Inflammation stimulates remyelination in areas of chronic demyelination

A. K. Foote and W. F. Blakemore

Department of Veterinary Medicine, University of Cambridge, UK

Correspondence to: W. F. Blakemore, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK
E-mail: wfb1000@cam.ac.uk

Summary

A major challenge in multiple sclerosis research is to understand the cause or causes of remyelination failure and to devise ways of ameliorating its consequences. This requires appropriate experimental models. Although there are many models of acute demyelination, at present there are few suitable models of chronic demyelination. The talep rat is a myelin mutant that shows progressive myelin loss and, by 1 year of age, its CNS tissue has many features of chronic areas of demyelination in multiple sclerosis: chronically demyelinated axons present in an astrocytic environment in the absence of acute inflammation. Using the talep rat and a combination of X-irradiation and cell transplantation, it has been possible to address a number of questions concerning remyelination failure in chronic multiple sclerosis lesions, such as whether chronically demyelinated axons have undergone changes that render them refractory to remyelination and why remyelination is absent when oligodendrocyte progenitor cells (OPCs) are present. Our experiments show that (i) transplanted OPCs will not populate OPC-containing areas of chronic demyelination; (ii) myelination competent OPCs can repopulate OPC-depleted chronically demyelinated astrocytosed tissue, but this repopulation does not result in remyelination—closely resembling the situation found in some multiple sclerosis plaques; and (iii) the induction of acute inflammation in this non-remyelinating situation results in remyelination. Thus, we can conclude that axonal changes induced by chronic demyelination are unlikely to contribute to remyelination failure in multiple sclerosis. Rather, remyelination fails either because OPCs fail to repopulate areas of demyelination or because if OPCs are present they are unable to generate remyelinating oligodendrocytes owing to the presence of inhibitory factors and/or a lack of the stimuli required to activate these cells to generate remyelinating oligodendrocytes. This non-remyelinating situation can be transformed to a remyelinating one by the induction of acute inflammation.

Keywords: multiple sclerosis; oligodendrocyte progenitor cell; remyelination; talep rat; transplantation

Abbreviations: FBS = fetal bovine serum; OPC = oligodendrocyte progenitor cell; PDL = poly-D-lysine

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Introduction

Remyelination failure is a feature of multiple sclerosis that probably contributes to the relentless progression of this disease. A major challenge in multiple sclerosis research is to understand the cause, or causes, of remyelination failure and devise ways of ameliorating its consequences. Amelioration could be achieved by the development of remyelination enhancing therapies. These might take the form of stimulating the brain’s inherent remyelinating potential or transplanting cells that have remyelinating potential. Evaluation of both approaches requires appropriate animal models and an understanding of why remyelination fails in multiple sclerosis.

It has been demonstrated that transplantation of myelin forming cells can result in extensive myelin sheath formation when undertaken during development or in association with acute demyelination (reviewed, Duncan et al., 1997; Blakemore and Franklin, 2000; Baron-Van Evercooren and Blakemore, 2004). Such environments can be regarded as physiological recipient environments since either myelination or remyelination is occurring, and thus the introduced cells are exposed to an environment that is conducive to myelin formation. Deciding how to approach a pathological environment such as pertains in areas of chronic demyelination, where oligodendrocyte progenitor cells (OPCs) with the potential
for remyelination may be present but fail to achieve remyelination (Wolswijk, 1998; Chang et al., 2000, 2002), has been hampered by a lack of suitable models. To be suitable, the experimental model should resemble chronic multiple sclerosis lesions in the following respects: (i) it should have axons that have remained without myelin sheaths for some considerable time; (ii) it should contain OPCs and an absence of remyelination; (iii) the demyelinated axons should be present in an area that contains astrocytes; and (iv) there should be an absence of acute inflammation. Results from experiments using such a model would have relevance for understanding how to enhance endogenous remyelination in areas of chronic demyelination, as well as potential application to cell therapy. Our study shows that such a model can be achieved using the *taiep* rat. The *taiep* rat has an autosomal recessive genetic defect which leads to an accumulation of microtubules in oligodendrocytes and altered intracellular transport (Song et al., 2003). This results in hypomyelination and progressive demyelination such that, by 1 year of age, all small diameter and many medium diameter CNS axons are without myelin sheaths (Duncan et al., 1992; Lunn et al., 1997; Foote and Blakemore, 2005). The loss of myelin sheaths is accompanied by an astrocytosis (Duncan et al., 1992; Foote and Blakemore, 2005) and an ongoing acute inflammatory response is absent. Therefore, the tissue has the features of chronic areas of demyelination in multiple sclerosis, with chronically demyelinated axons present in a non-inflammatory astrocytic environment.

