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Functional Repair of Rat Corticospinal Tract Lesions Does Not Require Permanent Survival of an Immunoincompatible Transplant

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Cell transplantation is one of the most promising strategies for repair of human spinal cord injuries. Animal studies from a number of laboratories have shown that transplantation of olfactory ensheathing cells cultured from biopsies of the olfactory bulb mediate axonal regeneration and remyelination and restore lost functions in spinal cord injuries. For translation from small laboratory experimental injuries to the large spinal cord injuries encountered in human patients the numbers of cells that can be obtained from a patient's own olfactory bulb becomes a serious limiting factor. Furthermore, removal of an olfactory bulb requires invasive surgery and risks unilateral anosmia. We here report that xenografted mouse bulbar olfactory ensheathing cells immunoprotected by daily cyclosporine restore directed forepaw reaching function in rats with chronic C1/2 unilateral corticospinal tract lesions. Once function had been established for 10 days, cyclosporine was withdrawn. Thirteen out of 13 rats continued to increase directed forepaw reaching. Immunohistochemistry shows that in all cases neurofilament-positive axons were present in the lesion, but that the grafted cells had been totally rejected. This implies that once grafted cells have acted as bridges for axon regeneration across the lesion site their continued presence is no longer necessary for maintaining the restored function. This raises the possibility that in the future a protocol of temporary immunoprotection might allow for the use of the larger available numbers of immunoincompatible allografted cells or cell lines, which would avoid the need for removing a patient's olfactory bulb.

Key words: Xenograft; Olfactory ensheathing cells (OECs); Spinal cord injury; Astrocytes

INTRODUCTION

A number of studies have shown that transplanted olfactory ensheathing cells (OECs) can induce axon regeneration, remyelination, and restoration of function in experimental spinal cord injuries (7,8,10,15,21,22,27,28). We have described a localized stereotaxic heat lesion completely destroying the adult rat corticospinal tract (CST) and partial adjacent medial gray matter unilaterally at the C1/2 level. Following this lesion, directed forepaw reaching (DFR) on the lesion side was totally absent. At 2 months after lesion, injection of a suspension of a mixture of cultured adult bulbar OECs and olfactory nerve fibroblasts (ONFs) restored DFR after around 10 days (12).

For translation to clinical application, removal of a patient's olfactory bulb necessitates a transdural endoscopic or intracranial approach, with the risk of unilateral anosmia. One way to obtain more cells and to avoid the problems of autograft biopsy would be to build up a bank of allogeneic cells or cell lines (16). As transplants, these would therefore have to cross an immune barrier. A number of studies with immunoincompatible grafts have shown that xenografted OECs immunoprotected with cyclosporine induce axon regeneration and remyelination in the spinal cord (10,11,18,19,29).

Clinically, permanent immunosuppression carries many disadvantages. In the present experiment we used a mouse to rat xenograft paradigm to test whether functions restored under transient immunosuppression would remain after the immunosuppressive regime was discontinued.

MATERIAL AND METHODS

Experimental Design

A total of 58 locally maintained strain of adult female Albino Swiss rats (locally bred, London, UK, body weight 200–220 g) were used.

Group 1: Twenty unlesioned rats received mouse OEC/ONF xenografts without immunosuppression. Histology was carried out at 4 days ($n=5$), 1 ($n=5$), 2 ($n=4$), 3 ($n=3$), and 4 weeks ($n=3$).

The remaining 38 rats had CST lesions and were tested for DFR.

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Group 2: In 11/38 of these rats DFR had not been abolished, and they were sacrificed for histology.

Group 3: In 8/38 rats in which DFR was abolished, they were left for 10–12 months (as a long-term control to check that DFR would not return) before sacrifice and histology.

The remaining 19/38 rats all received mouse OEC/ONF xenografts at 8 weeks after the CST lesions. Cyclosporine administration was applied immediately and continued afterward with daily injections.

Group 4: In 6/19 rats that showed no return of DFR at 5 weeks after receiving mouse OEC transplants, they were terminated for histology**.**

Group 5: In the remaining 13/19 rats where DFR had returned after receiving mouse OEC transplants, cyclosporine injection was continued for a further 10 days after the day when DFR had returned. Ten days later, cyclosporine was stopped, and DFR testing continued for two to three times a week until 8 weeks after receiving the transplants.

