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Alginate Encapsulated BDNF-Producing Fibroblast Grafts Permit Recovery of Function after Spinal Cord Injury in the Absence of Immune Suppression

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ABSTRACT

Encapsulation of cells has the potential to provide a protective barrier against host immune cell interactions after grafting. Previously we have shown that alginate encapsulated BDNF-producing fibroblasts (Fb/BDNF) survived for one month in culture, made bioactive neurotrophins, survived transplantation into the injured spinal cord in the absence of immune suppression, and provided a permissive environment for host axon growth. We extend these studies by examining the effects of grafting encapsulated Fb/BDNF into a subtotal cervical hemisection on recovery of forelimb and hindlimb function and axonal growth in the absence of immune suppression. Grafting of encapsulated Fb/BDNF resulted in partial recovery of forelimb usage in a test of vertical exploration and of hindlimb function while crossing a horizontal rope. Recovery was significantly greater compared to animals that received unencapsulated Fb/BDNF without immune suppression, but similar to that of immune suppressed animals receiving unencapsulated Fb/BDNF. Immunocytochemical examination revealed neurofilament (RT-97), 5-HT, CGRP and GAP-43 containing axons surrounding encapsulated Fb/BDNF within the injury site, indicating axonal growth. BDA labeling however showed no evidence of regeneration of rubrospinal axons in recipients of encapsulated Fb/BDNF, presumably because the amounts of BDNF available from the encapsulated grafts are substantially less than those provided by the much larger numbers of Fb/BDNF grafted in a gelfoam matrix in the presence of immune suppression. These results suggest that plasticity elicited by the BDNF released from the encapsulated cells contributed to reorganization that led to behavioral recovery in these animals and that the behavioral recovery could proceed in the absence of rubrospinal tract regeneration. Alginate encapsulation is therefore a feasible strategy for delivery of therapeutic products produced by non-autologous engineered fibroblasts and provides an environment suitable for recovery of lost function in the injured spinal cord.

Key words: alginate; axonal growth; encapsulation; functional recovery; spinal cord injury; transplantation

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INTRODUCTION

INJURY TO THE ADULT mammalian spinal cord results in loss of motor and sensory function. Recovery is often limited because the regenerative response elicited from injured CNS neurons is abortive (Murray, 2001; Plunet et al., 2002), axotomized neurons may die or atrophy (Himes and Tessler, 2001), the adult CNS contains molecules inhibitory to regeneration (Condic and Lemons, 2002), and glial scars and cysts prevent growth past the injury site (Fawcett and Asher, 1999). Individual strategies targeted to these obstacles have stimulated regeneration and partial recovery of function in rodent models of spinal cord injury (SCI). These interventions (Murray and Tobias, 2003) include the direct application of neurotrophic factors (Bregman et al., 1997; Ye and Houle, 1997) or proteases such as chondroitinase ABC (Bradbury et al., 2002), elevation of cAMP or blockade of the rho pathway (Dergham et al., 2002; Neumann et al., 2002) and transplantation of fetal tissue (Reier et al., 1986; Bregman et al., 1993; Tessler et al., 1997), Schwann cells (Xu et al., 1997), olfactory ensheathing cells (Ramon-Cueto et al., 2000), embryonic stem cells (McDonald et al., 1999), restricted precursor cells (Lepore et al., 2004), marrow stromal cells (Hofstetter et al., 2002; Himes et al., 2005) and genetically modified fibroblasts (Grill et al., 1997a; Grill et al., 1997b; Liu et al., 1999; Kim et al., 2001).

One of the more successful strategies for treatment of SCI has been the grafting of primary fibroblasts that are genetically modified *ex vivo* to secrete BDNF or NT-3. Grafts of BDNF-producing fibroblasts (Fb/BDNF), placed acutely into a cervical SCI site in immune suppressed adult rats, stimulate regeneration of rubrospinal axons, partially protect axotomized red nucleus neurons from loss and atrophy, and elicit recovery of forelimb and hindlimb behavioral deficits (Liu et al., 1999; Kim et al., 2001). A similar graft placed in a more chronic lesion site also elicited rubrospinal regeneration and protection of axotomized red nucleus neurons, but to a much more limited extent (Tobias et al., 2003). Immune suppression was, however, needed to prevent rejection of grafts placed into the out-bred Sprague-Dawley rats that were used in these experiments.

While immune suppression would be unnecessary if autologous grafts were used (Grill et al., 1997a), it is unlikely that autologous grafts will be widely available in clinical settings. Immune suppression with cyclosporin A (CSA) is used clinically to prevent rejection of organ and bone marrow transplants in humans (Simpson, 2001; Cohen, 2002). CSA is also commonly used in animal models of SCI for optimal survival and functioning of grafted cells, but prolonged usage can make the recipient vulnerable to infections, and increase the likelihood of tumorigenesis of the transplanted cells.

The reported effects of CSA on the injured host spinal cord have been inconsistent. CSA has been described as promoting axonal growth with behavioral recovery (Palladini et al., 1996) and decreasing tissue loss by inhibiting lipid peroxidation (Diaz-Ruiz et al., 1999; Diaz-Ruiz et al., 2000). No effects on tissue sparing and behavioral recovery were seen when more sensitive tests were applied (Rabchevsky et al., 2001). Studies from our laboratories have shown that CSA and another immunosuppressant drug (FK 506) permit survival of implanted non-neurotrophin secreting fibroblasts and that this therapy can support some degree of axonal growth and neuroprotection, but without modifying secondary injury or improving functional recovery (Hayashi et al., 2005). Nevertheless, because the repair processes associated with immune suppression are not as robust as those supported by interventions such as transplantation or trophic factor delivery, some treatment is necessary to prevent rejection of transplanted cells in order to promote behavioral recovery. To circumvent the need for immune suppression we have developed a method of alginate encapsulation for grafting non-autologous Fb/BDNF into the injured spinal cord (Tobias et al., 2001). Alginate is a water soluble, biocompatible poly-saccharide that forms a hydrogel in the presence of calcium ions. When alginate capsules are coated with a polycation such as poly-L-ornithine, a size exclusion barrier forms that limits the size of substances that pass into and out of the gel so that growth factors diffuse out but immune cells cannot target the foreign cells within the capsule. Using this model we were able to show that alginate encapsulated Fb/BDNF survived in culture for one month, maintained transgene expression, produced a bioactive neurotrophin, survived in the injured spinal cord and created a permissive environment for host axon growth in the absence of immune suppression (Tobias et al., 2001).

In the present investigation we extend our studies to determine (1) if alginate encapsulation can replace immune suppression by protecting the Fb/BDNF for 2 months after grafting; (2) if encapsulated Fb/BDNF will elicit recovery of forelimb and/or hindlimb function; and (3) if the encapsulated Fb/BDNF will stimulate axon growth.

MATERIALS AND METHODS

Retroviral Vector and Cell Line

Construction, characterization, and isolation of the BDNF-producing Sprague-Dawley primary fibroblasts (Fb/BDNF) used in this study have been described pre-

viously (Liu et al., 1999; Kim et al., 2001; Tobias et al., 2001; Liu et al., 2002). Briefly, Sprague-Dawley abdominal skin fibroblasts were isolated and infected with an MMLV retroviral vector containing the human BDNF cDNA and a fusion gene of β-galactosidase and neomycin phosphotransferase positioned down-stream of an internal ribosomal entry site (IRES). The Fb/BDNF were isolated by addition of G418 to the growth medium. These cells were previously shown to produce 12.8 ng/10^6 cells/24 h *in vitro*, and also to produce biologically active BDNF in an E8 chicken DRG assay (Liu et al., 1999; Tobias et al., 2001).

Alginate Encapsulation Procedure

The detailed procedure for encapsulation of Fb/BDNF has been described previously (Tobias et al., 2001). Briefly, Fb/BDNF were harvested while growing in culture, washed with sterile saline and resuspended at a concentration of 5×10^6 cells/mL of low viscosity (1.5% w/v) sodium alginate (Keltone-LV). The solution was sprayed from a 5-mL syringe attached to a pump, and a coaxial stream of air was used to shear the spray as it exited the needle. Alginate beads formed microspheres ($\sim$400–600 μm in diameter) in a 1.3% w/v solution of $CaCl_2$. The procedure was performed on "cell-free" alginate to create alginate filled cell-free control capsules, and again with alginate containing Fb/BDNF to create experimental capsules. The interior of all capsules is a calcium cross-linked hydrogel matrix of alginate with upwards of 80% aqueous space and when Fb/BDNF are present, they are embedded in the matrix. Alginate capsules were then coated with poly-L-ornithine (Sigma, St. Louis, MO) to create the semipermeable membrane and were washed with sterile saline, then coated with 0.12% sodium alginate to neutralize the residual positive charge. Capsules were then placed in standard culture medium (DMEM + 10% FBS) in a tissue culture incubator. Capsules designated for grafting were transplanted 48 h after preparation. Additional sets of both "cell-free" capsules and Fb/BDNF capsules were kept in culture for 8 weeks to observe transgene expression, capsule integrity, and cell growth.

Quantitation of Fb/BDNF within Alginate Capsules

The method for determining the number of cells within a capsule was adapted from the methods of Chang and colleagues (Liu et al., 1993; Chang et al., 1994). At 48 h after alginate encapsulation, twelve capsules were retrieved from tissue culture, placed individually in separate solutions of saline containing trypan blue, and covered with a glass coverslip. The pressure of the coverslip ruptured the capsule and the Fb/BDNF were counted by exclusion of the vital dye trypan blue under phase microcopy.