By using the *taiep* rat in combination with X-irradiation to deplete the endogenous OPC population and transplantation of remyelination competent OPCs, it has been possible to examine a number of suggested reasons for remyelination failure in multiple sclerosis and to predict the likely outcome of transplanting OPCs into areas of chronic demyelination. In particular, our studies show that (i) transplanted OPCs will not populate OPC-containing areas of chronic demyelination; (ii) OPCs can repopulate OPC-depleted chronically demyelinated astrocytosed tissue, but this repopulation by myelination competent cells does not result in remyelination, closely resembling the situation found in some multiple sclerosis plaques; and (iii) the induction of acute inflammation in this non-remyelinating situation stimulates remyelination. Thus, axonal changes induced by chronic demyelination are unlikely to contribute to remyelination failure in multiple sclerosis. Rather, remyelination fails either because OPCs fail to repopulate areas of demyelination or because if they are present they are dysfunctional because of the presence of inhibitory factors and/or a lack of the stimuli required to activate these cells to generate remyelinating oligodendrocytes. It is therefore significant that a non-remyelinating situation can be transformed to a remyelinating one by the induction of acute inflammation.

**Methods**

**Animals**

*Taiep* rats were obtained from Dr M. Roncagliolo (University of Valparaiso, Chile) and bred by mating either affected to affected animals or affected to non-affected heterozygotes. Control animals used for transplantation were heterozygous littermates of the *taiep* rats. Sprague–Dawley rats carrying the autosomal dominant GFP gene on an actin promoter were obtained from Professor M. Okabe (Osaka University, Japan) and heterozygous males were mated to normal Sprague–Dawley females. All animal experiments were performed under a Home Office licence and with ethical approval from the University of Cambridge.

**Progression of pathology**

To study the progression of pathology in the *taiep* rats, animals were perfused with 4% glutaraldehyde at 3, 7, 10, 14 and 18 months of age (at least two animals for each time point) and tissue from the spinal cord was processed into resin. Sections 1 μm thick were cut and examined using light microscopy following toluidine blue staining. For electron microscopy, 90 nm sections were cut, stained with uranyl acetate and lead citrate and examined using a Hitachi Model H600 transmission electron microscope.

**Transplantation**

Using halothane anaesthesia and aseptic surgical techniques, a laminectomy was performed at the level of T13 to expose the dorsal spinal cord for cell transplantation. With the aid of a three-way micromanipulator, the tip of a glass micropipette attached to a 10 μl Hamilton syringe was inserted into the dorsal funiculus and 1 μl of cell suspension (106 cells) was injected. Some animals (as indicated in Table 1) were immunosuppressed with cyclosporin A (Sandoz, 15 mg/kg s/c daily) from 1 day before transplantation. All animals used as transplant recipients were between 11 and 14 months of age.

**Cell preparations**

Unlabelled OPC enriched preparation

Mixed glial cell cultures were prepared from the cerebral hemispheres of 2–3 day old Sprague–Dawley rat pups. Briefly, cerebral hemispheres were removed, stripped of their meninges and mechanically dissociated in MEM containing 20 mM HEPES (Invitrogen). Cells were harvested by filtration through a 20 μm nylon mesh followed by centrifugation (500 × g for 5 min) and plated on

