsA is a standard treatment to prevent rejection. More recently another drug, FK506 (Tacrolimus), has been used clinically. Both FK506 and CsA are immunophilin-ligands, although they are structurally different and bind to different binding proteins. In addition to their immunosuppressive properties, they have been described as promoting axonal growth (Teichner et al., 1993; Gold et al., 1997; Steiner et al., 1997a,b; Wang et al., 1997; Sugawara et al., 1999; Wang and Gold, 1999) and as being neuroprotective (Scheff and Sullivan, 1999; Winter et al., 1999; Singleton et al., 2001).

In this study, we compared the effects of 2 or 8 weeks of treatment with CsA or FK506 in outbred Sprague-Dawley rats that received transplants of fibroblasts into a lateral funiculus lesion at the C3/C4 level. All animals survived 8 weeks. The fibroblasts were obtained from transgenic Fischer rats that express alkaline phosphatase (Fb/AP). The use of Fb/AP enabled us to reliably identify the grafted cells. Our outcome measures were (1) the survival of fibroblasts, to evaluate effects on protection from rejection; (2) the presence of inflammatory cells within or near the lesion/transplant site, to evaluate effects on the immune response; (3) the size of the lesion, to identify differences in secondary degeneration; and (4) the presence of axons within the graft, to assess whether axonal growth was promoted.

MATERIALS AND METHODS

Animal Groups

Forty female Sprague-Dawley rats (200–250 g; Taconic, Germantown, NY) were given free access to

food and water and housed under a 12-h light/dark cycle (lights on at 07:00). All animal procedures were approved by Drexel University College of Medicine's Institutional Animal Care and Use Committee in accordance with NIH guidelines for animal use.

All rats received a right lateral funiculotomy and a transplant of fibroblasts genetically modified to produce alkaline phosphatase (Fb/AP) into the lesion cavity. The fibroblasts were delivered in a Vitrogen Collagen (Cohesion Technology, Encinitas, CA) matrix. All rats received one bolus intravenous injection of methylprednisolone (30 mg/kg BW; Pharmacia & Upjohn Company, Kalamazoo, MI) through the tail vein within 5 min of the conclusion of surgery and another injection 2 h post-surgery, as in our previous studies (Liu et al., 1999).

Rats were divided into six groups. The control group ($n = 7$) received no CsA (Novartis Pharmaceutical Corp., East Hanover, NJ) or FK506 (Fujisawa Pharmaceutical Corp., Osaka, Japan) treatment. Of the experimental groups, one received a limited course of immunosuppression: 2 weeks of daily CsA injections (10 mg/kg), beginning three days before grafting and continuing for 2 weeks after grafting (2W CsA; $n = 7$), for a total of 17 days. This is the dose that we have used in our previous studies (Liu et al., 1999, 2002), based on an earlier study (Ibarra et al., 1996). Its comparison group received a limited course of immunosuppression: daily subcutaneous injections (1 mg/kg) of FK506, beginning 3 days before grafting and continuing for 2 weeks after grafting (2W FK506; $n = 8$). We chose the FK506 dose based on personal communication from the manufacturer, Fujisawa Pharmaceuticals. Another group of rats was treated with our standard immunosuppression regimen, as previously described (Liu et al., 1999), and given daily subcutaneous injections of CsA beginning 3 days before grafting and continuing for 2 weeks after grafting, followed by oral CsA, resuspended in the drinking water at a final concentration of 50 μg/mL, for 6 weeks (8W CsA inj + oral; $n = 6$). Another group received 8 weeks of subcutaneous CsA injections (8W CsA inj; $n = 6$) without oral CsA administration. This group was compared to one that received 8 weeks of daily subcutaneous injections of FK506 injections, beginning 3 days before grafting and continuing for 8 weeks (8W FK506; $n = 6$). Thus, immunophilin-ligand treatment was absent in the control group, discontinued after 2 weeks in the next two groups, and administered for the 8-week duration of the experiment in the remaining three groups. All animals were sacrificed at 8 weeks post-injury.

Surgical Procedures

Rats were anesthetized by an intraperitoneal (i.p.) injection of acepromazine maleate (0.5 mg/kg; Fermenta Animal Health Co., Kansas City, MO), ketamine (63 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA), and xylazine (6.3 mg/kg; Bayer Co., Shawnee Mission, KS). A laminectomy at the C3–4 level exposed one spinal cord segment. The dura over the right dorsal root entry zone was opened with a 30-gauge needle, and a shallow incision was made in the right dorsal spinal cord. A glass-pulled fine-tipped microaspiration device was then used to extend the lesion laterally and ventrally. This lesion completely disrupted the lateral funiculus, partially removed the ipsilateral ventral white matter and gray matter, but left the dorsal columns intact. The rostrocaudal extent of the lesion cavity was approximately 2–3 mm. When hemostasis was achieved, fibroblasts in a Vitrogen matrix were injected into the lesion cavity, using a 10-μL Hamilton syringe (26-gauge), until the cavity was filled completely. A total of 2×10^5 μL (in 2 μL) was injected. The dura was closed with interrupted 9-0 sutures (Ethicon), the muscle closed in layers with 4-0 sutures, and the skin closed with surgical staples. After surgery, rats were kept on heating pads and observed until fully awake, and then returned to their cages.

Cell Culture

Fb/AP cells were isolated from a transgenic strain of Fischer 344 adult rats (Kisseberth et al., 1999; Han et al., 2002) and cultured as described previously (Liu et al., 1999; Tobias et al., 2001). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 2 mM L-glutamine on uncoated tissue culture dishes (Falcon, Franklin Lake, NJ) in standard humidified 5% CO_2 at 37°C.

On the day of surgery, confluent cultures of cells were collected and counted. The cell suspension was centrifuged and the supernatant removed. The cell pellet was gently mixed 1:1 with a solution of Vitrogen Collagen to a final concentration of 1×10^5 cells/μL. The Vitrogen solution was prepared on the day of surgery by mixing 1 mL of Vitrogen Collagen with 0.125 mL of fibroblast cell culture medium; pH was adjusted to 7.4, and the solution was placed on ice to prevent gelation. Cells were kept on ice during surgery. After surgery, the cells not used for transplantation were cultured overnight to confirm that their viability was not compromised by storage on ice for 4–6 h during the surgical procedures or by suspending the cells in the Vitrogen matrix for transplantation. In all cases, Fb/AP cells survived and proliferated.

Tissue Preparation and Histology

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg, Abbott Laboratories, North Chicago, IL) and transcardially perfused

with 200 mL of physiological saline, followed by 500 mL of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Spinal cords were dissected, and the segment containing the transplant was washed with PB for 2 h, then placed in 0.1 M PB containing 30% sucrose for 72 h. Specimens were then frozen in OCT compound (Tissue Tek, Sakura Inc., Tokyo, Japan) and serially sectioned in the transverse plane on a freezing microtome at 20 μm. The collection method used resulted in sets of five slides containing four 20-μm sections per slide at 100-μm intervals. These adjacent serial sections were systematically stained for Nissl/myelin to identify the lesion/transplant site, alkaline phosphatase (AP) histochemistry, to identify the transplanted fibroblasts, and immunocytochemistry with antibodies against ED-1 to identify the extent of the immune response within and adjacent to the lesion/transplant; and RT97 and CGRP to identify axons that have grown into the transplant.

Immunocytochemistry

The procedures for immunocytochemistry have been described previously (Liu et al., 1999; Chow et al., 2000). Antibodies used and conditions for use are shown in Table 1. ABC Standard Kit (Vector Laboratories, Burlingame, CA) was used as specified by the manufacturer, and peroxidase activity was visualized, as previously described, using 3,3′-diaminobenzine tetrahydrochloride (DAB) as a chromagen (Kim et al., 1999). Comparable sections through the graft were stained at the same time using highly standardized methods to avoid issues of inter-run variability.