X-Gal Staining of Encapsulated Fb/BDNF In Vitro

Capsules containing Fb/BDNF were stained for β-galactosidase activity after growing in culture for 2 days, 4 weeks, or 8 weeks. The method of X-gal staining has been used previously for Fb/BDNF growing in culture (Liu et al., 1999) and Fb/BDNF encapsulated in alginate (Tobias et al., 2001). Capsules were fixed with 0.5% glutaraldehyde in PBS for 10 min, washed three times with PBS, then reacted with X-gal reagent at 1 mg/mL in X-gal mixer (35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 2 mM $MgCl_2$) in PBS overnight at 37°C. Capsules were then washed in PBS and examined with light microscopy.

Surgical Procedure, Immune Suppression, and Treatment Groups

Thirty-six female Sprague-Dawley rats, weighing 225–250 g (Taconic, Germantown, NY), received a subtotal hemisection (dorsolateral funiculotomy) at the level of the C4 nerve root. The surgical procedure has been described in detail previously (Liu et al., 1999; Chow et al., 2000; Tobias et al., 2001). Rats were anesthetized with a ketamine, xylazine, acepromazine mixture and a surgeon uninformed as to the treatment group performed a laminectomy at the C4 vertebral body to expose the spinal cord. A longitudinal incision was made in the dura to gain access to the spinal cord and the right dorsolateral funiculus was removed by gentle aspiration with a finely pulled glass pipette. The lesion cavity was $\sim$1.5–2 mm in rostral/caudal length. A second surgeon then examined the lesion site for completeness and placed a graft into the cavity. The grafted groups were (1) the experimental group, alginate encapsulated Fb/BDNF ($n = 12$), (2) the capsule control group, cell-free control capsules ($n = 12$), or two non-capsule control groups, both with Fb/BDNF soaked in gelfoam and (3) one that received CSA ($n = 6$) and (4) one that did not receive CSA ($n = 6$). Six of the twelve animals that received Fb/BDNF in gelfoam were immune suppressed with Cyclosporin A (CSA, Novartis, Pharmaceutical Corp., East Hanover, NJ) by daily subcutaneous injections of 1 mg/100 g body weight beginning three days before injury and continuing for 2 weeks after injury. This group of rats then received oral CSA (Novartis) resuspended in their drinking water at a final concentration of 50 μg/mL for the duration of the study. The other six animals in this group, and all animals in the other groups, were not

immune suppressed. Grafts of Fb/BDNF in gelfoam contained approximately 500,000 cells, and grafts of cell-free capsules or encapsulated Fb/BDNF contained approximately 10–12 capsules. After the lesion cavities were filled with the grafted material, the dura was closed with 10-0 sutures (Ethicon) and the muscle and skin closed in layers. The rats received bolus injections of Methylprednisolone (30 mg/kg, i.v.) at the end of surgery and two hours post-surgery. They were kept on heating pads and observed until fully awake, then returned to their home cages. Rats were kept under conditions of controlled temperature and humidity on a 12-h light/dark cycle with lights on at 07:00. All procedures were carried out in accordance with a protocol approved by Drexel University College of Medicine's Institutional Animal Care and Use Committee and followed NIH guidelines for the care and use of laboratory animals.

Anterograde Tracing of the Rubrospinal Tract with BDA

Biotinylated dextran amine (BDA) was injected 2 weeks before the animals were sacrificed in capsule recipients. Rats were anesthetized and placed into a stereotaxic apparatus. Labeling of the rubrospinal tract was achieved by injecting 0.5 μL of 10% BDA (Molecular Probes, Eugene, OR) through a 1-μL Hamilton syringe into the left red nucleus (RN) of each animal in groups in which capsules were implanted. The following stereotactic coordinates were used: anterior-posterior 5.8 mm, medial-lateral 0.7 mm, and dorsal-ventral (from dural surface) 7.0 mm, with Bregma as the zero point. After injection over 5 min, the Hamilton syringe was left in place for an additional 5 min to increase diffusion of the BDA into the brain. Because the rubrospinal tract is 99% crossed (Brown, 1974), injections were made into the red nucleus contralateral to the injury to label regenerated axons. After injections were completed, the skin was closed with 4-0 sutures (Ethicon) and the rats were kept on heating pads and observed until fully awake, then returned to their home cages.

Immunocytochemical Staining of Spinal Cord Tissue

Eight weeks post-transplantation, rats were deeply anesthetized with Nembutal, transcardially perfused with 200 mL of physiological saline, then 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Spinal cords were removed, washed in 0.1 M PB and cryoprotected in 30% sucrose at 4°C for 72 h. Spinal cord tissue was then frozen in OCT compound (Tissue Tek, Sakura Inc., Tokyo, Japan) and serially sectioned on a freezing microtome at 20 μm in either the transverse or parasagittal plane. Sectioned tissue was then stained with cresyl violet or processed for fluorescent immunocytochemical staining. The detailed procedures for immunostaining spinal cord tissue have been described previously (Liu et al., 1999; Chow et al., 2000; Tobias et al., 2001). Primary antibodies used were monoclonal RT-97 (1:100; Boehringer Mannheim, GmbH, Germany), monoclonal GFAP (1:100; Boehringer Mannheim), polyclonal CGRP (1:5000; Peninsula Laboratories, Inc., San Carlos, CA), monoclonal ED-1 (1:500; Harlan Bioproducts for Science, Indianapolis, IN), polyclonal TNF-α (1:100; R&D Systems, Minneapolis, MN), polyclonal 5-HT (1:10,000; Eugene Tech., Ridgefield, NJ), polyclonal MAP-2 (1:10,000; Fischer et al., 1991) and polyclonal GAP-43 (1:2,500; a gift kindly provided by Dr. Larry Benowitz). A mouse monoclonal antibody recognizing the extracellular domain of PO (clone 18, PO7) was kindly provided by Dr. Juan J. Archelos. Fluorescent secondary antibodies (diluted 1:200) were either FITC or Rhodamine conjugated goat-anti-mouse (Jackson ImmunoResearch, West Grove, PA) for monoclonal antibodies or FITC or rhodamine conjugated goat or donkey-anti-rabbit or sheep (Jackson ImmunoResearch) for polyclonal antibodies. Slides were coverslipped with Vectashield (Vector, San Diego, CA) containing the nuclear counterstain DAPI.

BDA labeled rubrospinal axons were visualized using an ABC elite kit (Vector Labs, Burlingame, CA) with DAB as the chromagen. This procedure has been described in detail previously (Liu et al., 1999). Slides containing spinal cord tissue were rinsed three times for 30 min each with TBST (50 mM Tris buffered saline containing 0.5% Triton X-100, pH 10.0), then incubated with the avidin-biotinylated-complex (ABC kit) at room temperature for two hours. Slides were rinsed in TBST, then in 50 mM Tris buffer, and finally reacted with Sigma Fast-DAB compound (Sigma Chemical Co., St. Louis, MO) in the dark for 30 min. The reaction was stopped by placing the slides in dH_2O, and the slides were dehydrated and coverslipped with DPX (Fluka Chemie AG, Buchs, Switzerland).

Behavioral Analysis

All behavioral tests were performed by observers trained to score with greater than 95% inter-rater reliability. Observers were unaware of the animal treatment groups while testing and scoring.

Open-field locomotion (BBB scale). This is a widely used behavioral measure to study hindlimb function during open-field locomotion after spinal cord injury (Basso et al., 1995). The BBB test was developed for use after

a bilateral contusion injury at the thoracic level and evaluates hindlimb function and coordinated locomotion. Because rats in the present study received unilateral cervical subtotal hemisections, only the BBB score of the affected (right) side is reported. This test was performed weekly by placing rats in an enclosed space approximately 5 feet by 2 feet. Rats that have received an acute cervical subtotal hemisection recover from an initial deficit to baseline values (Kim et al., 2001).

Vertical forelimb exploration (cylinder test). This test evaluates forelimb usage while a rat spontaneously explores within a cylinder. Rats are placed in a clear Plexiglas cylinder (20 cm in diameter and 30 cm in height) and videotaped for three minutes and the tapes used for scoring. The rats explore by rearing and supporting themselves by placing their forepaws on the cylinder walls. The number of times the forelimbs (right, left, or both) make contact with the cylinder walls while exploring was counted and expressed as a percentage of total placements. Percentage of forelimb contacts with the right forelimb and with both forelimbs was added to reflect the full usage of the affected (right) limb. Counts were made from videotapes. Initial baseline values showed no bias in forelimb preference in any of the animals used in the study. Testing was performed once a week for 5 weeks after injury and grafting. This test has been used previously to demonstrate partial recovery of forelimb function after an acute cervical subtotal hemisection and transplantation of fibroblasts genetically modified to secrete BDNF in immune suppressed adult rats (Liu et al., 1999; Schwartz et al., 2003)

Horizontal rope walking (rope test). This test evaluated the rats' ability to traverse a suspended rope 2.5 cm in diameter and 83 cm in length to reach their home cage. Horizontal rope walking (Kim et al., 2001) assesses the rat's ability to locomote and make postural adjustments on a challenging terrain. Rats were trained to walk across the rope before surgery to obtain baseline values. Postoperatively they were videotaped traversing the rope in five consecutive trials each week for 5 weeks following injury and grafting. The number of hindpaw slips with either paw and falls on either side were counted over five consecutive trials. Falls from the rope were counted as two slips (simultaneous slips by both hindlimbs). The number of slips and falls in the hindlimbs over five consecutive trials was divided by the total number of steps and expressed as % errors. This test has shown deficits after acute cervical lateral funiculus lesions and partial recovery in immune suppressed animals that received transplants of Fb/BDNF (Kim et al., 2001).