<table>
<thead>
<tr>
<th>Table 1 Number of animals, nature of transplanted cells and methods of tissue processing</th>
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<tbody>
<tr>
<td><strong>Number of animals</strong></td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td><em>taiep</em> 1</td>
</tr>
<tr>
<td><em>taiep</em> 5</td>
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<tr>
<td>X-<em>taiep</em> 3</td>
</tr>
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<td>Normal 3</td>
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<tr>
<td><em>taiep</em> 3</td>
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<tr>
<td>X-normal 3</td>
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<tr>
<td>X-<em>taiep</em> 4</td>
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<tr>
<td>X-<em>taiep</em> 5</td>
</tr>
<tr>
<td>X-<em>taiep</em> + saline/charcoal 5</td>
</tr>
</tbody>
</table>

X = 40 Gy X-irradiation of the spinal cord before transplantation.
poly-D-lysine (PDL) coated 80 cm$^2$ tissue culture flasks containing DMEM (Invitrogen) supplemented with glucose (6 mg/ml) and 10% heat inactivated feline bovine serum (FBS; Invitrogen). Each flask contained cells from two animals. Every 3–4 days 60% of the culture medium was replaced. After 10 days in vitro, cultures were shaken on an orbital shaker (200 r.p.m. for 2 h) to remove loosely adhered microglia; the medium was replaced and then the cultures were shaken again (375 r.p.m. overnight) to harvest OPCs. The detached cells were collected, the more adherent contaminating cells were allowed to adhere to non-tissue culture coated petri dishes for 30 min at 37°C and the less adherent OPCs were collected, centrifuged and resuspended in MEM–HEPES at a concentration of $10^5$ cells per microlitre for transplantation. The mixed glial cultures were maintained in culture for a further 7 days before shaking for a third time to harvest, in the same way as before, additional OPCs for transplantation.

**GFP labelled OPC enriched preparation**

OPC enriched preparations were prepared as described for unlabelled cells using 2–3 day old rat pups heterozygous for GFP, as indicated by green fluorescence under UV light.

**LacZ labelled OPCs**

OPC enriched cell preparations were prepared as described for unlabelled cells, using 2–3 day old rat pups. Before transplantation, the OPC enriched preparation was plated onto PDL coated 80 cm$^2$ tissue culture flasks containing 70% DMEM/F12 (with l-glutamine; Invitrogen) and 30% medium conditioned by the B104 neuronal cell line, prepared using methods described by Fok-Seang (1995). The medium was supplemented with N2 (10 μM; Invitrogen), BSA (0.66 mg/ml; Sigma), glucose (2.25 mg/ml) and the growth factors FGF-2 (20 ng/ml; Sigma) and PDGF-AA (10 ng/ml; Sigma). Fresh growth factors were added every 24 h, and 60% of the medium was replaced with fresh medium every 48 h.

Replication deficient Maloney murine leukaemia virus, lacZ (with nuclear localization sequence), was collected from an MFG pack-

**Experiments and tissue processing**

Four transplantation experiments were performed (summarized in Table 1). (i) Ten adult taeip rats were transplanted with unlabelled cells. After 4 weeks these animals were perfused with 4% glutaraldehyde and the tissue examined in resin sections for evidence of remyelination. (ii) Five taeip and three irradiated taeip rat spinal cords were transplanted with lacZ labelled cells and perfused after 4 weeks with 3% paraformaldehyde/1% gluteraldehyde. The cord was cut into 1.5 mm transverse blocks and the tissue was reacted with X-gal. The blocks were processed for light and electron microscopy to examine remyelination and the distribution of transplanted cells. (iii) GFP labelled cells were transplanted into control spinal cord (n = 3), taeip spinal cord (n = 3), irradiated control spinal cord (n = 3) and irradiated taeip spinal cord (n = 4). The tissue from these animals was analysed in longitudinal cryostat sections after 4 weeks to examine the distribution and immunophenotype of transplanted cells. (iv) Ten irradiated taeip spinal cords were transplanted with GFP cells, and five of them received two unilateral injections of saline and sterile charcoal into the lateral funiculus 3 weeks after implantation of cells. These animals were perfused with 4% paraformaldehyde 6 weeks after transplantation. The spinal cord of each animal was cut into 1.5 mm transverse blocks and alternate blocks were processed for either resin (postfixed in 4% glutaraldehyde followed by 2% osmium tetroxide and then resin embedded) or cryostat sectioning. The animals in experiments 1 and 4 were not immunosuppressed, whereas animals in experiments 2 and 3 were given immunosuppressant doses of cyclosporin.