Directed Forepaw Reaching (DFR) and Microsurgery

All animals were handled according to the UK Home Office regulations ASPA 5(2) PIL 70/9580 for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act 1986 with the ethical approval of the UCL Institute for Neurology.

Presurgery. DFR was carried out as previously described (12). Briefly the rats were trained to use the forepaws to retrieve small pieces of noodles through a slit in the front of the cage, with 50 retrievals each session. Rats that used both right and left paws (more than 15 times each) were selected for making the CST lesions.

CST Lesions. Rats were anesthetized with isoflurane (Abbott Laboratories Ltd., Maidenhead, and Berkshire, UK). Unilateral CST lesions were made between C1 and C2 vertebrae, using an electrode (LCE; Cosman Medical Inc., Burlington, MA, USA). The CST and adjacent gray matter were destroyed by a heat lesion produced by a radiofrequency lesion device (RFG-3C RF; Radionics Inc., Burlington, MA, USA) operating at 10 V for 1 min as previously described (32).

Postsurgery. DFR testing was resumed 3 days after the lesion, two to three times a week. Rats that showed complete absence of DFR for 8 weeks were selected to receive mouse OEC transplants (Groups 4 and 5). Eight rats with complete absence of DFR were maintained up to 12 months as a long-term control (Group 3).

OEC Culture

The tissue from the outer nerve and glomerular layers of the olfactory bulbs of (n=40) 5-month-old female C57BL/6 (Harlan Laboratories, Bicester, Oxfordshire, UK) was trypsinized (0.1%; Worthington Biochemical Corporation, Lakewood, NJ, USA) for 15 min at 37°C and plated on to 35-mm Nunc™ dishes (Thermo Fisher Scientific UK, Hertfordshire, UK) coated with poly-D-lysine (Sigma-Aldrich, Dorset, UK). The cells were maintained for 15–16 days in DMEM-F12 (Life Technologies Ltd, Paisley, UK) medium including with 10% fetal calf serum (3133-028; Gibco, Paisley, UK). The OECs were identified by p75 immunostaining [anti-nerve growth factor receptor antibody, extracellular, clone 192-IgG (MAB365); Millipore Ltd Hertfordshire, UK], and the ONFs by fibronectin (FN) immunostaining [polyclonal rabbit anti-human fibronectin (A0245; Dako Ltd, Cambridgeshire, UK)].

Transplantation

Two days before transplantation the cells were transfected with a GFP gene harboring lentiviral construct (4,17,24). The cell suspension was made up at a final concentration of 25×10^6/ml in DMEM-F12 without FCS. Four microliters of the suspension (around 100,000 cells) were injected into the lesion site via a glass micropipette with a 100- to 120-μm internal tip diameter. Immediately after transplantation, cyclosporine (Novartis, Surrey, UK) made up at 15 mg/ml in 20% Tween 80 solution (Sigma-Aldrich) at a dosage of 6 mg/kg with 100% ethanol (VWR, Fontenay Sous Bois, France) was delivered intraperitoneally (IP) via Microfine insulin syringes (BD Ltd, Dun Laoghaire, Ireland) and repeated daily. DFR testing was restarted 3 days after the transplantation and maintained two to three times weekly.

Immunohistochemistry

Rats were perfused with 4% paraformaldehyde (TAAB Laboratories Equipment Ltd, Reading, UK) under terminal anesthesia [for details, see Keyvan-Fouladi et al. (12)]. Coronal or horizontal cryostat sections, 16 μm, were cut and immunostained for GFP/NF (neurofilament, rabbit anti-H+L chain NF 1:500; Invitrogen, Paisley, UK); glial fibrillary acidic protein (GFAP/NF) (mouse anti-GFAP 1:1,000, rabbit anti-H+L chain NF 1:500; Invitrogen); CD45 (mouse anti-CD45 1:500; BD Pharmingen, Oxford, UK). The sections were incubated with either Alexa Fluor 488-, or 546-conjugated goat anti-rabbit, anti-mouse, or goat anti-chicken secondary antibodies (1:400; Invitrogen) for 2 h at room temperature. Sytox Orange, 0.1 μM (Life Technologies Ltd) was used for cell counterstaining. Fluorescent images were captured using a Leica confocal microscope TCS SP (Milton Keynes, UK).

