24. For immunochemistry, cell extracts were prepared as described (*11*). Antibodies used are as follows: PC10 (anti-PCNA) from Santa Cruz; anti-GST, Protein-G$^{\gamma}$ purified rabbit antibodies raised against GST from pGEX2T; mAb D12 raised against GST-(F323–1616); anti-MCMT, GST-(F323–1616) affinity column–purified rabbit antibodies raised against GST-(F122–1616); mAb Cip1 (anti-p21^{WAF1}), from Transduction Lab. For immunoprecipitation, cell extract (2 mg) was incubated with anti-MCMT (13 μg) in IP buffer [equal volume of 50 mM tris (pH 7.5), 5% glycerol, and 0.2% Triton X-100] at 4°C for 1 hour. Protein G$^{\gamma}$–Sepharose (60 μl) beads were added for 2 hours. After washing three times with 500 μl of IP buffer (with 0.1 M NaCl), samples were boiled in Laemmli buffer for immunoblot.
25. For PCNA-binding, CEM cell extract (*11*) or rPCNA were added to binding buffer [100 μl, 50 mM tris (pH 7.5), 0.1% Triton X-100, 28 μM $ZnCl_2$, 10% glycerol, and 0.22 M KCl)] with GSH–Sepharose bead–bound fusion proteins. After shaking at 4°C for 40 min, the beads were recovered and washed three times with 500 μl of binding buffer before boiling in Laemmli buffer for immunoblot with PC10 antibody. In peptide competition assays, high-performance liquid chromatography (HPLC)–purified dodecapeptides (Research Genetics) were reconstituted to 2 mg/ml with argon-treated water and used directly.
26. For transient transfection assay (*22*), the WT GST-(F122–207) construct was obtained from F122–1616 by PCR and cloned into Not I–Kpn I sites of pXJ41neo. This was used for site-directed mutagenesis to create the H170V mutant (confirmed by dideoxy sequencing). After transfection, MRC5SV cells were labeled with BrdU (cell proliferation kit, Amersham) before staining (*7, 22*).
27. Methylase activity assays were performed as described (*9*) but at 25°C with 10 min preincubation at 37°C. Aliquots (100 μl) were analyzed for tritiated poly(dI-dC) at 10-min intervals.
28. For [^{3}H]SAM labeling, cells in a 175-cm^2 (surface area) flask (75% confluent) were treated with O^6-benzylguanine [17 ml of 10 μM in serum-free media (SFM)] for 1 hour followed by addition of NMU (1 ml of 2.7 mM in SFM) or SFM with dimethyl sulfoxide as control (*11*). After 40 min, the media were removed and 11 ml of labeling mixture, containing 1.1 ml of dialyzed fetal bovine serum and 100 μCi of [^{3}H]SAM (75 Ci/mmol, Amersham) in amino acid–free modified Eagle's medium, was added for 6 or 16 hours. Genomic DNAs were isolated as described (*22*) and digested with venom phosphodiesterase (15 U) and shrimp alkaline phosphatase (10 U) in digestion buffer [25 mM tris (pH 8.0) with 2.5 mM Mg^{2+}, 300 μl per flask] for 16 hours at 37°C. The deoxynucleosides were analyzed by HPLC on a C-18 reversed-phase cartridge as described (*9*) and were quantified using standard 2′-deoxynucleosides (Sigma). [^{3}H]5meC was determined by scintillation counting of 0.5-ml fractions collected from each HPLC run and normalized to 1 mmol of total nucleosides detected. Two independent labeling experiments were performed.
29. We thank E. Manser for critical reading of the manuscript; Y. H. Tan and T. J. Lam for stimulating this collaboration; and R. B. Ali, A. Teo, and H. K. Oh for excellent assistance. Supported by the National Science and Technology Board of Singapore. This paper is dedicated to P. F. Swann (University College, London) for his constant encouragement to B.F.L.L.