In addition, in order to examine the effect of irradiation on the endogenous OPC population, the spinal cords of 4 adult taeip rats were irradiated over a 7 mm length. Animals were perfused 4 weeks after irradiation and in situ hybridization for PDGFRα was performed on cryostat sections. A saline/charcoal injection was also made into the dorsolateral funiculus of three irradiated taeip spinal cords (which had not received transplanted OPCs) and the effect of this injection on the tissue was examined in cryostat and resin sections 3 days post injection.

As well as transplantation into taeip spinal cord, all cell preparations were injected into X-irradiated ethidium bromide lesions in normal adult rat spinal cord 3 days after lesion induction, and the animals were killed 1 month later to evaluate the extent of remyelination (Blakemore and Crang, 1992).

**Immunostaining and ISH**

Samples of the cell preparation used for transplantation were plated onto CC2-coated chamber slides (Labtec) for 12 h in DMEM/F12 containing 1% FBS, then fixed with 4% paraformaldehyde and characterized by immunocytochemistry using antibodies to A2B5 (mouse IgM, Roche, 1:200), O4 (mouse IgM hybridoma supernatant, a gift from Dr Mark Noble), GalC (mouse IgG, Chemicon 1:100), GFAP (polyclonal rabbit IgG, Dako, 1:150) and CD11b (OX42, mouse IgG, Serotec, 1:200). Cell nuclei were labelled with Hoescht (Calbiochem, 1:500).

For immunohistochemistry, cryostat sections were stained using antibodies to NG2 proteoglycan (rabbit IgG, Chemicon, 1:150), GFAP (rabbit IgG, Dako, 1:150), APC (CC2, mouse IgG, Oncogene, 1:100), CD45 (LCA, mouse IgG, Chemicon, 1:100), rat B cells (mouse IgG, Serotec, 1:100), CD4 (mouse IgG, Serotec, 1:100), CD8 (mouse IgG) and GFP (rabbit IgG or mouse IgG, Molecular Probes, 1:300). Appropriate FITC, TRITC or Cy3 conjugated secondary antibodies were used (Jackson Laboratories). PDGFRα...
in situ hybridization was performed on cryostat sections using a DIG-labelled riboprobe (cDNA, a gift from Professor W. D. Richardson).

Analysis
The repopulation of non-irradiated and irradiated tissue by transplanted cells after 4 weeks was observed in transverse resin sections for lacZ labelled cells and in parasagittal sections for GFP labelled cells. The total length of repopulation was measured from the latter, as was the length of the area of increased GFP cell density containing APC+GFP+ cells (APC being a marker of mature oligodendrocytes). The lengths were measured from a minimum of three sections for each animal, using sections that passed through the centre of the transplant site.

In resin sections, transplant mediated remyelination was assessed by the detection of areas in which all axons were myelinated. For lacZ labelled cells, remyelination was confirmed as being transplant derived by its association with labelled cells in closely adjacent sections. For GFP labelled cells, regions of high cell density containing GFP+APC+cells could be detected in the cryostat blocks either side of resin blocks showing complete remyelination. For quantification, the cross-sectional area of white matter showing evidence of complete remyelination in the resin sections was outlined and the area measured using a live capture digital imaging system (MCID). By maintaining the craniocaudal order of the resin blocks in relation to the transplant site, the area of remyelination was compared at different levels of the cord and a volume of remyelination estimated for each animal.

Results
Examination of the spinal cord of taiep rats of different ages confirmed that there was progressive loss of myelin with age, as previously observed (Lunn et al., 1997; Foote and Blakemore, 2005). By 10 months of age, all small diameter axons and most medium sized axons lacked myelin sheaths, while large diameter axons were surrounded by thinner than normal myelin sheaths. In particular, almost all the axons in the corticospinal tract, gracile tract and the tracts in the dorsolateral funiculi were without detectable myelin sheaths in toluidine blue stained resin sections and at the ultrastructural level (Fig. 1).