Statistical Analysis of Behavioral Tests

Data for all behavioral tests were analyzed by two-way ANOVA between transplant groups and time, with time taken as the repeated measure. Following identification of significant interactions, we performed planned comparisons between the following groups: (1) encapsulated Fb/BDNF grafts versus cell-free capsule grafts to identify effects of the encapsulated transplanted cells; (2) encapsulated Fb/BDNF versus unencapsulated Fb/BDNF + immune suppression to compare methods of protection; (3) unencapsulated Fb/BDNF + immune suppression versus unencapsulated Fb/BDNF without immune suppression to identify the effects of immune suppresion; and (4) encapsulated Fb/BDNF versus unencapsulated Fb/BDNF without immune suppression to test effects of encapsulation. Post hoc analysis included two-way ANOVA between specific transplant groups and time, with time as the repeated measure, followed by one-way ANOVA at individual weeks. Statistical analyses were performed using Stat View software (SAS Institute Inc., Cary, NC). Significance levels for all tests were set at 0.05.

RESULTS

Analysis of Behavioral Recovery

Open field locomotion (BBB). Rats were analyzed with a 21-point BBB scale (Basso et al., 1995), modified by focusing on the hindlimb ipsilateral to the injury (right hindlimb), to determine deficits and recovery in open field locomotion. Immediately after surgery all rats displayed forelimb deficits in open-field locomotion, but recovered fully to preoperative levels by 3 weeks, with no significant differences among groups. These results are similar to those reported previously using grafts of unencapsulated Fb/BDNF, gelfoam alone, or unmodified fibroblasts in the same injury model but with immune suppression (Kim et al., 2001).

Vertical forelimb exploration (cylinder test). When placed in a Plexiglas cylinder, rats normally explore by rearing and supporting their weight against the cylinder with one or both forelimbs. Before injury rats used each forelimb (left or right) ~25%, and used both forelimbs for exploration ~50% of the time (data not shown). Therefore, total use of the right forelimb before injury (% right + % both) was ~75% with no significant differences among groups. A failure to use the affected limb would result in a score of 0%, indicating that all exploration was achieved by the left limb alone (Fig. 1). Interestingly, only the group that received CSA (Fb/BDNF + CSA)

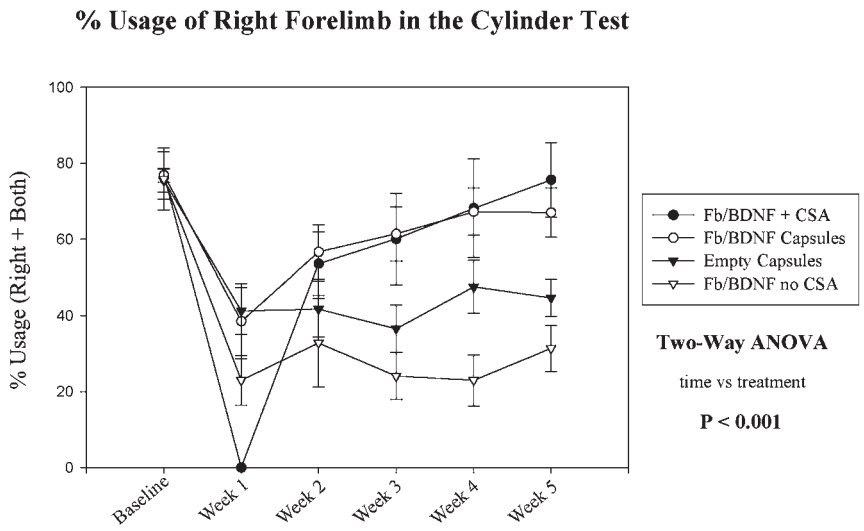

FIG. 1. Usage of the affected (right) forelimb in the cylinder showed deficits followed by significant behavioral recovery during the 5-week recovery period. Preoperative baseline performance showed ~75% usage of the right forelimb (% right + both) and was not different among groups. Overall two-way ANOVA showed significant interactions [$F(15,155) = 5.744$, $p < 0.001$] indicating that recovery occurred differently among treatment groups over time. Data are expressed as mean % use ± SEM.

showed a complete absence of use of the right limb and that was only at the first week postoperative. This transient response probably reflects the more lethargic state of CSA recipients.

We counted the frequency of use of the affected (right) forelimb during spontaneous vertical exploration post operatively. Overall analysis of the behavioral data from all groups by two-way ANOVA showed significant interactions ($p < 0.001$) for treatment groups over time (Fig. 1). All groups showed significant deficits at week 1 (Fig. 1). Animals that received Fb/BDNF capsules without CSA or Fb/BDNF in gelfoam + CSA recovered partial use of the forelimb over the 5 weeks of behavioral testing, but animals that received cell-free capsules or Fb/BDNF in gelfoam without CSA did not. Recovery of affected forelimb usually reflected use of both limbs for exploratory support, rather than the affected limb alone, and thus results in scores closer to 50%. In order to explain these differences in recovery among groups we conducted four post-hoc comparisons (Table 1).

1. *Encapsulated Fb/BDNF grafts versus cell-free capsule control grafts*. Analysis of each group by ANOVA indicated that rats that received encapsulated Fb/BDNF partially recovered forelimb use ($p < 0.001$) while rats that received cell-free capsules did not. Comparison of both groups by ANOVA showed a trend toward significant differences between treatment groups ($p = 0.076$). Post-hoc analysis showed that encapsulated Fb/BDNF grafts had significantly greater recovery ($p < 0.05$) at weeks 3–5 after grafting, compared to grafts of cell-free

Table 1. Statistical Results Comparing Selected Groups for Forelimb Usage During Vertical Forelimb Exploration in the Cylinder Test (% Right + Both), and Hindlimb Performance on the Horizontal Rope Test (% Errors) During the 5-Week Recovery Period After Grafting

	Fb/BDNF capsule vs. cell-free capsule	*Fb/BDNF capsule vs. Fb/BDNF + CSA*	*Fb/BDNF + CSA vs. Fb/BDNF no CSA*	*Fb/BDNF capsule vs. Fb/BDNF no CSA*
Cylinder Test	Fb/BDNF cap > cell-free cap	NSD	CSA > no CSA	Fb/BDNF cap > no CSA
Rope Test	NSD	NSD	CSA > no CSA	Fb/BDNF cap > no CSA

[a]Significant comparisons (ANOVA, $p < 0.05$, time vs. treatment) are represented as group > group. NSD, no significant difference.