RESULTS

Group 1 (n = 20): Nonlesioned Rats Receiving Mouse OEC/ONF Xenografts Without Immune Protection

After 15–16 days in culture the biopsied mouse olfactory bulbs yielded a mixture of 60–70% $p75^+$ spindle-shaped bipolar or tripolar OECs and 30–40% FN^+

flattened, short-processed multipolar ONFs. This mixture was transplanted into the same C1/2 position in intact CST as that where the lesions were carried out in the other groups.

At 4 days the transplanted cells, identified by the GFP labeling, survived well. The transplant area was occupied by a compact ovoid hypercellular mass about 1 mm along the rostrocaudal axis of the host CST and around 0.3 mm wide. Within the transplant the cells were elongated to around 100 μm and, presumably following the surrounding mechanical forces, were orientated in parallel to the host CST (Fig. 1A).

At 1 week the numbers of GFP-labeled cells were reduced to around 30% of those seen at 4 days. The majority of cells had lost their elongated longitudinal pattern and appeared to be breaking up into irregular blob-like shapes (Fig. 1B). The background was densely hypervascular with hollow cuffed areas suggesting an influx of host immunocytes and perivascular cuffing.

At 2, 3, and 4 weeks no GFP-labeled cells were present in any part of the transplanted area, which retained the hypercellularity and the hollow vascular areas (Fig. 1C). At 2 weeks immunostaining of CD45 (leukocyte common antigen) showed cells intimately associated with the vessel walls, suggesting diapedesis of immunocytes infiltrating the entire transplant mass. Thirty-eight rats (Groups 2–5) were chosen for DFR on the basis that on 50 trials the DFR was comparable on the two sides (maximum imbalance 35/15). Unilateral C1/2 CST lesions were performed. DFR was tested twice weekly from 3 days after operation.

*Group 2: Rats With Failed CST Lesions (*n = *11)*

In 11 of these rats DFR started to return within 2 weeks. These rats were sacrificed, and the histology confirmed that in all 11 cases the destruction of the CST was incomplete. The remaining 27 rats (Groups 3–5) showed complete loss of DFR at 8 weeks after operation.

*Group 3: Rats Confirming Long-Term Persistence of Loss of DFR (*n = *8)*

In the eight rats of this group DFR testing was continued two to three times a week for 10 months ($n = 3$) or 12 months ($n = 5$). At no time was there any spontaneous recovery of DFR on the lesioned side in any of these eight rats. Even at the longer survivals none of the eight rats showed any diminution of DFR on the unlesioned side. Immunostaining confirmed that all these rats had complete destruction of the CST with partial destruction of the adjacent gray matter and in two cases spreading into part of the ventral column. Immunostaining for astrocytes (GFAP) and axons (neurofilament, NF) showed that the lesion center was completely devoid of any trace of NF staining and was completely walled off from the NF$^+$ axons of the surrounding spinal tissue by a thick layer of scarred GFAP$^+$ astrocytes (Fig. 1E). The remaining 19 rats with no DFR at 8 weeks after lesion received mouse OEC/ONF mixtures transplanted into the lesion site followed by immunoprotection by daily cyclosporine (Groups 4 and 5). DFR testing was resumed 3 days after receiving the transplants and carried out two to three times a week.

Group 4: Rats With Grafts Failing to Restore DFR

Six of these cyclosporine-treated rats in which DFR had not returned after 5 weeks were terminated. Immunohistochemistry showed that all six had complete destruction of the CST and partial destruction of the adjacent gray matter, but no transplanted cells and no NF staining were present in the CST lesion center, which was walled off by a heavy GFAP$^+$ astrocytic scar.