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Repair of Adult Rat Corticospinal Tract by Transplants of Olfactory Ensheathing Cells

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The upper cervical corticospinal tract was transected on one side in adult rats. A suspension of ensheathing cells cultured from adult rat olfactory bulb was injected into the lesion site. This induced unbranched, elongative growth of the cut corticospinal axons. The axons grew through the transplant and continued to regenerate into the denervated caudal host tract. Rats with complete transections and no transplanted cells did not use the forepaw on the lesioned side for directed reaching. Rats in which the transplanted cells had formed a continuous bridge across the lesion exhibited directed forepaw reaching on the lesioned side.*

The failure of damaged nerve fibers to regenerate is the underlying cause of the permanent disabilities experienced by patients after spinal injury. Transplants of peripheral nerves (*1*) or Schwann cells cultured from peripheral nerves (*2*) have been used to induce reparative growth of axons in the adult rat central nervous system (CNS). The corticospinal tract (CST) is one of the main motor pathways of the spinal cord. After transection, the cut ends of the CST axons branch extensively but do not advance through the damaged area (*3, 4*). Transplanted Schwann cells enhance the growth of cut CST axons, but there is little indication that the CST axons reenter host pathways (*3, 5*).

Neurosensory cells of the adult olfactory system are continuously replaced (*6*), and their newly formed axons grow into the olfactory bulb to form functional connections. The pathway from the olfactory epithelium is made up of specialized ensheathing cells that share both Schwann cell and astrocytic characteristics (*7–9*). Unlike Schwann cells, which are confined to the peripheral nervous system, the olfactory ensheathing cells (OECs) accompany the olfactory axons into the CNS (*10*). We therefore examined whether injection of OECs cultured from adult olfactory bulb can improve the ability of regenerating CST axons to grow through a spinal cord lesion and reenter the host pathways.

OECs were obtained from the olfactory nerve and glomerular layers of syngeneic adult olfactory bulbs by the method of (*11*), but the final purification was omitted. Before transplantation the OECs were maintained in culture for 14 to 17 days. The cultures contained a variety of cell phenotypes (*8*). The CST was destroyed unilaterally by focal electrolytic lesions in the medioventral part of the dorsal columns between the first and second cervical segments (*3, 12*) (Fig. 1). At 6 days after transplantation of OECs into the lesion site, biotin dextran (BD) anterograde labeling of the CST showed that the thickened shafts of the cut axons were prolonged into single, fine, unbranched, regenerating processes with a few small varicosities close to a single, tapering, advancing tip. Many of the sprouts had already crossed the transplant and their tips could be seen in the caudal part of the host CST. From 10 days onward, the regenerating CST axons accumulated in parallel bundles of straight, unbranched axons that traversed the full rostrocaudal extent of the transplants (Fig. 2).

From the earliest time point studied (6 days after transplantation), OECs expressing p75 low-affinity neurotrophin receptor (*8*) had infiltrated the lesion area and extended beyond it for at least 2 to 3 mm caudally. Electron microscopy of the transplants showed that the lesion area contained fascicles of elongated pale, astrocyte-like cells (Fig. 3A) and dark, Schwann-like cells aligned with the longitudinal axis of the tract. Over 2 to 3 weeks, the Schwann-like OECs established a one-to-one, peripheral-type myelinating relationship with individual CST axons, and the astrocyte-like OECs formed outer sheaths around groups of myelinated axons (Fig. 3B). After reentry into the caudal part of the host CST, the axons became myelinated by oligodendrocytes (Fig. 3C).