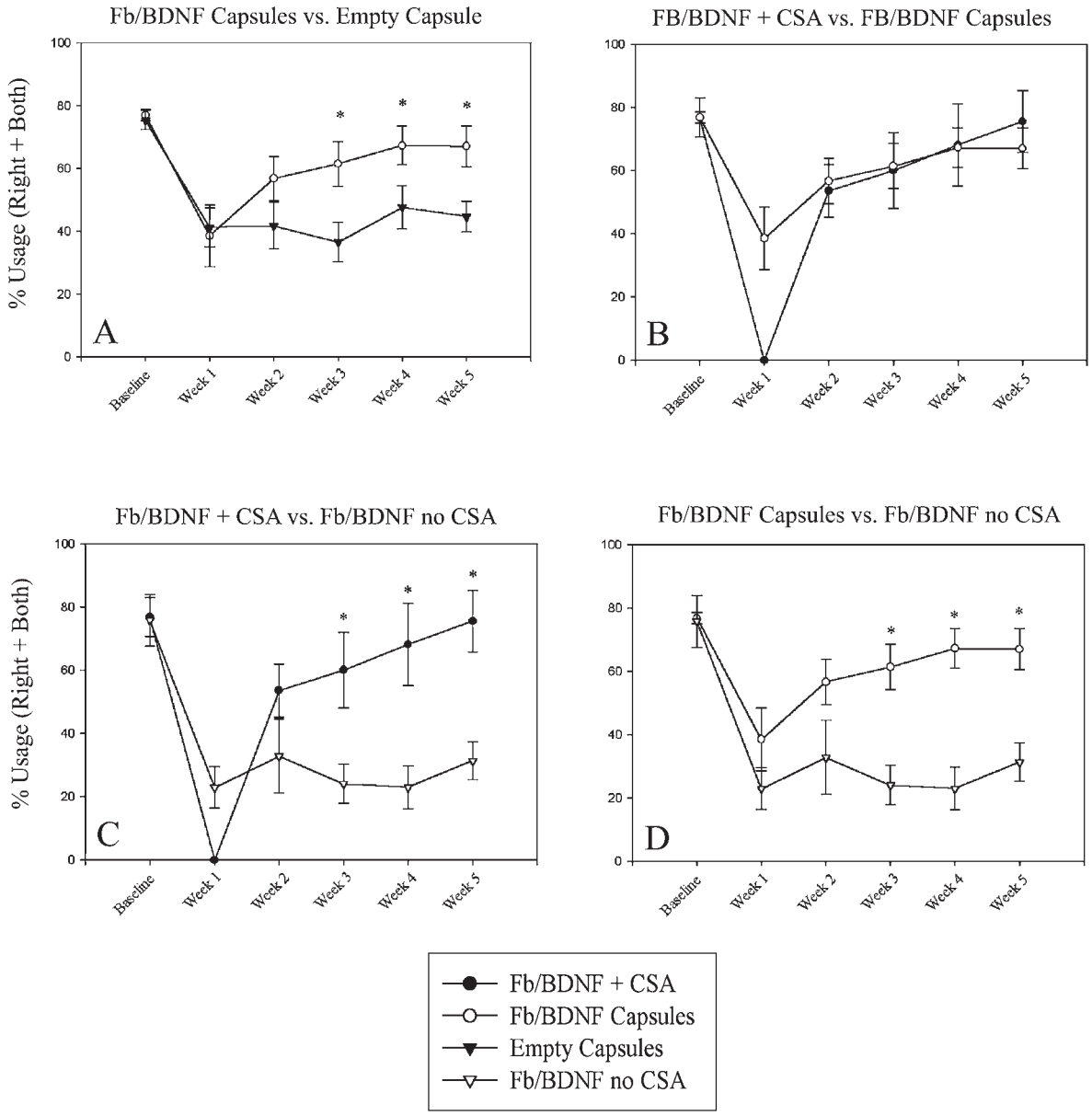

FIG. 2. Post hoc analysis (ANOVA) of forelimb recovery in the cylinder test comparing selected groups during the 5-week recovery period following grafting. (**A**) Animals that received Fb/BDNF capsules displayed a trend toward significantly better recovery than animals that received cell-free capsules by Week 2 [$F(1,21) = 3.418$, $p = 0.078$]. Significant recovery was noted at weeks 3–5 ($^*p < 0.05$). (**B**) Animals that received unencapsulated Fb/BDNF plus CSA recovered as well as animals that received encapsulated Fb/BDNF without CSA, with no differences between groups. (**C**) Animals that received unencapsulated Fb/BDNF plus CSA recovered significantly better than animals that received unencapsulated Fb/BDNF without CSA [$F(1,10) = 5.187$, $p < 0.05$]. Significant recovery was noted at weeks 3–5 ($^*p < 0.05$). (**D**) Animals that received Fb/BDNF capsules recovered significantly better than animals that received unencapsulated Fb/BDNF without CSA [$F(1,16) = 9.250$, $p < 0.01$]. Significant recovery was noted at weeks 3–5 ($^*p < 0.05$). Taken together, these findings suggest that alginate encapsulation or immune suppression with CSA is necessary for Fb/BDNF to elicit behavioral recovery of forelimb usage.

control capsules (Fig. 2A). Thus, there was a significant improvement associated with BDNF delivered from the alginate capsules, compared to the effects of the capsule alone.

2. *Encapsulated Fb/BDNF versus unencapsulated Fb/BDNF + immune suppression*. Analysis of each group by ANOVA indicated that both groups recovered ($p < 0.001$). Comparison of both groups by ANOVA showed no significant differences between these groups over the 5-week recovery period (Fig. 2B). Thus recovery of spontaneous forelimb usage was comparable in the groups receiving encapsulated Fb/BDNF and unencapsulated but immune suppressed Fb/BDNF grafts.

3. *Unencapsulated Fb/BDNF + immune suppression versus unencapsulated Fb/BDNF without immune suppression*. Analysis of each group by ANOVA indicated that rats that received unencapsulated Fb/BDNF + CSA recovered ($p < 0.001$), while rats that received unencapsulated Fb/BDNF without CSA did not. Comparison of both groups by ANOVA indicated significant differences between treatment groups ($p < 0.05$). Post-hoc analysis showed that immune suppressed rats showed significantly greater recovery ($p < 0.05$) at weeks 3–5 after grafting compared to non-immune suppressed rats (Fig. 2C). Immune suppression is therefore required for the unencapsulated Fb/BDNF grafts to enhance forelimb behavioral recovery in the cylinder test.

4. *Encapsulated Fb/BDNF versus unencapsulated Fb/BDNF without immune suppression*. Analysis of each group by ANOVA indicated that rats that received encapsulated Fb/BDNF recovered ($p < 0.001$), while rats that received unencapsulated Fb/BDNF without CSA did not. Comparison of both groups by ANOVA indicated significant differences between treatment groups ($p < 0.05$). Post-hoc analysis showed that rats with encapsulated Fb/BDNF grafts showed significantly greater recovery ($p < 0.05$) at weeks 3–5 than animals with unencapsulated Fb/BDNF grafts without immune suppression (Fig. 2D). Thus alginate encapsulation protected the Fb/BDNF and permitted their biological actions.

Horizontal rope walking (rope test). Rats were tested on their ability to traverse a horizontal rope by counting hindlimb slips and falls (expressed as % errors in five consecutive crossings). Before injury, rats made few errors while crossing the rope, with no significant differences among groups (Fig. 3). Overall analysis of all groups over time by two-way ANOVA showed significant interactions ($p < 0.01$) for treatment groups over time. There were significant deficits in all groups at week 1, but animals that received encapsulated Fb/BDNF or cell-free capsules or unencapsulated Fb/BDNF in gelfoam with CSA partially recovered from these impairments over the 5 weeks of behavioral testing. Animals that received unencapsulated Fb/BDNF without CSA did not. To explain these differences in recovery among groups we conducted the same post-hoc comparisons as we used for the cylinder test (Table 1).

1. *Encapsulated Fb/BDNF grafts versus cell-free capsule grafts*. Analysis of each group by ANOVA indicated

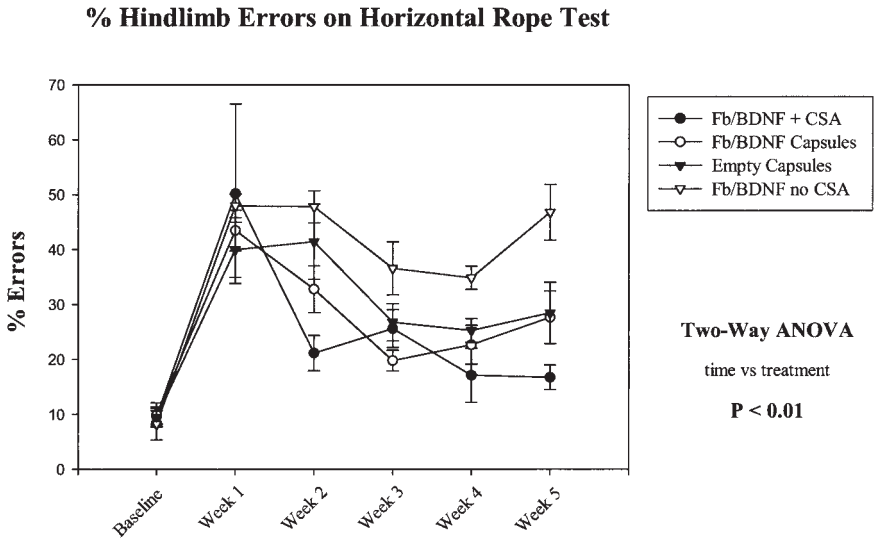

FIG. 3. Hindlimb function on the rope tested showed deficits followed by significant recovery during the 5-week recovery period. Preoperative baseline performance showed few errors (% slips + falls in both hindlimbs, ~10%) and was not different among groups. Overall two-way ANOVA showed significant interactions [$F(15,145) = 2.467$, ($p < 0.01$)] indicating that recovery occurred differently among treatment groups over time. Data are expressed as mean % use ± SEM.

that both groups recovered ($p < 0.01$). Comparisons of both groups by ANOVA showed no significant differences between groups in the 5-week recovery period after grafting (Fig. 4A). Thus, surprisingly, cell-free control capsules were as effective as alginate encapsulated Fb/BDNF in eliciting recovery in hindlimb function on the rope test.

2. *Encapsulated Fb/BDNF versus unencapsulated Fb/BDNF + immune suppression*. Analysis of each group by ANOVA indicated that both groups recovered ($p < 0.01$). Comparisons of both groups by ANOVA showed no significant differences between groups in the 5-week recovery period after grafting (Fig. 4B). Encapsulated Fb/BDNF grafts therefore elicit hindlimb recovery in the rope test similar to that seen in immune suppressed rats that received unencapsulated Fb/BDNF grafts (Kim et al., 2001).

3. *Unencapsulated Fb/BDNF + immune suppression versus unencapsulated Fb/BDNF without immune suppression*. Analysis of each group by ANOVA indicated that animals that received unencapsulated Fb/BDNF + CSA recovered ($p < 0.01$) while animals that received unencapsulated Fb/BDNF without CSA did not. Comparison of both groups by ANOVA indicated significant differences between treatment groups ($p < 0.05$). Post-hoc analysis showed that immune suppressed rats that received unencapsulated Fb/BDNF showed significant recovery from weeks 2 to 5 ($p < 0.05$ for weeks 2, 4, and 5; $p < 0.10$ for week 3) after grafting compared to non-immune suppressed rats (Fig. 4C). Immune suppression is therefore required for the unencapsulated Fb/BDNF grafts to promote hindlimb behavioral recovery in the rope test.

4. *Encapsulated Fb/BDNF versus unencapsulated Fb/BDNF without immune suppression*. Analysis of each group by ANOVA indicated that animals that received encapsulated Fb/BDNF recovered ($p < 0.01$) while rats that received unencapsulated Fb/BDNF without CSA did not. Comparison of both groups by ANOVA showed significant differences between treatment groups ($p < 0.05$). Post-hoc analysis showed that encapsulated Fb/BDNF grafts displayed significant recovery ($p < 0.05$) from weeks 2 to 5 compared to animals that received unencapsulated Fb/BDNF without CSA (Fig. 4D). Thus alginate encapsulation protected the Fb/BDNF, and allowed the BDNF secreted by the grafted cells to promote recovery, which was not observed with unencapsulated Fb/BDNF without CSA.