Alkaline Phosphatase Histochemistry

This procedure has been described in detail previously (Han et al., 2002). Sections were washed three times in PBS, heat-treated at 60°C in PBS for 1 h to inactivate endogenous alkaline phosphatase, washed in alkaline phosphatase buffer (100 mM Tris, 100mM NaCl, 50 mM $MgCl_2$; pH 9.5), and incubated at room temperature in the dark for 2 h with alkaline phosphatase solution containing 1.0 mg/mL nitro blue tetrazolium (NBT), 0.1 mg/mL 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP), 5 mM levamisole (Sigma, St. Louis, MO) in alkaline phosphatase buffer (Chow et al., 2000).

Quantification of Histological Sections

Nissl-myelin–stained sections were used to measure the lesion/graft size. The Vitrogen graft was closely apposed to the host tissue, and the borders of the graft were readily distinguished from host tissue in all graft recipients. The length and width of the lesion/graft were measured from sections in each animal to provide an estimate of total lesion volume to test for effects of treatment on secondary injury.

Sections stained for immunocytochemistry and histochemistry were examined using a Leica DMRBE microscope (Wetzlar, Germany), and images were captured using a DC-330 color video camera (DAGE-MTI, Inc., Michigan City, IN). Images were then processed on a Power Mac G4 computer with an IP-Lab program (Scanalytics Inc., Fairfax, VA) to quantitate AP positive or immunopositive staining.

For the purposes of the analysis, the Vitrogen matrix is referred to as the "graft" and the Fb/AP as the "transplanted cells." Three sections were examined for each staining procedure in each animal: one section through the center of the graft, and sections 100 microns rostral and caudal to the center. Because the total area occupied by Fb/AP, ED-1, and axonal staining within the graft differed according to the presence and duration of administration of CsA or FK506, we identified the center of the graft as the area with the densest cellular or axonal staining and captured images from the center of the graft and images 100 μm rostral and caudal to the graft at 100×, and then determined the area fraction of staining. RT-97 and CGRP immunoreactivity at the center and 100 μm rostral and caudal to the center of the graft were evaluated at 200×, and the area fraction occupied by immunolabeled axons was determined. Because grafts in animals that received no immunophilin-ligand treatment contained very few axons, we chose an area containing some staining as the

Table 1. Antibodies

Primary antibody	*Dilution*	*Secondary antibody*	*Dilution*
Mouse ED1, Serotec Inc.	1:500	Biotintylated horse anti-mouse IgG, Vector Laboratories	1:100
Mouse RT 97, Developmental Studies Hybridoma Bank	1:1009	Biotintylated horse anti-mouse IgG, Vector Laboratories	1:100
Rabbit CGRP, Peninsula Laboratories Inc.	1:5000	Biotintylated goat anti-rabbit IgG, Jackson ImmunoResearch Laboratories, Inc.	1:200

central portion. Thresholding values on video images were chosen so that only immunolabeled axons were measured, and nonspecific background labeling was not detected.

Statistical Analysis

The outcome measures in this study included fibroblast survival (AP histochemistry), lesion volume, and ED-1, RT97, and CGRP immunostaining. Planned compar-

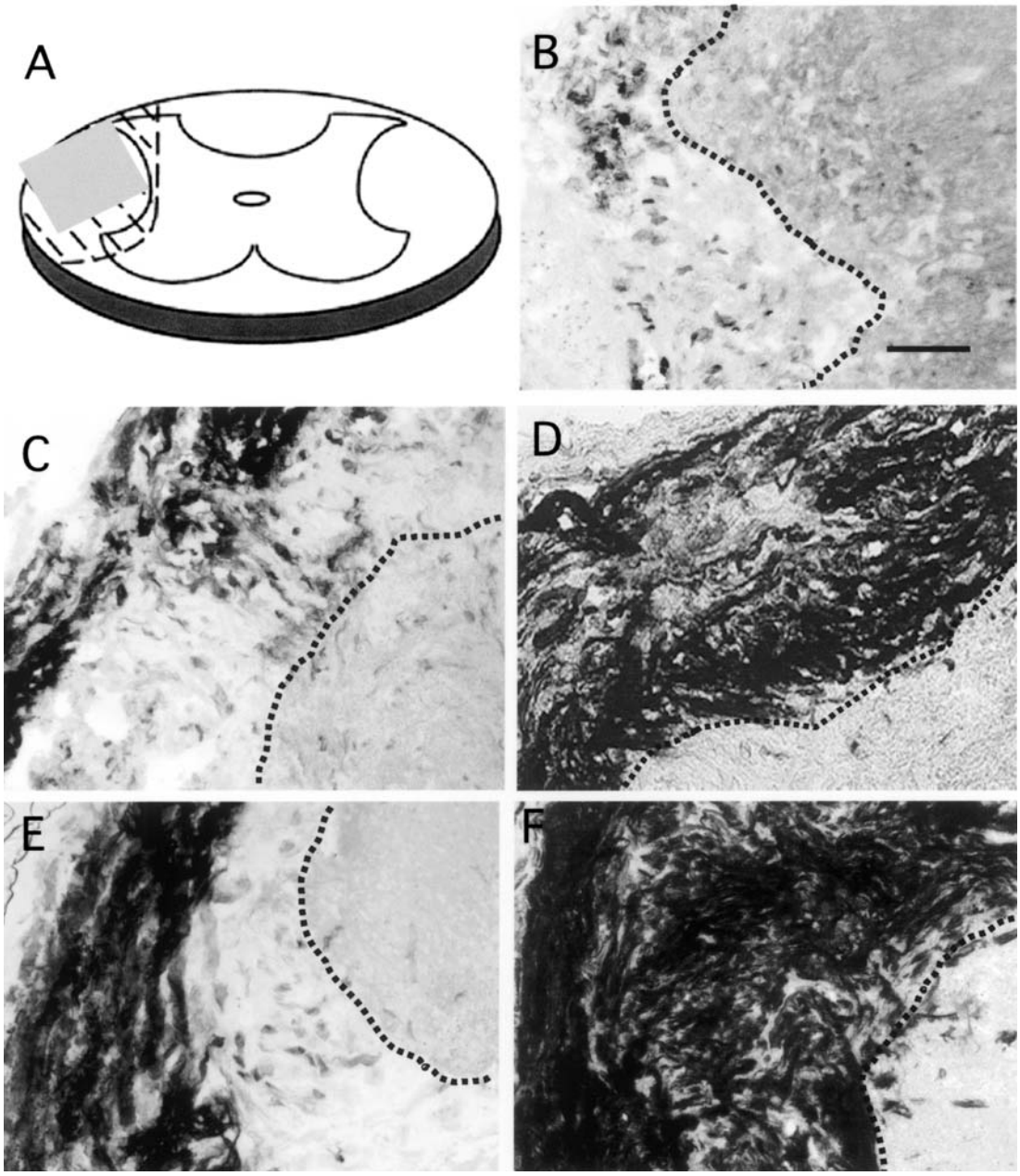

FIG. 1. Photomicrographs through the lesion–Fb/AP graft showing alkaline phosphatase (AP) histochemical staining of genetically modified fibroblasts under different immunosuppression conditions. All animals survived 8 weeks. (**A**) Diagram of injury (hatched lines) and transplant site for orientation of micrographs (gray box). (**B**) Cross section through the spinal cord of an animal, 8 weeks post-transplantation with no immunophilin-ligand treatment. (**C**) Cross section through the spinal cord of an animal that received cyclosporine A (CsA) for 2 weeks after transplantation. (**D**) Cross section through the spinal cord of an animal that received CsA for 8 weeks after transplantation. (**E**) Cross section through the spinal cord of an animal that received FK506 for 2 weeks after transplantation. (**F**) Cross section through the spinal cord of an animal that received FK506 for 8 weeks after transplantation. Dotted lines indicate the host/transplant interface. Bar = 100 μm.

isons were performed on the outcome measures to determine if there were any differences between (1) 8 weeks of FK506 and CsA (inj) administration, (2) 2 weeks of FK506 and CsA (inj) administration, (3) no immunophilin-ligand, 2 weeks of FK506 administration, and 8 weeks of FK506 administration, and (4) no immunophilin-ligand, 2 weeks of CsA administration, 8 weeks of CsA administration (inj + oral), and 8 weeks of CsA administration (inj). Comparisons were performed using one-way analysis of variance (ANOVA) with the Bonferroni test used for post hoc comparisons. Correlational analysis was performed between AP staining and ED-1 staining by simple linear regression. Data are expressed as mean ± SEM. Significance levels were set at 0.05 for all comparisons.