To explore whether these regenerating axons were capable of mediating useful function, we set up a pilot experiment to look for the presence or absence of a directed forepaw reaching function (DFR), in which the rats reach through an aperture to obtain a food reward (*13*). The rats were tested for one session a day on 10 successive days. During each session food pellets were presented until the rat had made 50 reaches through the aperture ("free" tests). For each

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*This paper is dedicated to Diana, Princess of Wales, who inaugurated the Norman and Sadie Lee Research Centre in 1988. She gave years of unstinting support to people in wheelchairs. Her untimely death prevented her seeing this result, with which she would have been so pleased.

reach, we recorded whether the right or left paw was used. A control group of five unoperated rats used both forepaws, three with varying degrees of preference for the right paw, and two for the left. Next, we restrained the preferred paw for the period of each food presentation by wrapping the forearm with a light sleeve (*13*), which prevented the rats from reaching through the aperture, and observed DFR daily for five successive days ("restrained" tests). All five normal rats readily made 50 reaches per day with the nonrestrained paw. These were scored as DFR present.

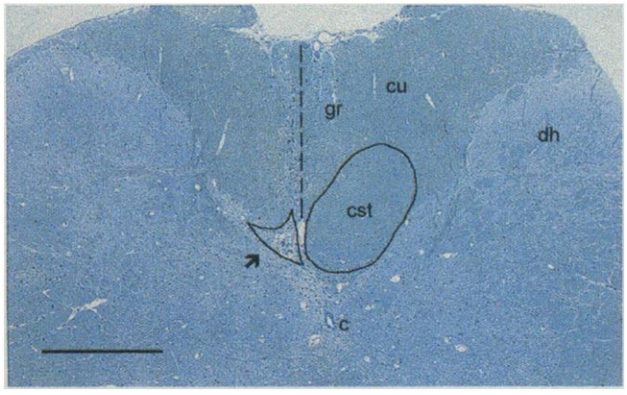

Fig. 1. Destruction of the left corticospinal tract (arrow). The coronal semithin section was stained with methylene blue and azur II. c, central canal; cst, intact right corticospinal tract (outlined); cu and gr, cuneate and gracile tracts; dh, dorsal horn of the spinal gray matter; and midline (dashed line). Survival time, 3 months. Scale bar, 0.5 mm.

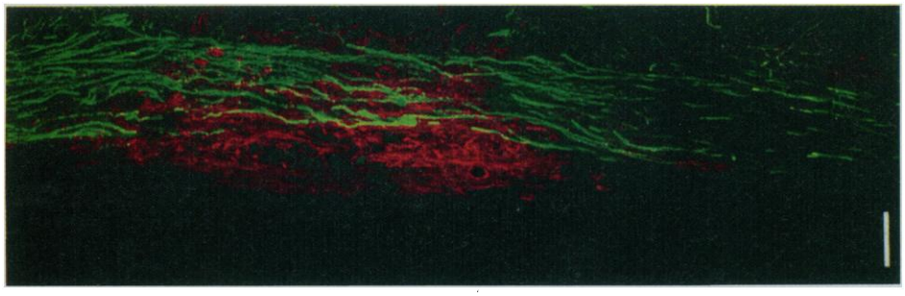

Fig. 2. A 100-μm-thick section showing the mass of transplanted OECs (red fluorescence, p75 low-affinity neurotrophin receptor) elongated along the rostrocaudal (left to right) axis of the host CST and traversed by a fascicle of parallel, unbranched, BD-labeled regenerating CST axons (green fluorescence), which have occasional varicosities and continue uninterrupted into the distal host CST caudal to the transplant. This confocal image shows all axons throughout the thickness of this section, which is the third of a series of six adjacent longitudinal sections through the transplant. The axons are traveling through the middle of the mass of transplanted cells. Survival, 4 weeks. Scale bar, 200 μm.

We then tested a group of 21 rats at 2 to 3 months after unilateral lesions of the CST. Of these, 14 resembled the normal control group, all showing a varying degree of paw preference in "free" tests, and all switching to the nonpreferred paw in "restrained" tests (that is, DFR was present). After testing, the extent of the lesion was determined from a complete series of coronal semithin sections (*14*). In 13 out of the 14 rats, the lesion had spared 20 to 50% of the cross-sectional area of the CST. In the 14th rat, only between 1 and 2% of the CST was spared (axon counts from an electron micrographic montage across this area showed that this rat had 698 out of a normal total of ~50,000 CST axons).