Analysis of Capsules In Vitro and In Vivo

Encapsulated Fb/BDNF in vitro. We examined the encapsulated fibroblasts in vitro over a period of 8 weeks to confirm cell survival and transgene activity. Immediately after formation of the capsules, cell-free capsules (Fig. 5A) and capsules containing Fb/BDNF (Fig. 5B) were uniform in size, and approximately 400–600 μm in diameter. Forty-eight hours later, encapsulated Fb/BDNF could be recognized as individual cells (Fig. 5B), and most stained positively for β-galactosidase (Fig. 5C,D), as in our previous report of encapsulated Fb/BDNF in culture (Tobias et al., 2001). The mean number of Fb/BDNF within a capsule, assessed using trypan blue exclusion, was 1154.3 ± 72.6 at this time. Encapsulated Fb/BDNF continued to divide, formed spheroid cell clusters, which filled the capsules by 4 weeks (Fig. 5E), and maintained β-galactosidase expression for at least 8 weeks (Fig. 5F). We have previously shown that BDNF produced by encapsulated Fb/BDNF is bioactive (Tobias et al., 2001).

Histological analysis of spinal cord. We next analyzed cervical spinal cord 8 weeks after lesion and grafting. The lesion completely ablated the dorsolateral funiculus in all rats, severing the rubrospinal, dorsal spinocerebellar, and at the level of the lesion, Lissauer's tract. In addition, some spinothalamic and propriospinal axons are included in the injury (Fig. 6C–F). The grafting sites in rats that received Fb/BDNF in gelfoam and were immune suppressed with CSA were filled with fibroblasts that were closely apposed to the host spinal cord tissue and maintained the normal shape of the spinal cord (Fig. 6C). The grafting sites of animals without immune suppression consisted of cysts containing few cells and the remnants of gelfoam (Fig. 6D). The grafting sites in non-immune suppressed rats that received cell-free capsules contained 10–12 capsules (Fig. 6E). Non-immune suppressed rats that received encapsulated Fb/BDNF also contained 10–12 capsules, but cells distributed as spheres and as sheets were present within the capsules. In both groups the capsules filled the lesion cavity, but in the encapsulated Fb/BDNF group, the capsules were surrounded by dense layers of host cells (Fig. 6F,G). The appearance of the capsules and the cells is similar to that reported previously when capsules were examined at one month after grafting (Tobias et al., 2001). Fb/BDNF appeared to fill the capsules less completely in histological sections than they do in culture, but this difference is due to the loss of alginate matrix within the capsules (in which centrally located Fb/BDNF are embedded) during tissue processing. Our results therefore show that encapsulated Fb/BDNF survive for 8 weeks in a spinal cord injury site in the absence of immune suppression, and suggest that alginate encapsulation can substitute for immune suppression to ensure survival of xenografts.

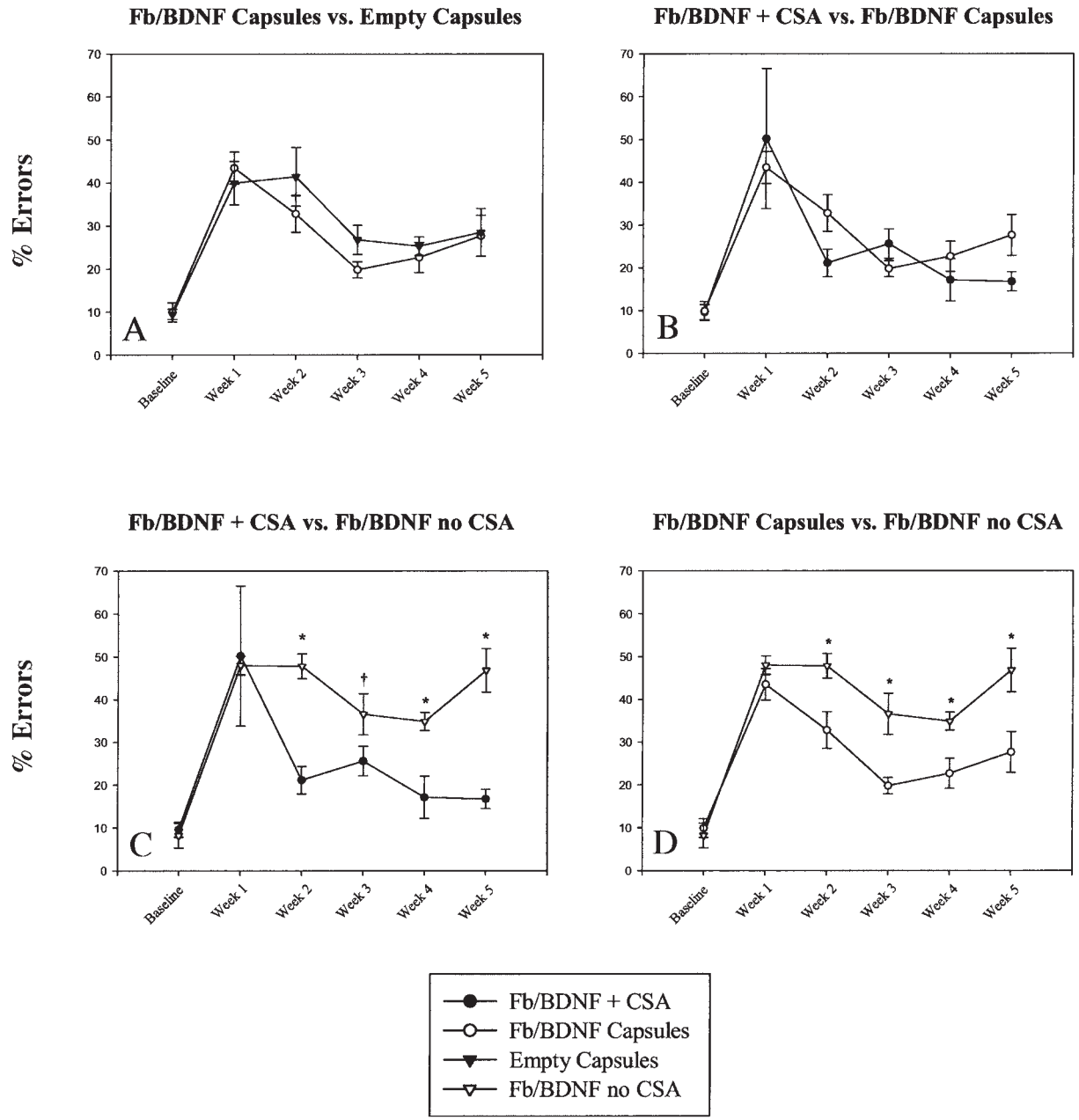

FIG. 4. Post hoc analysis (ANOVA) of hindlimb recovery in the horizontal rope test between selected groups during the 5-week recovery period following grafting. (**A**) Animals that received Fb/BDNF capsules recovered as well as animals that received cell-free capsules. (**B**) Animals that received unencapsulated Fb/BDNF plus CSA recovered as well as animals that received encapsulated Fb/BDNF without CSA. (**C**) Animals that received unencapsulated Fb/BDNF plus CSA recovered significantly better than animals that received unencapsulated Fb/BDNF without CSA [$F(1,10) = 8.011$, $p < 0.05$]. Significant recovery was noted at weeks 2–5 ($^{\dagger}p < 0.10$, $^{*}p < 0.05$). (**D**) Animals that received Fb/BDNF capsules recovered significantly better than animals that received unencapsulated Fb/BDNF without CSA [$F(1,16) = 7.052$, $p < 0.05$]. Significant recovery was noted at weeks 2–5 ($^{*}p < 0.05$).