RESULTS

Fibroblast Survival

Since the alkaline phosphatase reaction stains the cytoplasm intensely, survival of grafted cells is best evaluated by measuring the relative area of AP staining. These histological analyses showed little survival of fibroblasts in the lesion cavity 8 weeks after transplantation in animals that received neither CsA nor FK506 (Fig. 1A). Thus, the methylprednisolone treatment that all animals received was not sufficient to protect the fibroblasts from rejection. The best survival of fibroblasts was seen in animals that received 8 weeks of immunophilin-ligand treatment (Figs. 1 and 2). FK506-treated animals showed better graft survival than those that received CsA at both 2 weeks (Fig. 1B vs. 1D, $p < 0.05$) and 8 weeks (1C vs. 1E, $p < 0.001$). Statistical analysis of the area fraction occupied by Fb/AP after 0, 2, and 8 weeks of immunophilin-ligand treatment (Fig. 2) by ANOVA revealed that the amount of AP staining increased with duration of treatment, using either FK506 [$F(2,18) = 109.4$, $p < 0.001$] or CSA (both routes of administration) [$F(3,22) = 28.3$, $p < 0.001$]. These results support the conclusion that FK506 is superior to CsA in preventing rejection of the grafted cells and that a prolonged course of treatment is superior to a 2-week treatment. There was no difference in fibroblast survival using the two routes of CsA delivery for 8 weeks, indicating that both routes were equally effective. Interestingly few or no Fb/AP cells migrated into the host in animals with no immunophilin-ligand treatment or only 2 weeks of treatment. In these animals, the surviving transplanted fibroblasts tended to be clustered more superficially, leaving an area of cell poor Vitrogen matrix adjacent to host spinal cord. With 8 weeks of treatment, the trans-

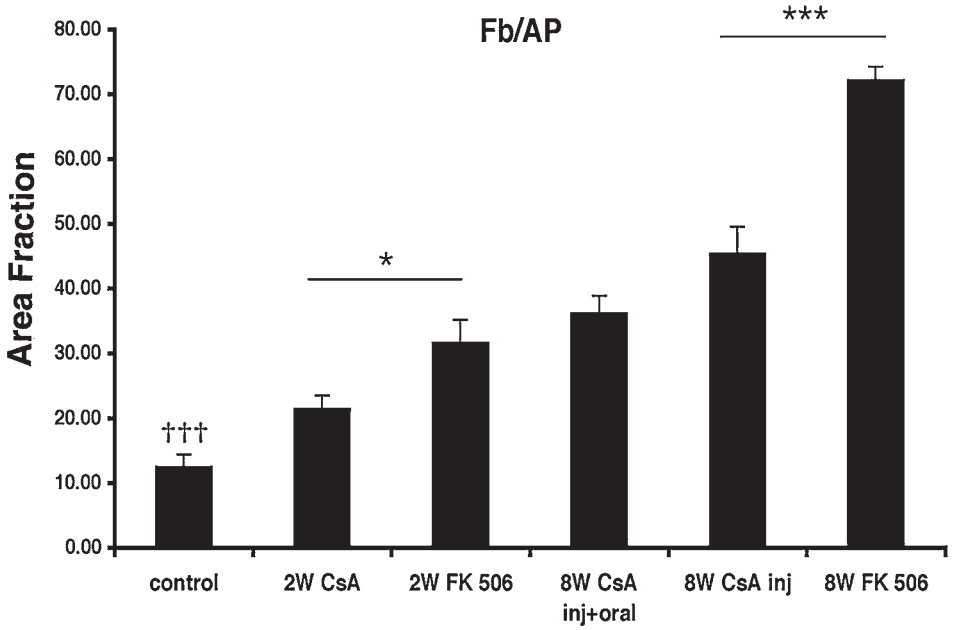

FIG. 2. Quantification of alkaline phosphatase (AP) histochemical staining of transplanted fibroblasts expressed as the area fraction (percent sampled area occupied by labeled cells), expressed as mean ± SEM. Planned comparisons showed that FK506 treatment supported greater Fb/AP survival than cyclosporine A (CsA) at both 2 weeks (*$p < 0.05$) and 8 weeks (***$p < 0.001$). Survival of Fb/AP after immunophilin-ligand treatment was significantly greater after 8 weeks of treatment using either FK506 or CsA (both routes of administration) compared to no treatment (***$p < 0.001$).