Only small focal areas of remyelination are found when OPCs are injected into areas of chronic demyelination
To determine whether axons that had been chronically demyelinated were receptive to remyelination we transplanted oligodendrocyte precursors into the dorsal funiculus of the taiep rat.

![Fig. 1](http://brain.cotonjournals.org) Progressive demyelination and astrocytosis in the taiep rat. (A and B) The appearance of the lateral columns of the spinal cord at 3 months (A) and 10 months (B) shows that the taiep spinal cord undergoes progressive myelin loss (toluidine blue stained resin sections). Small diameter fibres are most affected; larger diameter fibres remain thinly myelinated. (C and D) At the ultrastructural level, demyelination of small diameter fibres is evident in the corticospinal tract by 3 months of age (C) and demyelination has progressed to the majority of fibres in the corticospinal tract by 11 months (D). Note also the numerous astrocyte processes (a) and microtubule-laden oligodendrocyte processes (arrows), a feature of the defective oligodendrocytes in the taiep rat. Scale bar: A and B = 8 μm; C and D = 0.8 μm.
thoracolumbar spinal cord in adult (11–14 month old) taiep rats. Experiments were undertaken using cells from normal animals, cells transfected with the lacZ gene and cells derived from GFP expressing animals. The cell preparations used contained a similar proportion of OPCs (Table 2) and each showed the expected remyelinating capacity when injected into X-irradiated ethidium bromide lesions (data not shown). Four weeks after transplantation into the taiep rat spinal cord only small areas of remyelination could be detected in resin-embedded tissue (Fig. 2A–D). Using cells prepared from GFP expressing rats or lacZ labelled cells it was apparent that all the transplanted cells were restricted to a small area near to the point of cell implantation (Figs. 2E and 3E). The length of this region was consistent with the passive spread of cells that results from the injection of the cell suspension (Franklin et al., 1996; O’Leary and Blakemore, 1997). Therefore, repopulation of the chronically demyelinated tissue by the transplanted cells had not occurred, indicating that some property of the host tissue was inhibitory to widespread integration of the transplanted cells. However, although very restricted in its distribution, the presence of remyelination indicates that chronically demyelinated axons of the taiep rat can be remyelinated by myelination competent cells.

Table 2 Percentage of OPCs in the three cell preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>A2B5</th>
<th>O4</th>
<th>GalC</th>
<th>GFAP</th>
<th>OX42</th>
</tr>
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<tr>
<td>Unlabelled</td>
<td>45 ± 3</td>
<td>43 ± 3</td>
<td>5 ± 1</td>
<td>4 ± 2</td>
<td>n.a.</td>
</tr>
<tr>
<td>lacZ labelled</td>
<td>45 ± 5</td>
<td>23 ± 5</td>
<td>4 ± 1</td>
<td>7 ± 3</td>
<td>n.a.</td>
</tr>
<tr>
<td>GFP</td>
<td>42 ± 2</td>
<td>n.a.</td>
<td>6 ± 2</td>
<td>3 ± 1</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

The composition of the transplanted cell preparations at the time of transplantation indicates a similar percentage of oligodendrocyte progenitor cells in the three cell preparations used. A2B5 labels oligodendrocyte progenitor cells, O4 labels late oligodendrocyte progenitor cells and oligodendrocytes, GFAP labels astrocytes and OX42 labels microglia. Figures are % ± standard error of the mean; n.a. = not analysed.

Depletion of OPCs results in widespread repopulation of OPC-depleted tissue by transplanted OPCs but does not result in extensive remyelination

The probable explanation for the lack of migration away from the site of implantation is the presence of endogenous OPCs, since it has been shown in previous studies that transplanted OPCs fail to colonize normal tissue in adult animals, but will colonize OPC-depleted tissue (Franklin et al., 1996; O’Leary and Blakemore, 1997). To confirm this, the spinal cord of taiep and normal rats was exposed to 40 Gy of X-irradiation—the dose known to deplete rat tissue of its OPC population in normal (Hinks et al., 2001; Chari and Blakemore, 2002a) and taiep rats (Fig. 3A and B) (Foote and Blakemore, 2005). Transplantation of cells into taiep spinal cord exposed to X-irradiation resulted in extensive repopulation of the OPC-depleted tissue (Fig. 3C and E). A greater area of remyelination was also seen around the point of cell implantation.