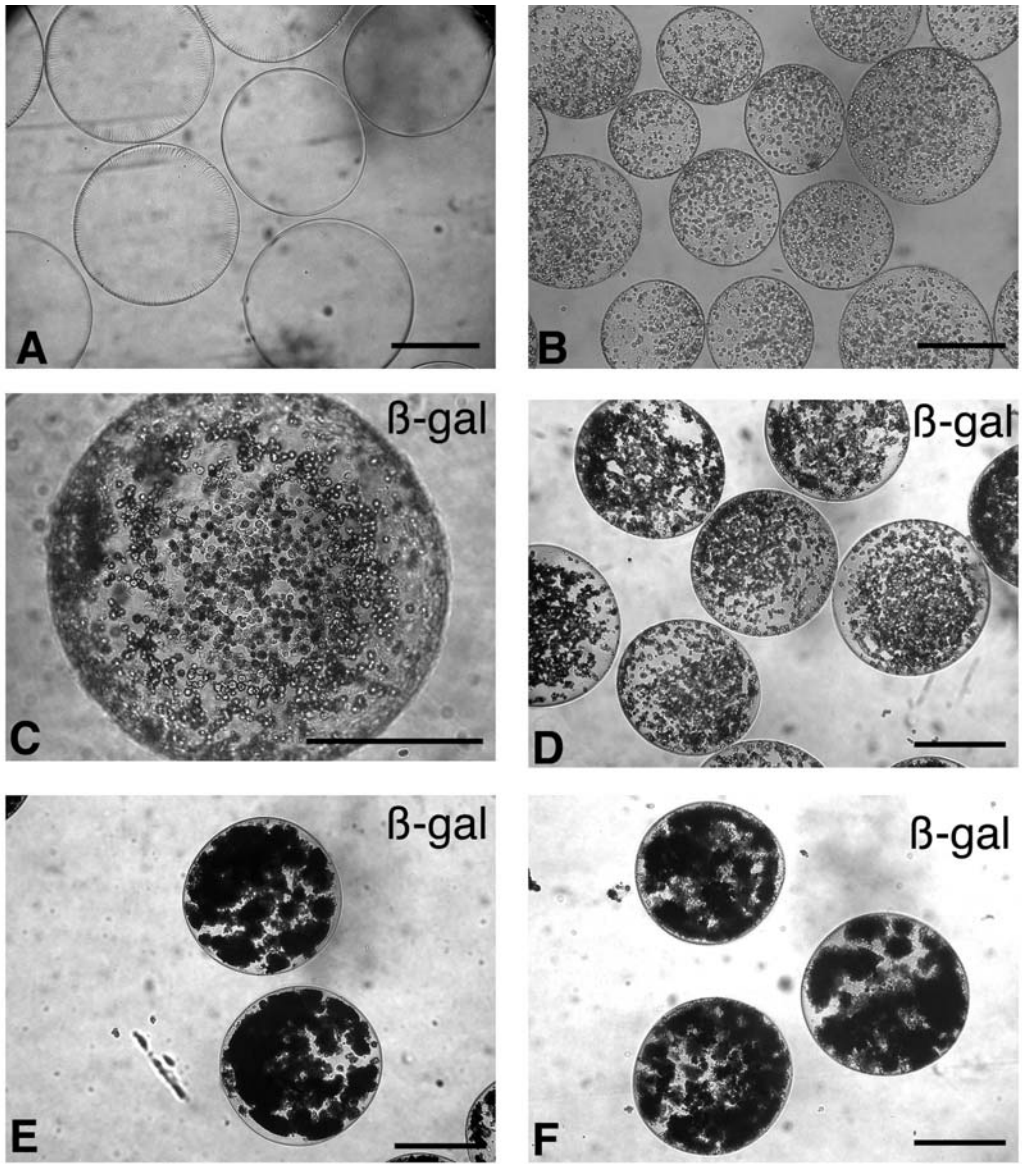

FIG. 5. Encapsulated Fb/BDNF growth and reporter gene expression over 2 months *in vitro*, phase contrast microscopy. Cell-free capsules (**A**) and encapsulated Fb/BDNF (**B**) immediately after encapsulation. High-power view (**C**) and lower-power view (**D**) of β-galactosidase staining 48 h after encapsulation of Fb/BDNF. β-galactosidase staining at 4 weeks (**E**) and at 8 weeks (**F**) after encapsulation of Fb/BDNF. Note that cells form spheroid aggregates over the 2-month period in culture, and that most of the cells continue to stain strongly for the reporter gene. Bars = 250 μm.

Immunocytochemical Analysis of Host Reaction to Grafts

The grafted region of cervical spinal cord was analyzed for the presence of axonal neurofilaments, dendrites, serotonin containing (raphe-spinal) and CGRP containing (dorsal root) axons, elongating axons, astrocytes, activated macrophages/microglia, TNF-α, and Schwann cells (utilizing RT-97, MAP-2, 5-HT, CGRP, GAP-43GFAP, ED-1, TNF-α, and PO antibodies, respectively). We elected to screen a number of antibodies to evaluate the effects of the treatments; the relatively small number of sections at the transplantation site precluded a useful quantitative analysis.

Neurofilament stained axons penetrated far into the cellular matrix that developed between the capsules containing Fb/BDNF (Fig. 7B) although no RT-97 immunoreactivity was found inside individual capsules. The

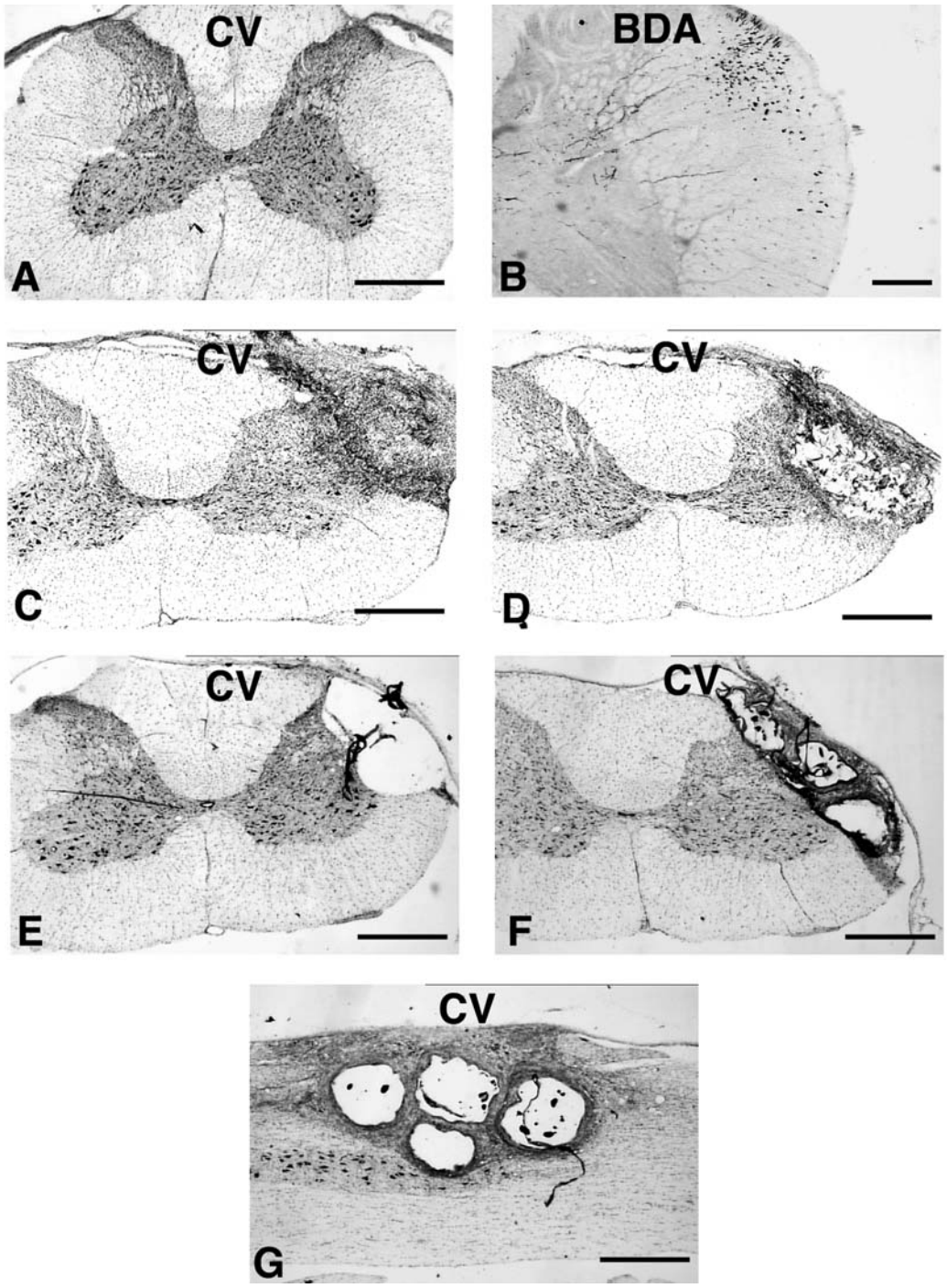

FIG. 6. Photomicrographs of uninjured and injured spinal cords in transverse (**A–F**) and sagittal (**G**) section 8 weeks after grafting. (**A**) Normal unoperated Nissl-stained cervical spinal cord. (**B**) Adjacent section showing BDA labeled rubrospinal tract fibers in the dorsolateral funiculus. (**C**) Nissl stained spinal cord of an immune suppressed rat containing a Fb/BDNF graft in gelfoam. (**D**) Non-immune suppressed rat containing a Fb/BDNF graft in gelfoam. Note the survival of the Fb/BDNF in the immune suppressed graft, while most areas of the non-immune suppressed graft are devoid of cells and contain only gelfoam and cyst-like structures. (**E**) Nissl-stained spinal cord of a non-immune suppressed rat containing a graft of cell-free capsules. (**F,G**) Encapsulated Fb/BDNF grafts. Note cellular infiltration of around capsules in F and G. The lesions and grafts in all groups (C–F) completely ablate the regions of rubrospinal tract tracing (B). Bar = 500 μm (A,D–G), 200 μm (B).

matrix surrounding the grafts of encapsulated Fb/BDNF also contained axons immunopositive for GAP-43 (Fig. 7C), and 5-HT (Fig. 7D), whereas the area surrounding cell-free capsules did not. Neurofilament staining (Fig. 7A) and occasional CGRP-containing axons (data not shown) were present at the lesion border surrounding the outer surface of cell-free capsules, but not within the central grafted region or inside individual capsules. Thus the cellular environment surrounding the grafts of encapsulated Fb/BDNF was permissive for growth of host axons (regenerating or sprouting) in the absence of immune suppression, similar to that seen one month after grafting (Tobias et al., 2001), and similar to that seen after grafting of Fb/BDNF in gelfoam in immune suppressed rats (Liu et al., 1999). In contrast little axonal growth was associated with grafts in cell-free capsules or in non-immune suppressed animals that received Fb/BDNF.

Strong GFAP immunostaining surrounded the lesion borders and extended to the outer surface of both cell-free control capsules (Fig. 7E) and capsules that contained Fb/BDNF (Fig. 7F). GFAP staining was not found in the interior of capsules in either group. GFAP staining was also found at the graft/host border in animals that received non-encapsulated Fb/BDNF grafts in gelfoam with and without immune suppression (data not shown). Therefore, a qualitatively similar astrocytic response developed and persisted at the lesion borders in all groups. The scar did not prevent growth of neurofilament, CGRP, GAP-43, or 5-HT labeled axons around the encapsulated Fb/BDNF within the graft.