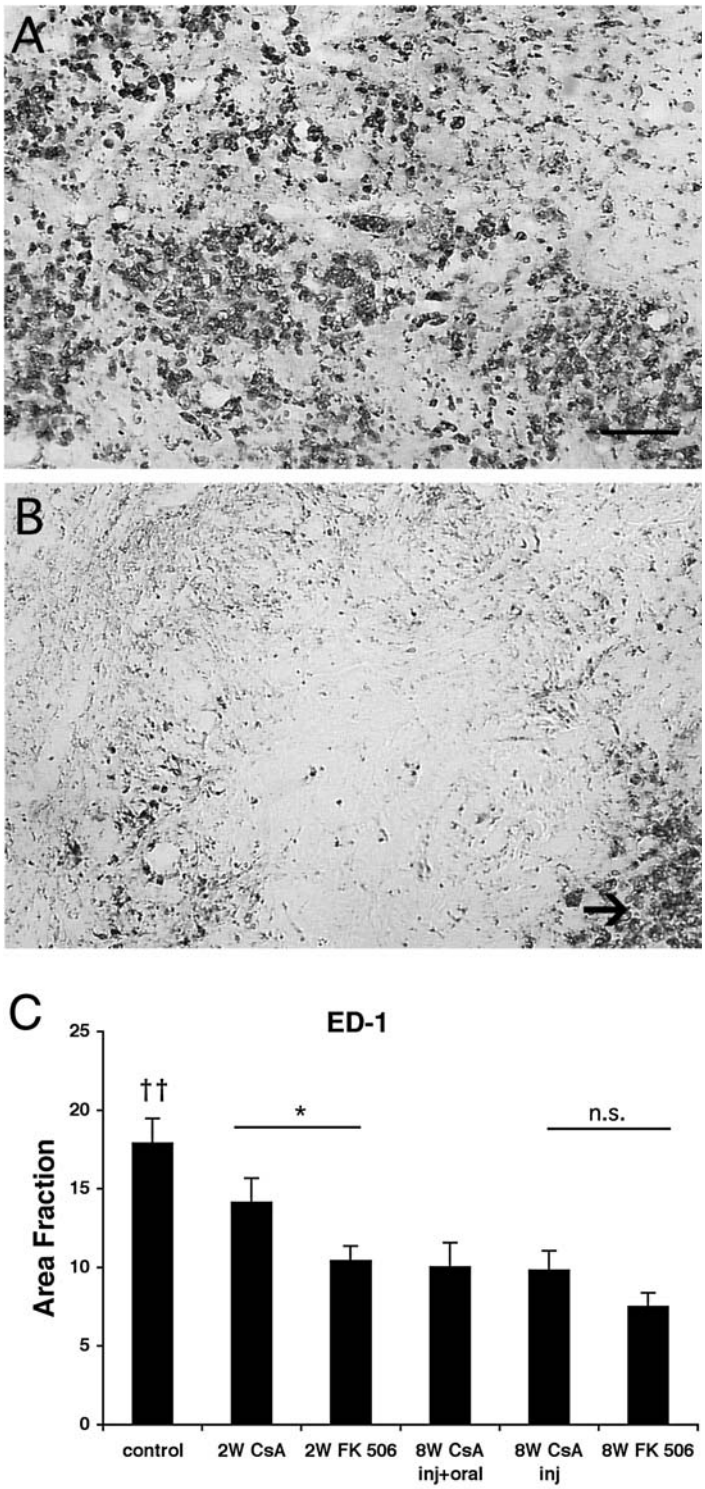

FIG. 3. (**A**) Photomicrograph of ED-1 immunocytochemical staining within the transplant. This animal received a graft of fibroblasts genetically modified to produce the marker alkaline phosphatase (Fb/AP) into a lateral funiculus lesion cavity with no subsequent immunophilin-ligand treatment. The high level of ED-1 staining indicates activated macrophages and microglia. Bar = 20 μm. (**B**) ED-1 immunocytochemical staining at the same magnification in an animal that received a graft of Fb/AP into the lesion cavity with daily subcutaneous injections of FK506 for 8 weeks. ED-1 staining is greatly decreased within the transplant, but a high density of ED-1 staining persists at the host/transplant interface (arrow). (**C**) Quantification of ED-1 immunocytochemical staining (mean ± SEM). Planned comparisons show that significantly fewer ED-1–positive cells persisted within the graft after 2 weeks of treatment with FK506 compared with cyclosporine A (CsA; $*p < 0.05$), with no significant differences (n.s.) between the two treatments after 8 weeks. There were many ED-1–positive cells within and surrounding the matrix in the absence of immunophilin-ligand treatment ($**p < 0.01$), and significantly fewer ED-1–positive cells persisted after 8 weeks of treatment with either CsA (both routes of administration) or FK506.

planted fibroblasts occupied the entire area of the Vitrogen matrix and abutted the host tissue. In these animals, some Fb/AP cells migrated into the host (data not shown). Thus, the matrix itself did not promote migration of fibroblasts, and entry of fibroblasts into the host occurred only when the grafted cells were in close apposition to the host tissue.

Immune Response

We evaluated the immune response to the grafts by immunocytochemical staining with ED-1 to identify activated macrophages and microglia within the graft. There were many ED-1–positive cells within and surrounding the matrix graft in the absence of immunophilin-ligands (Fig. 3A). ANOVA revealed that fewer ED-1–positive cells were present within the graft after 8 weeks of treatment using either FK506 [$F(2,18) = 18.9$, $p < 0.001$] or CsA (both routes of administration) [$F(3,22) = 6.4$, $p < 0.01$], compared to the no immunophilin-ligand controls (Fig. 3B). Fewer ED-1 positive cells persisted after 2 weeks of treatment with FK506 compared with CsA ($p < 0.05$), but there were no differences between the two treatments given for 8 weeks (Fig. 3C). Correlational analysis (Fig. 4) indicated that the density of AP staining was inversely related to the density of ED-1 staining ($R^2 = 0.48$; $p < 0.001$). At the host/transplant interface, there was a high density of ED-1 staining in all animals examined with no obvious differences among groups (data not shown). Thus, the treatment did not prevent a response to the injury by activated macrophages, but persistence of activated macrophages within the graft, and presumably their efficacy in eliminating fibroblasts, was dependent on the duration of treatment and on the agent.

Protective Effects

The fibroblasts were transplanted in a Vitrogen matrix; the matrix filled the cavity and remained recognizable in all cases. There was little or no cyst formation within the graft or in adjacent host tissue. The boundary between the Vitrogen/transplant and host tissue was clearly demarcated by the presence of small cells, mostly ED-1–positive cells, and by the absence of host neurons. ANOVA showed that there were no significant differences in lesion volume either between treatments or across time points. Lesion volume following 8 weeks of treatment with FK506 was no different from 8 weeks of treatment with CsA (2.34 ± 0.40 mm^3 vs. 2.45 ± 0.36 mm^3 [$F(1,10) = 0.04$, n.s.]). Lesion size following 2 weeks of treatment with FK506 was no different from 2 weeks of treatment with CsA (2.51 ± 0.17 mm^3 vs. 2.32 ± 0.17 mm^3 [$F(1,13) = 0.66$, n.s.]). Lesion size with neither CsA nor FK506 was 2.48 ± 0.27 mm^3. Comparisons across time (at 0, 2, and 8 weeks) were also not significant for FK506 [$F(2,18) = 0.10$, n.s.] or for CsA

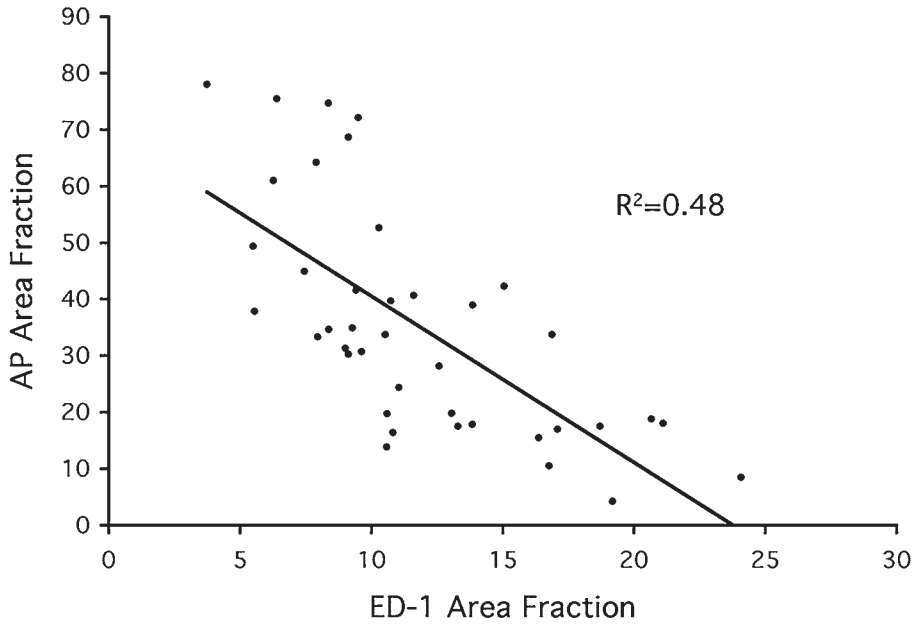

FIG. 4. Correlation analysis of survival of transplanted fibroblasts (area fraction of alkaline phosphatase [AP] histochemical staining) to density of activated macrophages and microglia (area fraction of ED-1 immunocytochemistry). There is an inverse relationship between density of activated macrophages and microglia and survival of Fb/AP ($R^2 = 0.48$, $p < 0.001$).

[F(2,17) = 0.11, n.s.]. These results indicate that the immunophilin-ligand treatments did not significantly modify the secondary damage that is believed to contribute to the final size of the lesion. Since the lesion site was filled with the Vitrogen matrix in all cases, the similarity in lesion size indicated that the graft size was also similar.

Axonal Growth into Graft

We used two methods to assess permissiveness to axonal growth into the graft: RT97 immunocytochemistry, which visualizes axons that express phosphorylated neurofilaments (Fig. 5), and CGRP, which visualizes small diameter dorsal root axons (Fig. 6). Density measurements were taken in the area of greatest ingrowth, defined here as the center, and in regions 100 μm rostral and caudal to the center. Both immunocytochemical methods showed axonal ingrowth into the graft even in the absence of immunophilin-ligands, indicating that the Vitrogen matrix itself is permissive for axonal growth. In the 2-week treatment groups, where fewer Fb/AP survived, ingrowing RT-97 immunoreactive axons were distributed homogeneously in the cell free matrix and in the part of the graft that contained surviving fibroblasts, supporting the permissive nature of the Vitrogen matrix. ANOVA revealed that significantly more axons entered the graft in both CsA [$F(3,22) = 26.6$, $p < 0.001$] and FK506 [$F(2,18) = 27.1$, $p < 0.001$] treated groups, compared to animals receiving neither CsA nor FK506, but only methylprednisolone. There were no differences in RT-97 labeling between treatments at 2 and 8 weeks, suggesting that short-term (2-week) treatment is effective in stimulating axonal growth and that these axons persist in the grafted region throughout the survival period, even after treatment was discontinued.