Fig. 2 Transplantation of OPCs into the chronically demyelinated taiep rat spinal cord results in only small areas of remyelination. (A and B) Following transplantation of unlabelled cell preparations containing OPCs into adult taiep rats, the distribution of remyelination is restricted to a small area close to the point of cell implantation. (B) is the boxed area in (A); the arrow in (A) indicates damage caused by the transplant needle and r in (B) indicates the area of remyelination, with n indicating normal appearing tissue. (C and D) When cells transduced with lacZ are transplanted, labelled cells are associated with the small area of remyelination at the transplant site, but no labelled cells are found outside the areas showing remyelination (consecutive toluidine blue stained and unstained resin sections). (E) Similar results are obtained using GFP labelled cells, with transplanted cells restricted to the transplant site (parasagittal section). Scale bar: A = 85 μm; B and D = 10 μm; E = 650 μm.
in irradiated animals than in non-irradiated animals, detected either on resin sections or, indirectly, as an area of increased GFP cell density containing many oligodendrocytes (APC positive cells) (Fig. 3D and F). However, in contrast to these areas of remyelination close to the injection site, transplanted cells repopulated the whole cross-sectional area of the spinal cord at the level of the implantation site and extended longitudinally cranially and caudally for some considerable distance (Fig. 3E). The length of repopulated tissue was similar in X-irradiated normal and taiep rats (Fig. 3E), and thus the astrocytosis and the chronically demyelinated axons present in the taiep rats were not significantly influencing the rate of repopulation. Nearly all the transplanted cells that established themselves away from the transplant site co-labelled with antibodies to the oligodendrocyte progenitor marker NG2 (Fig. 3K). It was noteworthy that the density of transplant derived OPCs in areas distant from the transplant site was similar to that for OPCs in normal tissue (50–60 cells/mm²) and there was no evidence of remyelination or generation of transplant derived oligodendrocytes (Fig. 3G–L) except in the area of remyelination associated with the transplant site (Fig. 3D, M–O). We had therefore achieved a situation in which OPCs were present adjacent to demyelinated axons without evidence of oligodendrocyte differentiation and remyelination. Thus, failure of remyelination at a distance from the implantation point cannot be attributed to a failure of OPCs to interact with demyelinated axons.

**Induction of inflammation stimulates remyelination in areas of chronic demyelination**

Since the widespread repopulation of the chronically demyelinated taiep spinal cord by OPCs resulted in remyelination only near to the transplant site, we hypothesized that the act of implantation of cells was inducing changes in the tissue that rendered it conducive to remyelination, whereas the mere repopulation of tissue by myelination competent OPCs was insufficient to bring about remyelination. To test this hypothesis, we conducted an experiment in which we first allowed repopulation of the irradiated taiep tissue by transplanted OPCs and then created, at a site distant from the cell transplant site, a situation that would mimic the injection of cells (Fig. 4A). This was achieved by injecting a small volume of saline (containing sterile charcoal to mark the site). The site chosen for this injection was the dorsal part of the lateral funiculus where there was an almost complete absence of myelin (Fig. 4D) and where remyelination had not been observed following transplantation of OPCs into the dorsal funiculus. In this experiment, unlike the previous experiments with genetically labelled cells, the recipient animals were not given cyclosporin, since we did not want to interfere with any inflammatory changes that may be associated with the saline injection. When animals were examined 3 weeks after the saline injection, remyelination was associated with the site of saline/charcoal injection in all five animals injected (Fig. 4C) and there was no evidence of remyelination in the same area on the contralateral side of the spinal cord (Fig. 4D). Since both areas contained transplanted OPCs, this result indicates that the injection of saline had stimulated the transplant established OPCs to remyelinate the tissue.

To examine the consequences of injecting saline and charcoal into the irradiated spinal cord, resin embedded and frozen sections of the injection site were examined 3 days after this injection. The injection resulted in a marked increase the number of macrophages and OX42 positive cells (Fig. 4B). Lymphocytes (CD4, CD8, B cell) were not detected in these areas using immunohistochemistry.