ED-1 staining of cell-free control capsule grafts (Fig. 7G) and Fb/BDNF containing capsule grafts (Fig. 7H) revealed a rim of macrophages and microglial cells at the lesion border surrounding the outer perimeter of the capsules. TNF-α immunolabeling was used to identify inflammatory cytokine involvement in and around the injury site. In both groups, double-immunostaining with TNF-α and ED-1 revealed the presence of macrophages and microglia expressing TNF-α as well as other host cells that stained positively only for TNF-α (data not shown). Thus a modest immune response persists for 2 months after injury and grafting of cell-free capsules and of encapsulated Fb/BDNF. ED-1 staining of grafts containing Fb/BDNF in gelfoam without immune suppression revealed similar staining at the lesion borders but much stronger staining around the few surviving fibroblasts within the grafted region (Fig. 7J), whereas immune suppressed animals with the same grafts had staining only at the borders of the injury (Fig. 7I). These results indicate a stronger and/or more persistent immune-mediated response to the non-autologous fibroblasts in the absence of immune suppression. To determine if Schwann cells contributed to the cellular matrix of encapsulated Fb/BDNF cell transplants, we immunostained sections with PO, which recognizes the major protein of peripheral nervous system myelin (Archelos et al., 1993). In tissue sections, PO specifically labeled myelin in peripheral roots but not myelin in parenchyma of the spinal cord. A low level of non-specific background staining was seen in the transplant region but was similar in all groups. Thus Schwann cell migration did not appear to account for the cellular composition of the matrix that developed around encapsulated neurotrophin secreting cells.

Regeneration of Rubrospinal Axons

Two weeks before sacrifice rats that received capsules were given injections of biotinylated dextran amine (BDA) into the magnocellular division of the left red nucleus to label regenerating rubrospinal axons on the right side of the cord. Labeled rubrospinal axons were present in the vicinity of the rostral edge of the injury site in rats that received cell-free capsules (Fig. 8A), but did not penetrate into the grafts or the caudal host cord. Labeled rubrospinal axons were also present in the area rostral to the lesion border, but did not grow into or through encapsulated Fb/BDNF grafts (Fig. 8B), or into grafts of Fb/BDNF in gelfoam in non-immune suppressed rats. Thus axons of cut red nucleus neurons did not regenerate either in the absence of immune suppression or when fibroblasts were encapsulated. In contrast, rubrospinal tract axons regenerate into, around, and through grafts of Fb/BDNF in immunosuppressed rats (Liu et al., 1999).

DISCUSSION

The *in vitro* results presented in this study show that alginate encapsulated Fb/BDNF survived and maintained transgene expression in culture for two months. Alginate encapsulation also protected non-autologous Fb/BDNF from rejection after transplantation into a cervical cord injury site and promoted behavioral recovery. The encapsulated Fb/BDNF elicited axonal growth, but rubrospinal axons did not regenerate. In contrast, grafted Fb/BDNF are lost without immune suppression or encapsulation and do not enhance recovery without protection from the host immune system.

Behavioral Recovery

All injured rats displayed initial deficits in open-field locomotion with full recovery subsequently. Greater and more persistent deficits were seen in vertical forelimb exploration in the cylinder test and in locomotion on the horizontal rope test. Similar results have been reported previously after this injury (Liu et al., 1999; Kim et al.,

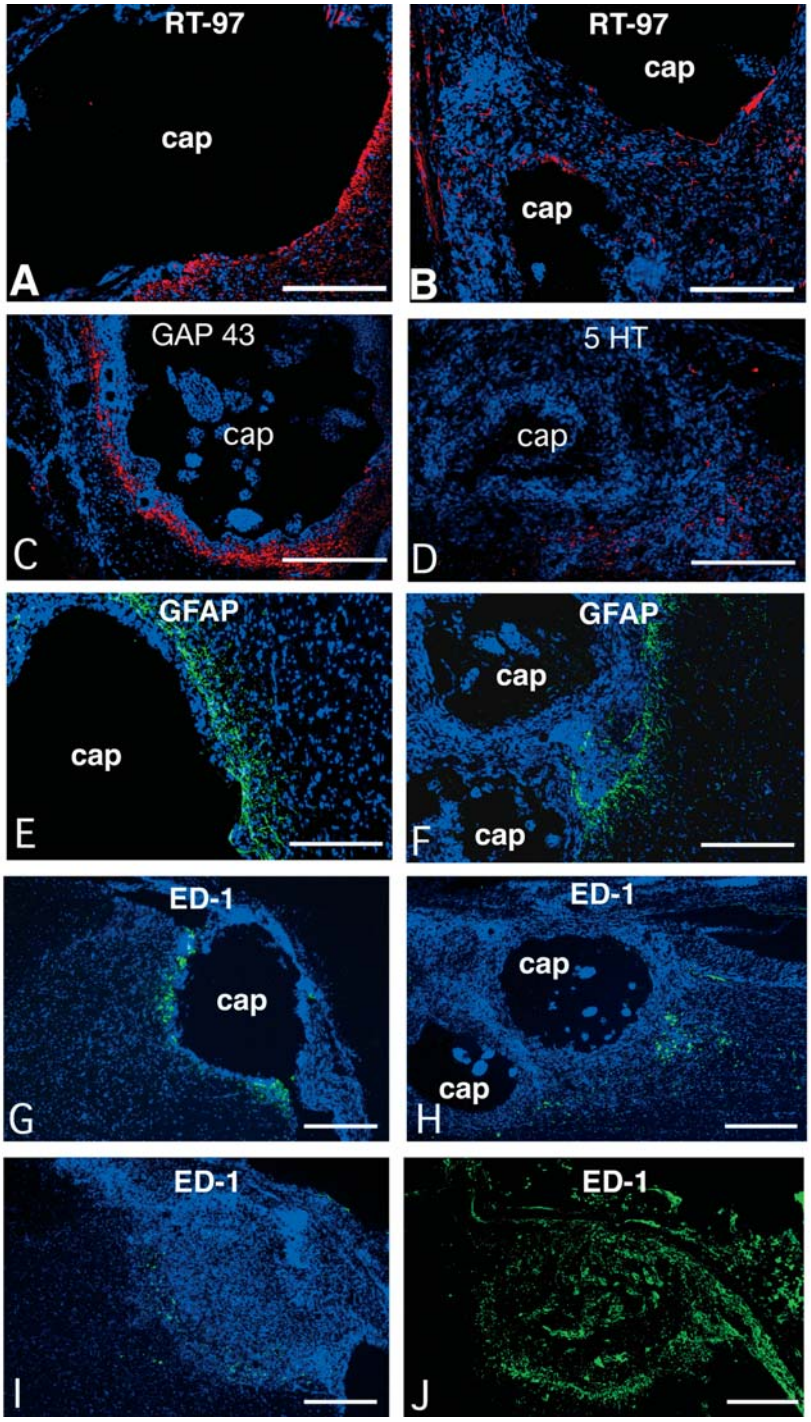

FIG. 7. Darkfield photomicrographs illustrating host response to grafts of cell-free capsules and Fb/BDNF capsules in cervical spinal cord sections 8 weeks after transplantation. Panels A–I were counterstained with DAPI (blue). Neurofilament (RT-97) staining shows axons growing to the lesion border in cell-free capsule grafts (**A**), but penetrating deep (**B**) within the Fb/BDNF capsule graft but not entering the interior of the capsule. GAP43 staining shows axons abutting the capsules (**C**) and 5–HT staining shows some axons also abutting the capsule (**D**). All axon staining is red. GFAP staining shows astrocytes surrounding exterior of capsule walls only at the graft border in cell-free capsule graft (**E**) and Fb/BDNF capsule graft (**F**). ED-1 immunostaining of cell-free capsule graft (**G**) in transverse section, Fb/BDNF capsule graft (**H**) in parasagittal section, and Fb/BDNF gelfoam graft (**I**) in an immune suppressed animal showing activated macrophages and microglia surrounding the injury site in these groups, but not entering the grafted region. (**J**) Strong ED-1 staining throughout a graft of Fb/BDNF in gelfoam without immune, suggesting a stronger reaction to the Fb/BDNF without immune suppression or encapsulation. ED-1 and GFAP staining is green. Bar = 250 μm. The symbol (cap) identifies capsules within a graft.