Fewer CGRP positive dorsal root axons entered the graft, compared to RT-97–positive axons, and they tended to be distributed at the superficial surface near the dorsal root entry zone rather than distributed homogenously throughout the Vitrogen matrix, as were the RT-97–labeled axons. ANOVA revealed that more axons entered the graft in both CsA [$F(3,22) = 4.9$, $p < 0.01$] and FK506 [$F(2,18) = 7.0$, $p < 0.01$] treated animals, compared to no immunophilin-ligand treatment (Fig. 6A). The density of CGRP-labeled axons, like that of RT-97–labeled axons, did not differ among the groups receiving immunophilin-ligands (Fig. 6B), supporting our conclusion that 2 weeks of treatment is as effective as 8 weeks in stimulating axonal growth.

The axonal ingrowth was not related to the density of ED-1–positive cells within the graft. Thus axonal growth was independent of macrophage invasion.

DISCUSSION

Immunophilin-Ligands

Two drugs commonly used in organ transplantation, the immunophilin-ligands CsA and FK506 (Tacrolimus), are effective in preventing rejection of transplanted cells (Gold, 2000). They have also been shown to have neurotrophic and neuroprotective properties. We show here that both are effective in preventing rejection and in supporting axonal growth into a graft placed in a spinal injury, but neither appeared to modify the secondary expansion of the injury. In our experimental design, we treated all animals at the time of injury with methylprednisolone and thus methylprednisolone may have interacted with either CsA or FK506. The role of methylprednisolone in improving repair or recovery appears to be modest at best (Rabchevsky et al., 2002; Takami et al., 2002, 2005; Pearse et al., 2004). While we cannot rule out an interaction between methylpredinosolone and either immunophilin-ligand that might have affected the results, previous studies have shown few or no interactions between methylprednisolone and CsA or FK506 (Borlongan et al., 2005; Mabon et al., 1999). Kaymaz et al. (2005), however, showed greater edema and polymorophonuclear leucocyte infiltration after spinal injury in animals treated with methylprednisolone compared to those given FK506, suggesting that FK506 alone might have given better results.

Fibroblast Survival

Our results show that both CsA and FK506 can protect an allogeneic graft of AP-fibroblasts from rejection by the host immune system. The grafted fibroblasts are derived from a transgenic strain of Fischer 344 rats that constitutively expresses human alkaline phosphatase and the hosts are adult Sprague-Dawley rats. The fibroblasts are therefore allogeneic because they are performed across rat strains and potentially xenogeneic if the alkaline phosphatase marker protein that allows identification of the grafted cells is encountered by the host immune system. The small amount of residual alkaline phosphatase staining that we observed at graft sites 8 weeks after transplantation in rats that received neither CsA nor FK506 may be due to the modest level of protection provided by the methylprednisolone that all recipient rats received as part of our basic transplantation protocol (Liu et al., 1999, 2002; Tobias et al., 2001, 2003, 2005). Both CsA and FK506, whether administered for 2 or 8 weeks, support significantly greater fibroblast survival than that seen in the absence of immunophilin-ligand treatment, with decreased invasion of the graft by activated microglia/macrophages or peripheral cells migrating into

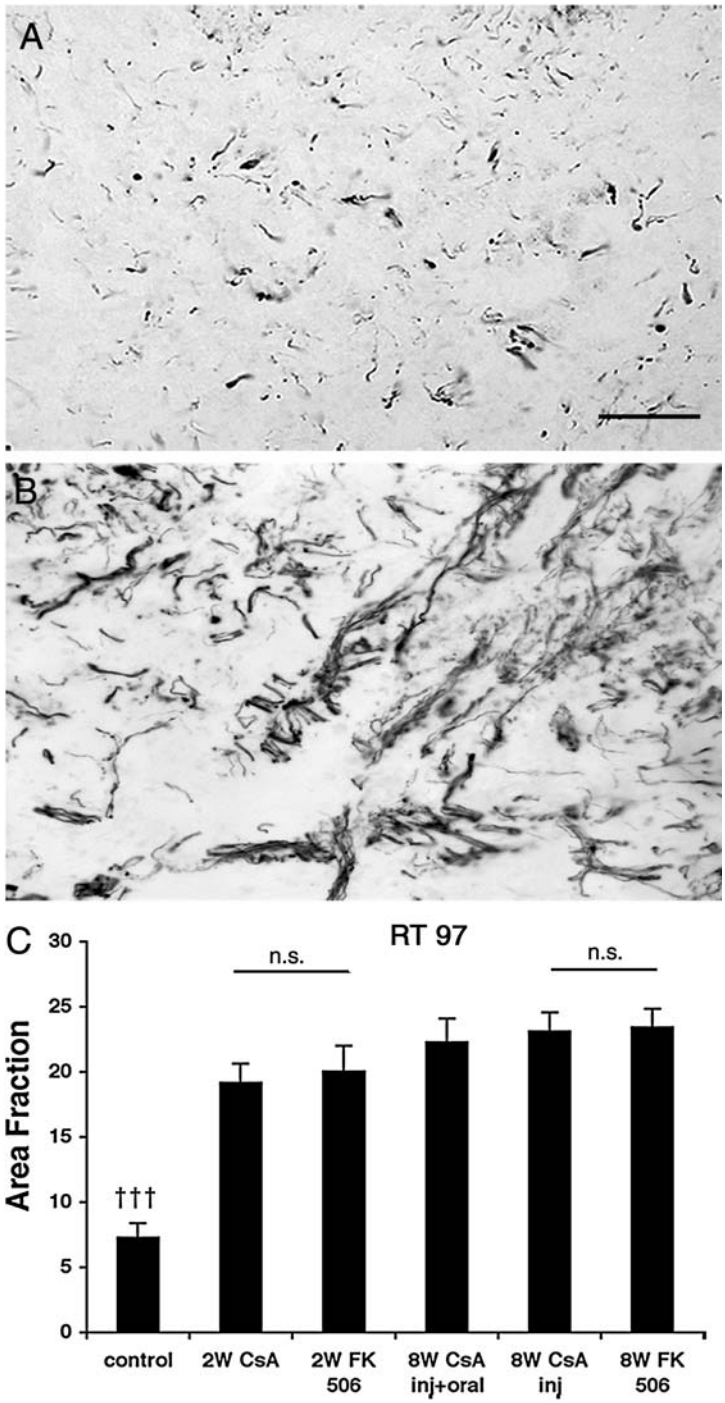

FIG. 5. (**A**) Photomicrograph of RT-97 immunocytochemical staining, visualizing axons containing phosphorylated neurofilaments, within the transplant. This animal received a graft of Fb/AP into a lateral funiculus lesion cavity with no immunophilin-ligand treatment. The low level of RT-97 staining indicates low axon density within the transplant. Bar = 20 μm. (**B**) RT-97 immunocytochemical staining at the same magnification in an animal that received a graft of Fb/AP into the lesion cavity with daily subcutaneous injections of CSA for 8 weeks. The density of labeled axons within the transplant was increased by this treatment. (**C**) Quantification of RT-97 immunocytochemical staining, expressed as area fraction occupied by labeled axons (mean ± SEM). Significantly more axons entered the graft in either CsA or FK506 treated animals compared to no immunophilin-ligand treatment (***$p < 0.001$). There were no significant differences between treatments at either time point (n.s.), suggesting that even short-term (2 week) treatment is effective in stimulating axonal growth and that these axons persist in the grafted region throughout the survival period.