In some of the transplant repopulated animals subjected to the saline/charcoal injection, as well as in some animals allowed to survive for 6 weeks without saline/charcoal injection, we also found extensive remyelination around the periphery of the spinal cord (Fig. 4E). It was noticeable that, in the animals showing the most extensive remyelination, inflammatory cells were present in the meninges in toluidine blue stained resin sections, and CD4 and CD8 positive cells could be detected by immunostaining within the

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**Fig. 3** Depletion of OPCs by exposing the spinal cord to 40 Gy of X-irradiation results in widespread colonization of tissue by transplanted OPCs, but remyelination is still restricted to a small area around the point of cell implantation. (A and B) Use of a riboprobe to platelet derived growth factor receptor alpha (PDGFRα) demonstrates the presence of OPCs in the adult taiep central nervous system (A) and the almost complete depletion of these cells following exposure to 40 Gy of X-irradiation (B). (C) Transplantation of cells obtained from rats expressing GFP under an actin promoter demonstrates that, following X-irradiation, there is extensive colonization of tissue by transplanted cells (E), with a region of high cell density near the injection point and low cell density elsewhere. The length of the high cell density region is significantly greater (one-way ANOVA; P < 0.01) in the irradiated taiep rats than in non-irradiated taiep rats (F), indicating that depletion of OPCs facilitates remyelination by the transplanted OPCs. (D) In resin sections, increased remyelination is seen close to the area of cell injection in irradiated taiep spinal cord compared with non-irradiated spinal cord. (E–L) The spread of low density cells is not significantly different in X-irradiated taiep rats and X-irradiated normally myelinated control animals (E), indicating that the presence of chronically demyelinated axons and an astrocytosis was not influencing the repopulation rate of OPC-depleted tissue. In the low density areas, lacZ labelled cells were present in the absence of remyelination (G–I). These cells contained only a narrow band of cytoplasm that lacked defining ultrastructural features and were, therefore, consistent with being OPCs. (H: EM image of the lacZ labelled cell highlighted by an asterisk in G, showing nuclear located X-gal reaction product in this unstained section. After staining with contrast agents, the X-gal reaction product is less apparent, but the same cell can be seen amidst numerous demyelinated axons and astrocyte processes.) Consistent with the electron microscope findings, the transplanted GFP positive cells away from the transplant site co-labelled with antibodies to the OPC marker NG2 (J and K), but not with the oligodendrocyte marker APC (L). (M–O) In contrast, the regions of high cell density at the transplant site contained transplant derived oligodendrocytes (M–O) and remyelination (D).

Scale bar: A and B = 100 μm; C = 1 mm; D = 50 μm; G = 25 μm; H = 1.5 μm; I = 2 μm; J–M = 90 μm; N = 25 μm; O = 10 μm. Error bars in E and F = standard error of the mean; ** = P < 0.01, *** = P < 0.001, one-way ANOVA.
CNS parenchyma and within the meninges (Fig. 4F). When the extent of complete remyelination was measured and compared between the animals with and without evidence of meningeal infiltration (as detected in resin sections), it was clear that the extent of remyelination in the animals with cell infiltration was significantly greater than in those without evidence of meningitis (Fig. 4G and H); in the sections showing the most extensive remyelination, up to 80% of the white matter was remyelinated. Our results therefore show that induction of inflammation in non-remyelinating...
OPC-populated areas of chronic demyelination stimulates remyelination.