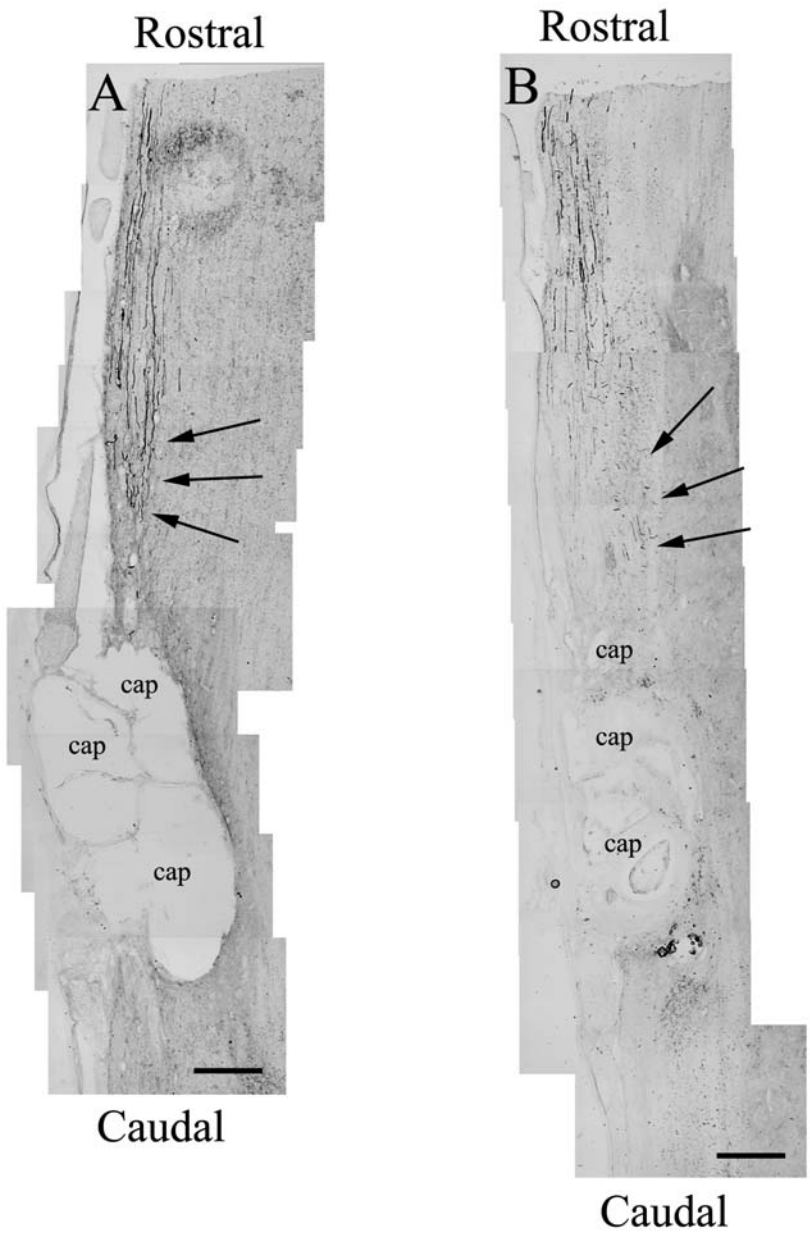

FIG. 8. Photomicrograph montages of rubrospinal tract axons labeled by BDA injections 8 weeks after grafting of cell-free capsules or encapsulated Fb/BDNF. Rubrospinal tract axons are present near the lesion border, but do not enter or grow through cell-free capsule grafts (**A**) or encapsulated Fb/BDNF grafts (**B**). Arrows indicated BDA labeled axons approaching the lesion border. Cap indicates capsule. Both panels are 100× magnification montage reconstructions of parasagittal cervical spinal cord sections. Bar = 250 μm.

2001). Behavioral deficits on the cylinder and rope tests partially recovered over 5 weeks, and these tests were therefore more useful than BBB score in evaluating the interventions. The rope test evaluates locomotion on a more challenging terrain by testing both forelimb and hindlimb paw placements as well as support and postural adjustments and thus is more sensitive to deficits in locomotion than the BBB test. The cylinder test, as employed here, measures spontaneous forelimb usage and is therefore a more sensitive test of this lesion model. Surprisingly, recovery in the rope test in animals with cell-free capsules was comparable to that in animals that received encapsulated Fb/BDNF or non-encapsulated Fb/BDNF with immune suppression. Since other forms of alginate (e.g. freeze-dried alginate) have been reported to support regeneration of axotomized neurons after

spinal cord injury (Suzuki et al., 1999a,b; Kataoka et al., 2001; Suzuki et al., 2002), and to decrease the extent of the glial scar (Suzuki et al., 2001), it is possible that the alginate capsules themselves may have offered some benefit to the injured spinal cord even though the populations of axons that we examined did not indicate growth into the region of the cell free capsules. While the contribution of alginate alone may account for the hindlimb recovery observed on the rope test, it was insufficient to enable recovery of forelimb function on the cylinder test. Recovery in the cylinder test was seen only in animals that received either alginate encapsulated Fb/BDNF without immune suppression or Fb/BDNF in gelfoam with immune suppression.

Alginate encapsulation of other cell types has been shown to provide therapeutic physiological benefits after grafting experiments in animal models of malignant glioma (Joki et al., 2001), liver failure (Hirai et al., 1993), hemophilia (Hortelano et al., 1996), diabetes (Sun et al., 1996), Parkinson's disease (Xue et al., 2001), and growth hormone deficiency (Cheng et al., 1998). To our knowledge, these are the first results documenting behavioral recovery due to alginate encapsulated genetically engineered cells after spinal cord injury. Our results indicate that neurotrophins provided in the encapsulated form have beneficial effects on behavioral recovery despite the fact that they need to be delivered through the capsule membrane and that the alginate capsule itself is embedded in a cellular matrix. These results also support the application of this technology to more complex models of spinal cord injury such as a transection, or the delayed grafting models.

Axonal Growth

After grafting, encapsulated Fb/BDNF provided a permissive environment for host axon growth as indicated by the presence of RT-97, CGRP, 5-HT, and GAP-43 immunostained axons within the central grafted region. These immunolabeled axons were found surrounding capsules, but never inside them, indicating that the capsule walls remained intact. In contrast, CGRP and neurofilament immunostained fibers were largely confined to the graft borders in cell free capsule grafts with only occasional CGRP-labeled axons within the central grafted region, indicating a less permissive environment for axon growth. Our labeling methods did not allow us to determine whether the RT-97, CGRP, and 5HT labeled axons had sprouted from intact axons, or were regenerating axons, or a combination of both.

When we looked for regeneration of the rubrospinal tract, BDA labeling provided no evidence that these axons regenerated in response to either encapsulated Fb/BDNF or cell-free capsule grafts. In a previous studies using the same and a similar injury models, we found rubrospinal regeneration elicited by unencapsulated Fb/BDNF transplants in immune suppressed hosts (Liu et al., 1999). We attribute the absence of rubrospinal regeneration when fibroblasts were encapsulated to the considerably smaller amounts of BDNF provided by the encapsulated Fb/BDNF than the Fb/BDNF gelfoam grafts. Ten to 12 capsules containing ~1,100–1,200 Fb/BDNF per capsule were grafted into each cervical spinal cord injury site. If approximately 12,000 encapsulated Fb/BDNF continued to double within the capsules after grafting for two passages, each graft would eventually contain ~50,000 cells. In contrast, grafts of Fb/BDNF soaked in gelfoam have been estimated to contain 500,000–1,000,000 cells (Liu et al., 1999) resulting in a 10–20-fold greater amount of BDNF secreted at the site of injury. Taken together, these results suggest that low concentrations of neurotrophins are sufficient to stimulate sprouting or beneficial reorganization after injury, but that higher concentrations are needed to stimulate regeneration of axotomized brainstem neurons.

Regeneration versus Sprouting

Behavioral recovery in both the cylinder and rope tests occurred in the absence of regeneration of rubrospinal axons. Previous results utilizing Fb/BDNF grafted in the presence of immune suppression in the same injury model showed that behavioral recovery was abolished by a second lesion just rostral to the graft which eliminates axons that had regenerated or sprouted into the rostral portion of the graft (Liu et al., 1999; Kim et al., 2001). The re-lesion experiments implied that axonal growth or neuroprotective effects elicited by the graft contributed to the recovery (Liu et al., 1999, 2002). Our results here suggest that the behavioral recovery was mediated by compensatory mechanisms other than regeneration of rubrospinal axons, which could include reorganization, neuroprotection, sprouting, or regeneration of descending systems that were not examined. In support of this hypothesis, it has been shown recently that uninjured descending motor systems sprout in response to incomplete spinal cord injuries (Weidner et al., 2001) and that sprouting can be enhanced by administration of neurotrophins (Jeffery and Fitzgerald, 2001) and that novel relays can develop (Bareyre et al., 2004). Sprouting of the uninjured ventral corticospinal tract (after dorsal corticospinal tract injuries) was at least partially responsible for behavioral recovery on a pellet retrieval task (Weidner et al., 2001). It is certainly possible in the present study that compensatory corticospinal tract sprouting may have occurred after the injury in response to the rubrospinal tract injury

and/or the application of BDNF via the grafted cells within capsules (Jeffery and Fitzgerald, 2001). In addition, sprouting indicated by the presence of CGRP containing axons as well as neurofilament, 5-HT, and GAP-43 immunostaining within and around these grafts could have contributed to the behavioral recovery.

Response of Non-Neuronal Cells

Analysis of immune mediating cells, utilizing ED-1 or TNF-alpha immunostaining, revealed activated macrophages and microglia surrounding the injury site in rats that received cell-free capsules or encapsulated Fb/BDNF, but staining was not detected within the interior of the capsules. The lack of ED-1–positive cells within capsules provides additional support for the notion that alginate capsules maintain their integrity for at least two months after grafting. Early time points were not examined but tissue evaluated 8 weeks post grafting did not appear to show differences in the density or distribution of ED-1–positive cells around the transplant sites among groups, although the types and amounts of cytokines released by these cells may have differed. Similarly, GFAP immunostaining demonstrated an astrocytic response at the lesion borders surrounding both cell-free and Fb/BDNF capsules.

The encapsulated Fb/BDNF grafts but not the cell-free capsules also elicited formation of a cellular matrix within which axons grew. These cells were presumably host cells since any grafted fibroblasts that might have escaped from ruptured capsules would have been subject to rejection. The absence of significant GFAP, ED-1, TNF-alpha, or PO immunoreactivity expressed by these cells suggests that they are not astrocytes, inflammatory cells or cells with a Schwann cell lineage. Their identify remains unknown. It is likely that this cellular response, stimulated by the alginate encapsulated Fb/BDNF grafts, favorably modified the environment of the host cord over the post-grafting period, by providing a permissive terrain for local axonal growth that contributes to the behavioral recovery. Thus the beneficial effects of the neurotrophic factors may include indirect mechanisms acting on the environment, as well as stimulatory effects on the injured neurons.

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