The remaining seven lesioned rats used only one paw throughout the 10 days of "free" testing. When this paw was restrained, they never reached through the aperture with the other paw. We continued to observe them until they had made 50 daily failed attempts to reach through the aperture with the restrained paw over a period of 5 days. These were scored as DFR absent. In all seven rats, subsequent serial coronal semithin sections showed that the CST had been completely destroyed on the side of the nonreaching paw. Thus, these

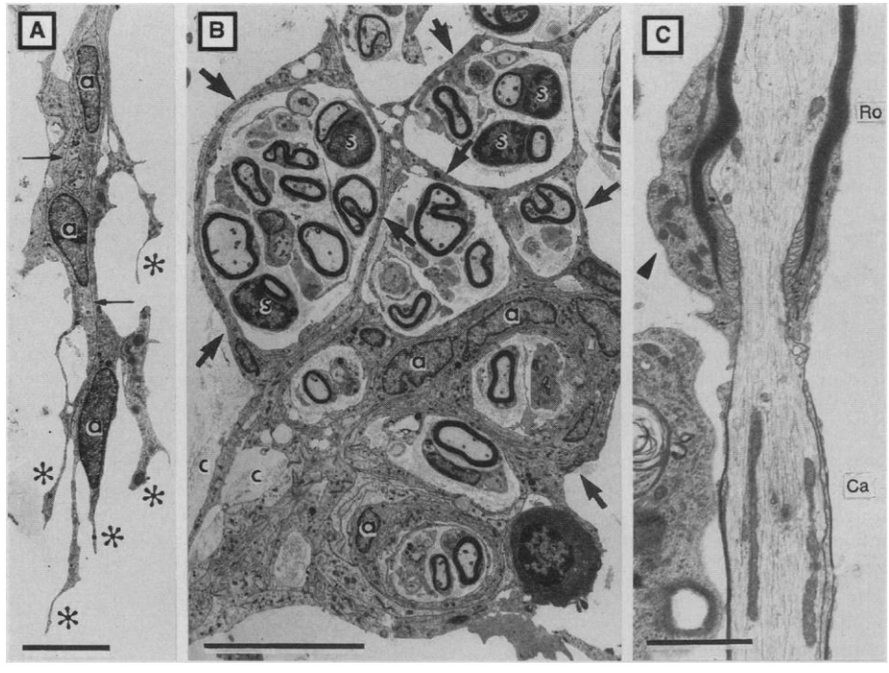

Fig. 3. (**A**) Electron micrograph of a column of pale, astrocyte-like OECs (a) aligned along the longitudinal axis of the transplant. The cells make plasma membrane contact with each other (arrows), and cytoplasmic filopodia (asterisks) extend caudally. (**B**) A cross section through a column of axons taken from the center of the lesion and myelinated by thick, peripheral-type myelin formed on a one-to-one basis by Schwann-like OECs (s). Bundles of 3 to 10 axons and their myelinating cells are wrapped by an outer sheath (arrows) consisting of several apposed layers of astrocyte-like OECs (a). c, collagen. (**C**) A "transitional" node at the point where the axon leaves the transplant caudally and reenters the host CST. The rostral internode (Ro, in the transplant) is myelinated by a Schwann-like OEC (arrowhead), which forms thick, peripheral-type myelin, and is invested by a basal lamina (not seen at this magnification). The caudal internode (Ca, in the host distal CST) is myelinated by thinner, central-type oligodendrocytic myelin and is not associated with basal lamina. Survival, 10 days (A), 4 weeks (B), and (C). Scale bar, (A) 5 μm, (B) 10 μm, and (C) 2 μm. Higher magnifications of the myelin sheaths (not shown) confirm that the periodicity of the peripheral myelin is about 10% greater than that of the central myelin.

preliminary results indicate that, under these test conditions, the presence of DFR depends on the presence of intact fibers in the CST ($P < 0.0001$; Fisher's exact probability test) and that as few as 1% of the CST fibers may be sufficient for DFR.