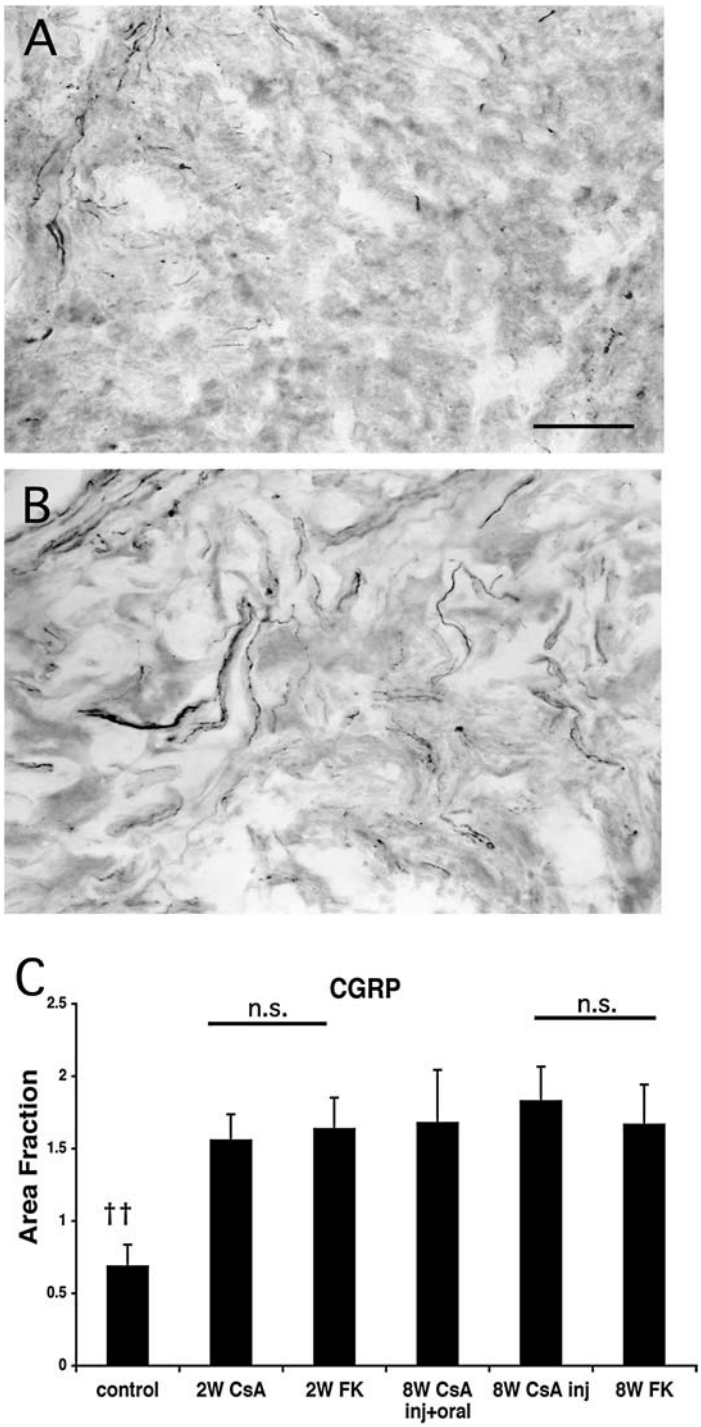

FIG. 6. (**A**) Photomicrograph of CGRP immunocytochemical staining, visualizing small diameter dorsal root axons, within the transplant. This animal received an Fb/AP graft into a lateral funiculus lesion cavity with no immunophilin-ligand treatment. Bar = 20 μm. (**B**) CGRP immunocytochemical staining at the same magnification as above in an animal that received a Fb/AP graft into the lesion cavity with daily subcutaneous injections of cyclosporine A (CsA) for 8 weeks. Fewer CGRP-labeled axons entered the graft compared to RT-97–positive axons, but the density of labeled axons within the transplant was increased by this treatment. (**C**) Quantification of CGRP immunocytochemical staining, expressed as area fraction occupied by labeled axons, mean ± SEM, revealed no significant differences (n.s.) between treatments at either time point. Significantly more axons entered the graft in either CsA- or FK506-treated animals compared to no treatment (**$p < 0.01$), suggesting that even short-term (2 week) treatment is effective in stimulating axonal growth.

the spinal cord, but this had little effect on the accumulation of ED-1 cells at the transplant/host boundary. Thus the rejection of fibroblasts appeared to be related to the immunophilin-ligand protocol but the response of ED-1–positive cells to the injury was not affected by the treatment. That the enhanced fibroblast survival results from a direct action on the host immune system is supported by our observation that the density of AP reactivity is inversely related to the number of ED-1–expressing cells that populate the graft. FK506 delivered for the entire 8-week postoperative period provides the best graft survival and FK506 is superior to CsA whether the agents are delivered according to a 2-week or 8-week protocol. This presumably reflects the greater potency of FK506 in preventing activation of T cells (Ochiai, 1989). Cessation of CsA or FK506 delivery after 2 weeks leads to a partial loss of the transplanted fibroblasts during the next 6 weeks. The survival of some cells at 8 weeks after a 2-week course of immunophilin-ligand treatment suggests either that rejection is a prolonged and gradual process or that the fibroblasts continue to proliferate if treatment is discontinued. Our data do not allow us to distinguish between these two alternatives.

Protection

Both drugs have been described as neuroprotective although FK506 is less effective than CsA in reducing hippocampal damage in a model of transient forebrain ischemia (Uchino et al., 2002). Both CsA and FK506 inhibit Il-2 production, prevent T cell activation by inhibiting calcineurin (Sewell et al., 1994; Uchino et al., 2002), and inhibit dephosphorylation of the pro-apoptotic protein BAD (Wang, 1997; Wang et al., 1999). These drugs therefore can diminish or delay apoptosis (Springer et al., 2000). CsA, in addition, blocks the mitochondrial permeability transition pore and formation of the mitochondria megapore (Sullivan et al., 1999), and is thus potentially more neuroprotective than FK506.

CsA and FK506 treatment protocols failed to modify the final size of the spinal cord lesion. We studied a partial transection of the C3/4 dorsolateral quadrant, but previous investigations similarly failed to find tissue sparing when either agent was used to treat other types of spinal cord injury (Madsen et al., 1998; Bavetta et al., 1999; Rabchevsky et al., 2001; Ibarra et al., 2003). The initial mechanical impact of a spinal cord injury initiates a complex series of secondary cellular and biochemical processes that cause the lesion to expand over a period of hours and weeks (Tator, 1995; Schwab and Bartholdi, 1996). The end result is the death of all cell types normally found at the injury site and in spinal cord adjacent to the injury where the secondary expansion of the injury occurs. Whether the cells that infiltrate and scavenge the lesion site contribute to the destruction is controversial, but most of the tissue destruction appears to precede the cellular infiltration (Dusart and Schwab, 1994). Our results suggest that immunophilin-ligands have little effect on these secondary effects at the lesion site and therefore that these drug treatments are ineffective in limiting necrotic processes or in prevention of the secondary expansion of the lesion.

In models of traumatic brain injury, for example, a cortical contusion injury, CsA administration does reduce the cortical lesion volume (Scheff and Sullivan, 1999; Sullivan et al., 1999), presumably through its actions in limiting formation of the mitochondrial megapore. One difference in response to traumatic brain injury and spinal cord injury appears to reside in intrinsic differences in brain and spinal cord mitochondria (Sullivan et al., 2004). FK506 was also found to be effective in decreasing the extent of axonal damage after traumatic brain injury (Winter et al., 2000; Singleton et al., 2001) but our results show that FK506 was ineffective after spinal cord injury. Parameters in addition to spinal cord mitochondria are likely to contribute to the difference in protection offered by immunophilin-ligands following traumatic brain injury and spinal cord injury.

Axonal Growth

There is conflicting and incomplete evidence as to whether both CsA and FK506 stimulate axon growth (Sugawara et al., 1999; Rabchevsky et al., 2001). CsA has been shown to inhibit GAP43 secondarily (Ibarra et al., 2003), which would be expected to counteract regeneration. FK506 and its binding proteins or their analogues have been reported to increase GAP43 mRNA in axotomized sensory neurons (Gold et al., 1998; Madsen et al., 1998; Udina et al., 2004) and to activate BDNF (Tanaka et al., 2003), which might enhance regeneration in the PNS and CNS and preserve damaged tissue in the CNS (Bavetta et al., 1999; Lee et al., 2000; Pan et al., 2003; Rosenstiel et al., 2003). FK506 accelerates regeneration in peripheral nerves to a significantly greater extent than CsA (Wang et al., 1997; Jost et al., 2000) and also promotes axonal growth in serotonergic, dopaminergic (Steiner et al., 1997a,b) and supraspinal and spinal (Wang and Gold, 1999) axons.