GFP labeling showed that under cyclosporine treatment the xenografted mouse cells had survived well, forming compact masses elongated along the rostrocaudal axis of the host spinal cord (as in the short-term animals of Group 1). These six transplants were located outside the scar walling off the CST lesion area and were therefore not in a position to bridge the lesion. Two transplants were located in the ascending dorsal column above the CST, one was in the ventral spinal cord, and three were in the gray matter adjacent to the CST (Fig. 1D). These transplants had induced the ingrowth of local NF$^+$ axons from the surrounding area.

*Group 5: Rats With Return of DFR (*n = *13) With Mouse OEC/ONF Xenografts Initially Protected by Cyclosporine, Which Was Later Withdrawn*

In 13 rats the cyclosporine-protected mouse OEC/ONF transplants had restored DFR within 2 weeks ($n = 11$) and 3 weeks ($n = 2$). Once DFR had been reestablished, cyclosporine and DFR testing were continued for a further 10 days, at which point cyclosporine administration was terminated and testing continued until 8 weeks after the time of transplantation. In all 13 rats DFR continued for 5–6 weeks after withdrawal of cyclosporine, showing a continuous increase (Fig. 2).

The histology showed that all the spinal cords in this group had complete destruction of CST and partial destruction of the adjacent gray matter as in Groups 3 and 4. As in the rats of Group 1 (Fig. 1C), at 3 weeks after transplantation, none of the xenografted cells had survived. However, double immunostaining of GFAP/NF showed that in every case NF$^+$ axons were seen traversing the lesion center (Fig. 1G–J). Numerically there were 317.14 ± 35.62 NF$^+$ fibers per section in the $n = 7$ spinal cords cut in cross section [this was surprisingly close to the estimate of 320 regenerating axons in our previous rat-to-rat OEC transplants into complete CST lesions (12)]. The GFAP$^+$ scar tissue was less dense than in the