To investigate the functional potential of the regenerating CST axons induced by OEC transplants, we examined DFR in seven rats at 2 to 3 months after OECs had been transplanted into the lesion sites immediately after lesioning. In "free" tests, four of these seven rats used both paws. When the preferred paw was restrained, each of these four rats switched to using the unrestrained paw for the 50 daily reaches over 5 days (that is, scored as DFR present). This behavior was indistinguishable from the normal, unoperated control animals. Subsequent histology showed that although all four rats had complete unilateral destruction of the CST, the transplants had formed a continuous column of OECs with their associated peripherally myelinated axons, which bridged the entire rostrocaudal length of the lesion in the host CST.

The remaining three rats with OEC transplants used only one paw in the "free" tests, and when that paw was restrained, they never reached through the aperture with the other paw over a period of 5 days, during which time we observed 50 failed reaches per day by the restrained paw (that is, scored as DFR absent). Subsequent histology showed that these three rats also had complete unilateral destruction of the CST on the side of the nonreaching paw. However, in two of these three rats there were no OECs or peripherally myelinated axons in the lesioned CST, and in the third rat OECs and axons were present but only in the caudal part of the CST lesion and did not bridge the full rostrocaudal extent of the defect in the CST. Thus, in this pilot group of seven animals with complete CST lesions, the presence of DFR was correlated ($P = 0.029$; Fisher's exact probability test) with the presence of a continuous bridge of OECs and axons across the lesion.

The suggestions arising from these preliminary behavioral findings will need numerical confirmation and an investigation of the wider behavioral consequences of CST lesions. However, the suggestion that transplanted cultured OECs are able to induce functionally useful regeneration of adult axons in the experimental situation provided by these acute and highly defined rat spinal tract lesions opens up the prospect that in the future they may also have a role in treatment of the more complex lesions caused by human spinal cord injury. The fact that OECs can be obtained from adult donors raises the possibility of autotransplantation for clinical situations.