We observed a baseline level of growth of RT97- and CGRP-containing axons in animals with a Vitrogen matrix alone, indicating that the matrix itself is permissive to axonal growth. Vitrogen (Cohesion Technology) is 99.9% pure native collagen (95–98% collagen type I) with hemostatic properties. In addition to its permissive properties, the Vitrogen matrix persisted within the le-

sion cavity for 8 weeks in all animals, and thus provided a bridging material that could support axonal growth. We have previously used Gelfoam as a matrix in which to suspend our cells. Gelfoam is derived from pork skin and thus is a heterogeneous mix of collagens and other compounds. Gelfoam grafts alone are not permissive to axonal growth (Liu et al., 1999). Our results suggest therefore that a pure collagen matrix is more effective than Gelfoam in providing a bridge, allowing axonal growth, and decreasing cyst formation.

CsA and FK506 enhanced host axon growth into the graft site to a similar extent. In the absence of experiments testing the effects of non-calcineurin inhibiting analogs (Madsen et al., 1998), we cannot determine whether the enhanced axon growth that we observed was due to direct or indirect effects on axon growth mechanisms, immunosuppression, or a combination of both types of action. Because we studied an incomplete lesion model rather than a complete spinal cord transection, we cannot determine whether the growth of axons into the transplant site represents collateral sprouting of spared axons or compensatory sprouting or regeneration of injured axons, nor can we evaluate the extent to which axons grow through the graft into the host. Nevertheless, it is clear that both drugs promote axonal growth.

Of more practical interest is our observation that a 2-week administration of either immunophilin-ligand is as effective in eliciting axonal growth into the graft as 8 weeks. This suggests that axonal growth occurs within the first two weeks and that the axons persist even after treatment is discontinued (Udina et al., 2004b). CsA and FK506 appear to be comparably effective in this model of spinal cord injury and transplantation, although prolonged treatment with FK506 is more effective in preventing rejection of the transplanted cells. Future experiments will test whether transplanting fibroblasts genetically modified to secrete neurotrophins with 2 weeks of immunosuppression treatment will allow recovery of function that is as robust as when immunosuppression is continued for the entire survival period (Liu et al., 1999, 2002).

ACKNOWLEDGMENTS

We wish to thank Amy Slater for her excellent technical assistance. We also thank Dr. Ramesh Raghupathi for his critical reading of the manuscript and helpful discussions, and Dr. B.T. Himes for his assistance in technical assessments. Fujisawa Pharmaceutical Corp. (Osaka, Japan) provided us with FK506. This research was supported by NS 24707 and United Spinal Association.

REFERENCES

ANDERSON, D.K., BRAUGHLER, J.M., HALL, E.D., WATERS, T.R., McCALL, J.M., and MEANS, E.D. (1988). Effects of treatment with U-74006F on neurological outcome following experimental spinal cord injury. J. Neurosurg. **69,** 562–567.

BARTHOLDI, D., and SCHWAB, M.E. (1995). Methylprednisolone inhibits early inflammatory processes but not ischemic cell death after experimental spinal cord lesion in the rat. Brain Res. **672,** 177–186.

BAVETTA, S., HAMLYN, P.J., BURNSTOCK, G., LIEBERMAN, A.R., and ANDERSON, P.N. (1999). The effects of FK506 on dorsal column axons following spinal cord injury in adult rats: neuroprotection and local regeneration. Exp. Neurol. **158,** 382–393.

BORLONGAN, C.V., YU, G., MATSUKAWA, N., et al. (2005). Acute functional effects of cyclosporine-A and methylprednisolone treatment in adult rats exposed to transient ischemic stroke. Life Sci. **76,** 1503–1512.

CHOW, S.Y., MOUL, J., TOBIAS, C.A., et al. (2000). Characterization and intraspinal grafting of EGF/bFGF-dependent neurospheres derived from embryonic rat spinal cord. Brain Res. **874,** 87–106.

DIAZ-RUIZ, A., RIOS, C., DUARTE, I., et al. (2000). Lipid peroxidation inhibition in spinal cord injury: cyclosporin-A vs methylprednisolone. Neuroreport **11,** 1765–1767.

DUSART, I., and SCHWAB, M.E. (1994). Secondary cell death and the inflammatory reaction after dorsal hemisection of the rat spinal cord. Eur. J. Neurosci. **6,** 712–724.

FINBERG, R., and FINGEROTH, J. (2004). Infections in transplant recipients [On-line]. Available: ⟨http://harrisons.accessmedicine.com/server-java/Arknoid/amed/harrisons/co_chapters/ch136/ch136_p01.html⟩.

GOLD, B.G. (2000). Neuroimmunophilin ligands: evaluation of their therapeutic potential for the treatment of neurological disorders. Exp. Opin. Invest. Drugs **9,** 2331–2342.

GOLD, B.G., ZELENY-POOLEY, M., WANG, M.S., et al. (1997). A nonimmunosuppressant FKBP-12 ligand increases nerve regeneration. Exp. Neurol. **147,** 269–278.

HADLEY, M.N. (2002). Pharmacological therapy after acute cervical spinal cord injury. Neurosurgery **50,** S63–S72.

HAN, S.S., KANG, D.Y., MUJTABA, T., RAO, M.S., and FISCHER, I. (2002). Grafted lineage-restricted precursors differentiate exclusively into neurons in the adult spinal cord. Exp. Neurol. **177,** 360–375.

IBARRA, A., REYES, J., MARTINEZ, S., et al. (1996). Use of cyclosporin-A in experimental spinal cord injury: design of a dosing strategy to maintain therapeutic levels. J. Neurotrauma **13,** 569–572.

IBARRA, A., CORREA, D., WILLMS, K., et al. (2003). Effects of cyclosporin-A on immune response, tissue protection and motor function of rats subjected to spinal cord injury. Brain Res. **979,** 165–178.

JOST, S.C., DOOLABH, V.B., MACKINNON, S.E., LEE, M., and HUNTER, D. (2000). Acceleration of peripheral nerve regeneration following FK506 administration. Restor. Neurol. Neurosci. **17,** 39–44.

KATSUBE, K., DOI, K., FUKUMOTO, T., FUJIKURA, Y., SHIGETOMI, M., and KAWAI, S. (1998). Successful nerve regeneration and persistence of donor cells after a limited course of immunosuppression in rat peripheral nerve allografts. Transplantation **66,** 772–777.

KAYMAZ, M., EMMEZ, H., BUKAN, N., et al. (2005). Effectiveness of FK506 on lipid peroxidation in the spinal cord following experimental traumatic injury. Spinal Cord **43,** 22–26.

KIM, D., SCHALLERT, T., LIU, Y., et al. (2001). Transplantation of genetically modified fibroblasts expressing BDNF in adult rats with a subtotal hemisection improves specific motor and sensory functions. Neurorehabil. Neural Repair **15,** 141–150.

KISSEBERTH, W.C., BRETTINGEN, N.T., LOHSE, J.K., and SANDGREN, E.P. (1999). Ubiquitous expression of marker transgenes in mice and rats. Dev. Biol. **214,** 128–138.

LEE, M., DOOLABH, V.B., MACKINNON, S.E., and JOST, S. (2000). FK506 promotes functional recovery in crushed rat sciatic nerve. Muscle Nerve **23,** 633–640.

LIU, Y., KIM, D., HIMES, B.T., et al. (1999). Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function. J. Neurosci. **19,** 4370–4387.

LIU, Y., HIMES, B.T., MURRAY, M., TESSLER, A., and FISCHER, I. (2002). Grafts of BDNF-producing fibroblasts rescue axotomized rubrospinal neurons and prevent their atrophy. Exp. Neurol. **178,** 150–164.

MABON, P.J., WEAVER, L.C., and DEKABAN, G.A. (1999). Cyclosporin A reduces the inflammatory response to a multimutant herpes simplex virus type–1 leading to improved transgene expression in sympathetic preganglionic neurons in hamsters. J. Neurovirol. **5,** 268–279.

MADSEN, J.R., MACDONALD, P., IRWIN, N., et al. (1998). Tacrolimus (FK506) increases neuronal expression of GAP-43 and improves functional recovery after spinal cord injury in rats. Exp. Neurol. **154,** 673–683.