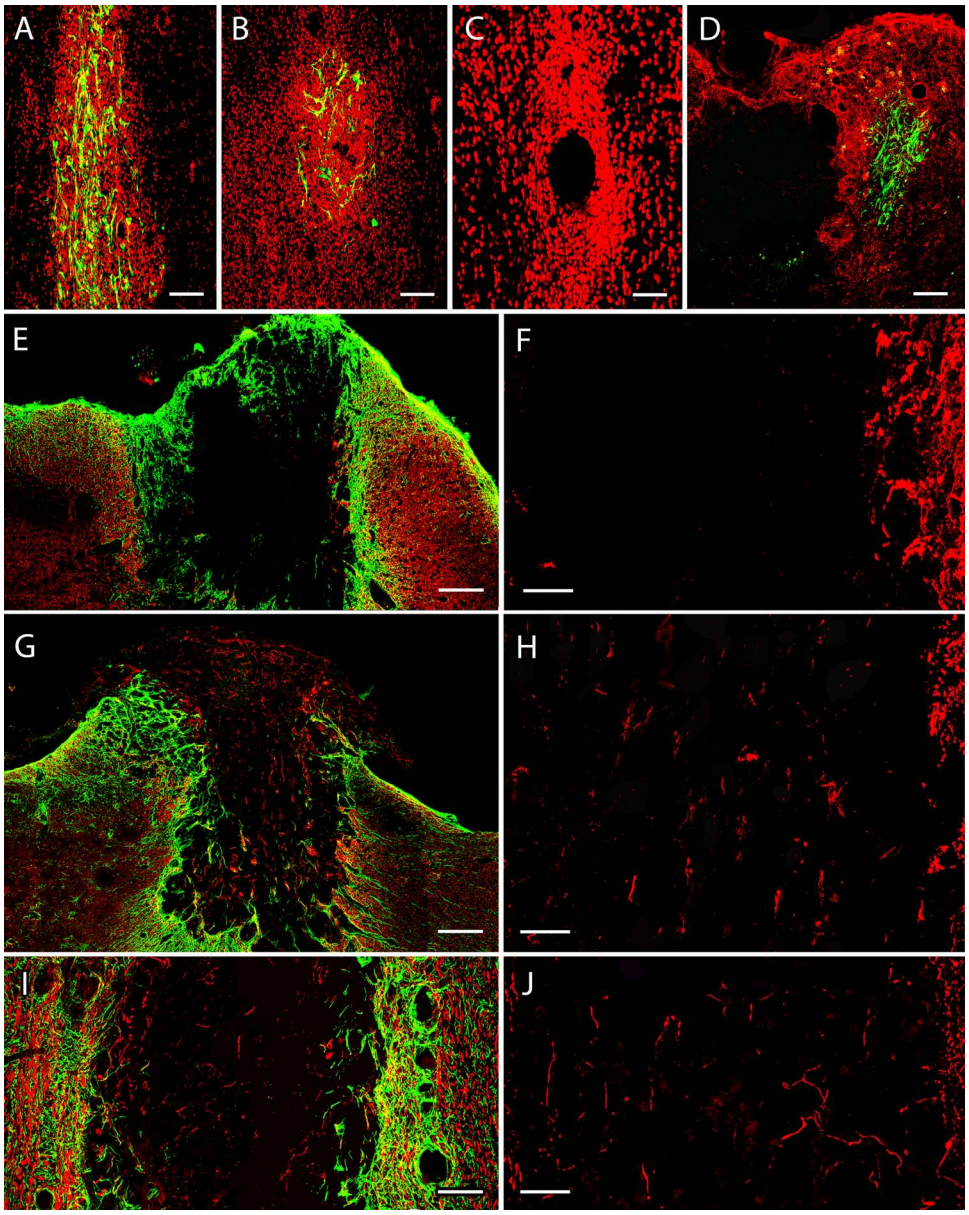

Figure 1. Lesions and transplanted OECs. (A, B, C) Group 1. Mouse OECs transplanted into rat CST, no cyclosporine, survival time, 4 days in (A), 1 week in (B), and 3 weeks in (C). GFP labeling, green; Sytox Orange nuclear counterstaining, red. (D) Group 4. Failed restoration of DFR, the OECs not located in the lesion. GFP labeling, green; GFAP immunostaining, red. Survival time, 13 weeks (8 weeks after CST lesion, 5 weeks after the transplantation) with daily cyclosporine injection. (E, F) Group 3. Long-term complete lesions showing no recovery of DFR. NF/GFAP double immunostaining. NF, red; GFAP, green. Survival time, 10 months. (F) High power. (G–J) Group 5. Restoration of DFR after withdrawal temporary immunoprotection. Complete destruction of the xenografted cells. NF/GFAP double staining. NF, red; GFAP, green. Survival time, 16 weeks (8 weeks after CST lesion, 8 weeks after the transplantation). (H) High power. (A–C, I, J) Horizontal sections; (D–H) cross sections. Scale bars: 100 μm (A, B, D), 50 μm (C, F, H, J), 200 μm (E, G, I).

cases with lesion alone (Group 3), with some astrocytes extending fine processes into the margins of the lesion.

DISCUSSION

Olfactory ensheathing cells accompany the olfactory nerves from their origin in the olfactory mucosa all the way through the cribriform plate to their intracranial termination in the olfactory bulb (5,23). Unfortunately, with our current procedures cultures from the mucosal area yield much smaller proportions of OECs (13) than those from the bulb. In straight comparisons with bulbar-sourced cultures, transplantation of mucosal-sourced