REFERENCES AND NOTES

1. M. Vidal-Sanz *et al.*, *J. Neurosci.* **7**, 2894 (1987).
2. T. J. Neuberger, C. J. Cornbrooks, L. F. Kromer, *J. Comp. Neurol.* **315**, 16 (1992); C. N. Montero-Menei *et al.*, *Brain Res.* **570**, 198 (1992); X. M. Xu, A. Chen, V. Guénard, N. Kleitman, M. B. Bunge, *J. Neurocytol.* **26**, 1 (1997).
3. Y. Li and G. Raisman, *J. Neurosci.* **14**. 4050 (1994).
4. ———, *Exp. Neurol.* **134**, 102 (1995).
5. K. R. Kuhlengel, M. B. Bunge, R. P. Bunge, H. Burton, *J. Comp. Neurol.* **293**, 74 (1990).
6. D. G. Moulton, *Ann. N.Y. Acad. Sci.* **237**, 52 (1974); P. C. Barber and G. Raisman, *Brain Res.* **141**, 57 (1978); K. C. P. Wilson and G. Raisman, *ibid.* **205**, 245 (1981); P. P. C. Graziadei and G. A. Montigraziadei, *J. Neurocytol.* **8**, 1 (1979).
7. P. C. Barber and R. M. Lindsay, *Neuroscience* **7**, 3077 (1982); R. Devon and R. Doucette, *Brain Res.* **589**, 175 (1992).
8. A. Ramón-Cueto and F. Valverde, *Glia* **14**, 163 (1995).
9. I. A. Franceschini and S. C. Barnett, *Dev. Biol.* **173**, 327 (1996).
10. G. Raisman, *Neuroscience* **14**, 237 (1985).
11. A. Ramón-Cueto and M. Nieto-Sampedro, *ibid.* **47**, 213 (1992).
12. Unilateral lesions of the CST were produced (on the side that offered access with minimal damage to pial vessels) by a current of 10 μA for 8 to 10 min passed through a stainless steel electrode inserted to a vertical depth of 1 mm, at 1 mm caudal to the obex, and 0.3 mm from the midline in 71 young adult female AS rats (6 to 8 months old, body weights 200 to 220 g). Immediately after withdrawing the lesioning electrode, 50 rats received 5 μl of a suspension containing about 125,000 cultured OECs injected into the lesion site over a period of 1 to 2 min through a glass micropipette (*3*). In 30 rats 10% BD was injected stereotaxically into the contralateral medullary pyramid (*4*) 6 days before they were killed to allow time for anterograde labeling of the CST axons. At survival times of 6 days ($n = 6$), 10 days ($n = 9$), 3 weeks ($n = 4$), 4 weeks ($n = 7$), and 9 weeks ($n = 4$) after the lesioning and transplantation, the rats were killed and perfusion fixed (*4*). Longitudinal Vibratome sections 100 μm thick were used to identify the OECs by immunofluorescence for p75 low-affinity neurotrophin receptor and the CST axons by immunofluorescence for BD, and the results were recorded by confocal microscopy of serial longitudinal sections through the operated area. The remaining 20 rats [after survivals of 10 days ($n = 3$), 20 days ($n = 3$), 4 weeks ($n = 7$), and 2 to 3 months ($n = 7$)] were killed and perfusion fixed for electron microscopy (*4*).
13. I. Q. Whishaw, R. B. O'Connor, S. B. Dunnett, *Brain* **109**, 805 (1986).
14. Perfusion-fixed spinal cords of all 28 tested rats were divided coronally, from the obex caudally, into 20 consecutive 0.7-mm-thick blocks; three blocks corresponded to one cervical segment (as ascertained from surface observation of the spinal nerve roots). The lesions damaged an area of tissue about 0.5 mm in cross section and 1 to 2 mm in a rostrocaudal direction. In semithin sections, the descending CST axons were distinguished from the larger ascending sensory fibers of the dorsal columns by their position as a well-delineated, compact bundle in the mediodorsal edge of the dorsal columns, by their smaller fiber diameter (about 1 μm), and by the denser packing of the interfascicular glial cells. The extent of the lesions, the position of the transplants, and the presence of transplanted OECs and OEC-myelinated axons were recorded in camera lucida drawings of 1-μm-thick coronal sections. The area of any remaining part of the CST was measured.

15. We thank D. Li for consultation and collaboration. Y. Ajayi adapted the method for culturing the OECs. Supported by the Medical Research Council, the British Neurological Research Trust, the International Spinal Research Trust, the Barnwood House Trust, and Smith's Charities.

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Requirement for Macrophage Elastase for Cigarette Smoke–Induced Emphysema in Mice

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To determine which proteinases are responsible for the lung destruction characteristic of pulmonary emphysema, macrophage elastase–deficient ($MME^{-/-}$) mice were subjected to cigarette smoke. In contrast to wild-type mice, $MME^{-/-}$ mice did not have increased numbers of macrophages in their lungs and did not develop emphysema in response to long-term exposure to cigarette smoke. Smoke-exposed $MME^{-/-}$ mice that received monthly intratracheal instillations of monocyte chemoattractant protein–1 showed accumulation of alveolar macrophages but did not develop air space enlargement. Thus, macrophage elastase is probably sufficient for the development of emphysema that results from chronic inhalation of cigarette smoke.

Pulmonary emphysema is a major component of the morbidity and mortality of chronic obstructive pulmonary disease (COPD), a condition that afflicts more than 14 million persons in the United States and has become the country's fourth leading cause of death (*1*). Cigarette smoking is the main risk factor for the development of COPD. With the rapid rise in cigarette smoking occurring now in many countries, COPD may become epidemic worldwide in years to come.

Emphysema is defined as the enlargement of peripheral air spaces of the lung, including respiratory bronchioles, alveolar ducts, and alveoli, accompanied by destruction of the walls of these structures (*2*, *3*). Before the development of emphysema, smokers' lungs show an accumulation of macrophages, lym-

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