McCARTHY, P.W., and LAWSON, S.N. (1990). Cell type and conduction velocity of rat primary sensory neurons with calcitonin gene–related peptide-like immunoreactivity. Neuroscience **34,** 623–632.

OCHIAI, T., NAKAJIMA, K., SAKAMOTO, K., et al. (1989). Comparative studies on the immunosuppressive activity of FK506, 15-deoxyspergualin, and cyclosporine. Transplant. Proc. **21,** 829–832.

PAN, Y.A., MISGELD, T., LICHTMAN, J.W., and SANES, J.R. (2003). Effects of neurotoxic and neuroprotective agents on peripheral nerve regeneration assayed by time-lapse imaging *in vivo*. J. Neurosci. **23,** 11479–11488.

PEARSE, D.D., MARCILLO, A.E., OUDEGA, M., LYNCH, M.P., WOOD, P.M., and BUNGE, M.B. (2004). Transplantation of Schwann cells and olfactory ensheathing glia after spinal cord injury: does pretreatment with methylpredisolone and interleukin-10 enhance recovery? J. Neurotrauma **21,** 1223–1239.

RABCHEVSKY, A.G., FUGACCIA, I., SULLIVAN, P.G., and SCHEFF, S.W. (2001). Cyclosporin A treatment following spinal cord injury to the rat: behavioral effects and stereological assessment of tissue sparing. J. Neurotrauma **18,** 513–522.

ROSENSTIEL, P., SCHRAMM, P., ISENMANN, S., et al. (2003). Differential effects of immunophilin-ligands (FK506 and V-10,367) on survival and regeneration of rat retinal ganglion cells *in vitro* and after optic nerve crush *in vivo*. J. Neurotrauma **20,** 297–307.

SCHEFF, S.W., and SULLIVAN, P.G. (1999). Cyclosporin A significantly ameliorates cortical damage following experimental traumatic brain injury in rodents. J. Neurotrauma **16,** 783–792.

SCHNELL, L., and SCHWAB, M.D. (1990). Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature **343,** 269–272.

SCHWAB, M.E., and BARTHOLDI, D. (1996). Degeneration and regeneration of axons in the lesioned spinal cord. Physiol. Rev. **76,** 319–369.

SEWELL, T.J., LAM, E., MARTIN, M.M., et al. (1994). Inhibition of calcineurin by a novel FK-506-binding protein. J. Biol. Chem. **269,** 21094–21102.

SINGLETON, R.H., STONE, J.R., OKONKWO, D.O., PELLICANE, A.J., and POVLISHOCK, J.T. (2001). The immunophilin ligand FK506 attentuates axonal injury in an impact-acceleration model of traumatic brain injury. J. Neurotrauma **18,** 607–614.

SPRINGER, J.E., AZBILL, R.D., NOTTINGHAM, S.A., and KENNEDY, S.E. (2000). Calcineurin-mediated BAD dephosphorylation activates the caspase-3 apoptotic cascade in traumatic spinal cord injury. J. Neurosci. **20,** 7246–7251.

STEINER, J.P., HAMILTON, G.S., ROSS, D.T., et al. (1997a). Neurotrophic immunophilin ligands stimulate structural and functional recovery in neurodegenerative animal models. Proc. Natl. Acad. Sci. USA **94,** 2019–2024.

STEINER, J.P., CONNOLLY, M.A., VALENTINE, H.L., et al. (1997b). Neurotrophic actions of nonimmunosuppressive analogues of immunosuppressive drugs FK506, rapamycin, and cyclosporin A. Nat. Med. **3,** 421–428.

SUGAWARA, T., ITOH, Y., and MIZOI, K. (1999). Immunosuppressants promote adult dorsal root regeneration into the spinal cord. NeuroReport **10,** 3949–3953.

SULLIVAN, P.G., THOMPSON, M.B., and SCHEFF, S.W. (1999). Cyclosporin A attenuates acute mitochondrial dysfunction following traumatic brain injury. Exp. Neurol. **160,** 226–234.

SULLIVAN, P.G., RABCHEVSKY, A.G., KELLER, J.N., et al. (2004). Intrinsic differnces in brain and spinal cord mitochendria. J. Comp. Neurol. **474,** 524–534.

TAKAMI, T., OUDEGA, M., BETHEA, J.R., WOOD, P.M., KLEITMAN, N., and BUNGE, M.B. (2002). Methylprednisolone and interleukin-10 reduce gray matter damage in the contused Fischer rat thoracic spinal cord but do not improve functional outcome. J. Neurotrauma **19,** 653–666.

TANAKA, K., FUJITA, N., and OGAWA, N. (2003). Immunosuppressive (FK506) and non-immunosuppressive (GPI1046) immunophilin ligands activate neurotrophic factors in the mouse brain. Brain Res. **970,** 250–253.

TATOR, C.H. (1995). Update on the pathophysiology and pathology of acute spinal cord injury. Brain Pathol. **5,** 407–413.

TEICHNER, A., MORSELLI, E., BUTTARELLI, CARONTI, B., PONTIERI, F.E., VENTURINI, G., and PALLODINI, G. (1993). Treatment with cyclosporine A promotes axonal regeneration in rats submitted to transverse section of the spinal cord. J. Hirnforsch. **34,** 343–349.

TOBIAS, C.A., DHOOT, N.O., WHEATLEY, M.A., TESSLER, A., MURRAY, M., and FISCHER, I. (2001). Grafting of encapsulated BDNF-producing fibroblasts into the injured spinal cord without immune suppression in adult rats. J. Neurotrauma **18,** 287–301.

TOBIAS, C.A., SHUMSKY, J.S., SHIBATA, M., et al. (2003). Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy and provides limited regeneration. Exp. Neurol. **184,** 97–113.

TOBIAS, C.A., HAN, S.S., SHUMSKY, J.S., et al. (2005). Alginate encapsulateed BDNF-producing fibroblast grafts permit recovery of function in the absence of immune suppression. J. Neurotrauma **22,** 138–156.

UCHINO, H., MINAMIKAWA-TACHINO, R., KRISTIAN, T., et al. (2002). Differential neuroprotection by cyclosporin A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. Neurobiol. Dis. **10,** 219–233.

UDINA, E., GOLD, B.G., and NAVARRO, X. (2004). Comparison of continuous and discontinuous FK506 administration on autograft or allograft repair of sciatic nerve resection. Muscle Nerve **29,** 812–822.

UDINO, E., RODRIGUEZ, F.J., VERDU, E., ESPEJO, M., GOLD, B.G., and NAVARRO, X. (2004). FK506 enhances regeneration of axons across long peripheral nerve gaps repaired with collagen guides seeded with allogeneic Schwann cells. Glia **47,** 120–129.

WANG, M.S., and GOLD, B.G. (1999). FK506 increases the regeneration of spinal cord axons in a predegenerated peripheral nerve autograft. J. Spinal Cord Med. **22,** 287–296.

WANG, M.S., ZELENY-POOLEY, M., and GOLD, B.G. (1997). Comparative dose-dependence study of FK506 and cylcosporin A on the rate of axonal regeneration in the rat sciatic nerve. J. Pharm. Exp. Ther. **282,** 1084–1093.

WANG, H.G., PATHAN, N., ETHELL, I.M., et al. (1999). $Ca2^{+}$-induced apoptosis through calcineurin dephosphorylation of BAD. Science **284,** 339–343.

WINTER, C., SCHENKEL, J., BURGER, E., EICKMEIER, C., ZIMMERMANN, M., and HERDEGEN, T. (2000). The immunophilin ligand FK506, but not GPI-1046, protects against neuronal death and inhibits c-Jun expression in the substantia nigra pars compacta following transection of the rat medial forebrain bundle. Neuroscience **95,** 753–762.

Address reprint requests to:
Marion Murray, Ph.D.
Department of Neurobiology and Anatomy
Drexel University College of Medicine
2900 Queen Lane
Philadelphia, PA 19129

E-mail: mm72@drexel.edu