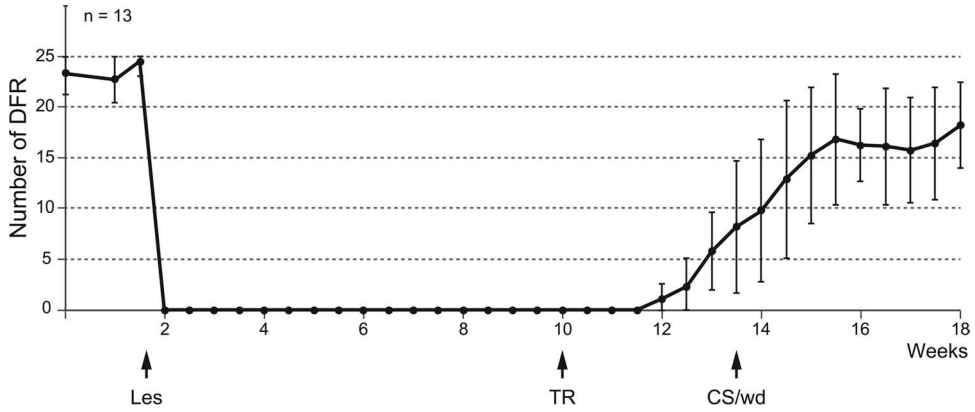

Figure 2. Maintained function after rejection of xenografts. Number of directed forepaw reaches (DFR, ordinate, mean ± SD) in the 13 rats of Group 5, which had complete absence of DFR for 8 weeks after an ipsilateral corticospinal tract lesion (Les) and return of DFR after mouse OEC/ONF xenografts (TR) initially protected by daily cyclosporine. DFR continued to increase in all rats after withdrawal of cyclosporine (CS/wd) at 10 days after the first return of DFR. Time scale, abscissa in weeks.

cultures was ineffective in inducing regeneration of cut axons in rat CST (31), rat dorsal roots (9), and in three cases in a human clinical trial (25), even after expansion by added growth factors (3,6). It is not clear whether this reflects the lower proportions of OECs or an intrinsic difference between the OECs at these two levels (30).

To the best of our knowledge the only spinal injured patient who has received autologous bulbar OECs showed a pattern of functional recovery highly suggestive of fiber regeneration across the injury site (26). This patient had a near complete severance of the midthoracic spinal cord with stump separation of 7–8 mm. In his case 500,000 cells were available from culture of a single olfactory bulb. As this number of cells was insufficient to form a bridge across an injury of this size, the cells were injected into the margins of the stumps, and bridging was provided by grafting peripheral nerve strips.

The mechanics of future clinical cell transplantation would be greatly enhanced if larger numbers of cells were available. Allografts using banks of human bulbar OECs or cell lines would provide an attractive possibility to address this problem and would also avoid the drawbacks of having to source cells from the patient's own olfactory bulb (16).

Protection of immunoincompatible grafts involves all the side effects of requiring the use of immunosuppressive drug regimes. Such permanent immunosuppression is unavoidable in situations where the grafted tissue must survive to carry out an essential function (as heart, kidney, or pancreatic insulin-secreting cells). However, the recovery of function in the rat CST lesion model depends on new fiber connections being formed by the *host* axons. Although this recovery is mediated by the transplanted OECs, it is not clear whether the OECs need to be present beyond the period of providing a bridge for fiber regeneration.

To examine this we here describe a mouse to rat xenograft paradigm. We confirm (12,32) that after total unilateral destruction of the dorsal CST and the adjacent medial gray matter at the C1/2 level there was no return of DFR for up to 12 months [cf., e.g., (2,20)]. The present series also confirms that unless the transplants form a bridge across the lesion area there is no return of function.

In all 13 rats with CST lesions leading to loss of DFR the transplantation of immunoprotected mouse OEC/ONF xenografts mediated recovery of DFR. This confirms a number of previous studies showing that reparative OEC signaling mechanisms are conserved across widely different species (10,11,18,19,29).

In all these rats the restored function was maintained after the grafted cells had been completely rejected by an immune attack following removal of the immunosuppressive regime. Thus, once the OECs have restored function, their continued presence is not required for the function to be maintained.

Despite the intimacy of the association between the transplanted OECs (14) and the regenerating fibers, the rejection mechanism acts with such precision that a functionally effective number of regenerating CST fibers survived an immune attack completely destroying the xenografted mouse OECs. Histological analysis shows that the return of function had been mediated by the 2–300 fibers present in the lesion center after rejection of the xenografted mouse cells. This is comparable to the number of CST fibers, which restored DFR in a previous study (12).

In a similar study (1) researchers showed that rat Schwann cells xenografted into mouse peripheral nerve under immunosuppressive conditions myelinated the host fibers and that the host fibers survived subsequent rejection of the foreign myelinating cells (Aguayo personal communication).

The importance of our present finding is that it opens the possibility of using transient immunoprotection of allografted cells. For the surgical repair of large human spinal cord injuries the ability to use allografted cells or cell lines could provide an approach to getting round the numerical restriction of autologously sourced cells.

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