Discussion

Our studies represent the first in which extensive remyelination has been achieved using a model of chronic demyelination in adult animals. As a consequence, they establish a number of principles that have relevance both to understanding the reasons for remyelination failure in multiple sclerosis and to evaluating remyelination enhancing strategies for this disease. First, by transplanting OPCs into this model of chronic demyelination, we have confirmed the recent observation on the retina that axons that have been without myelin for a considerable time can be myelinated (Setzu et al., 2004) and thus axonal changes resulting from demyelination are unlikely to play a role in remyelination failure (Chang et al., 2002). Second, by replacing taeip OPCs with normal OPCs we have demonstrated that cells capable of undertaking remyelination can be present in tissue containing receptive demyelinated axons without consequent remyelination. Thus, the OPCs found in some chronic multiple sclerosis lesions and considered by some to be non-functional (Greenwood and Butt, 2003) may not in fact be so, since they, like the transplanted OPCs in this study, may simply require induction of inflammation to enable their proliferation and subsequent differentiation into remyelinating cells. Third, by showing that transplanted OPCs established away from the implantation site have a similar density to that found in normal tissue, our study extends to pathological tissue a conclusion drawn from studies on normal tissue that, in the absence of inflammation, OPC numbers are tightly regulated (Dawson et al., 2003; Woodruff et al., 2004). This has implication for attempts to introduce transplanted OPCs into areas of chronic demyelination as well as normal tissue, since it indicates that transplanted OPCs can be established only by either deleting tissue of endogenous OPCs, or changing the environment so that it can support these supernumary cells. Finally, our studies demonstrate that although transplanted OPCs can be established in OPC-depleted areas of chronic demyelination, remyelination is dramatically stimulated only when the environment is made conducive to OPC expansion and differentiation; this can be achieved by establishing acute inflammation.

In agreement with a number of previous studies using longer-lived mature myelin mutants (Archer et al., 1997; Milward et al., 2000; Zhang et al., 2003), we found very limited remyelination following transplantation of myelination competent cells into the spinal cord of taeip rats. In the shaking pup the extent of remyelination decreased with the age of the recipient and was enhanced when late embryonic rather than neonatal cells were transplanted in adult animals (Archer et al., 1997), indicating that donor age influences the extent of remyelination. The poor results in the Long Evans rat were attributed to the presence of activated microglial cells, as down-regulation of this response by minocycline enhanced cell survival and remyelination (Zhang et al., 2003). Our study clearly shows that transplanted OPCs, as in normal tissue (Franklin et al., 1996; O’Leary and Blakemore, 1997), do not enter tissue containing endogenous OPCs. Since there can be no remyelination without repopulation of tissue by myelination competent cells, a major reason for the failure to achieve significant remyelination following transplantation into these long-lived myelin mutants is the presence of endogenous OPCs.

The rate of OPC repopulation of OPC-depleted tissue by transplanted OPCs was not significantly different in taeip and normal rats, indicating that the chronically demyelinated environment found in the taeip rat does not significantly inhibit or enhance the OPC repopulation rate. The repopulation rate of the transplanted OPCs was slightly slower in the taeip than in normal animals, which may suggest an inhibitory role for astrocytes in vivo, as has been demonstrated in vitro (Fok-Seang et al., 1995). In a previous study, we found no difference in the rate of endogenous OPC repopulation between normal and taeip rats of similar age (Foote and Blakemore, 2005). However, failure to detect a difference in that study may have been a consequence of the...
very slow OPC repopulation rate in old animals. Despite a possible slight negative influence on the rate of repopulation, it is clear that transplanted OPCs colonize OPC-depleted chronically demyelinated tissue at a significantly faster rate than endogenous cells [1.5 mm/week (present data) compared with 0.2 mm/week (data from Foote and Blakemore, 2005)]. Thus, where tissue is depleted of OPCs, transplanted neonatal OPCs can repopulate normal and chronically demyelinated tissue more rapidly than endogenous cells.

More important than the identification of how to achieve OPC repopulation in areas of chronic demyelination was the finding that this widespread repopulation resulted in only a modest increase in remyelination. The increased remyelination was restricted to the transplant area, and away from this area there was no evidence of remyelination despite repopulation of the tissue by the transplanted myelination competent OPCs. Thus, repopulation and remyelination are distinct processes, with the former not necessarily resulting in the latter. The non-remyelinating OPC-repopulated chronically demyelinated tissue that we have created in the taipep rat is comparable to the situation observed in non-remyelinating OPC-populated multiple sclerosis plaques (Chang et al., 2002). Since we were able to demonstrate that the chronically demyelinated axons of the taipep rat could be remyelinated under some circumstances, we can conclude that chronically demyelinated tissue has properties that either inhibit or fail to stimulate remyelination. Our studies show a clear relationship between acute inflammation and successful remyelination. This adds to the growing body of evidence that acute inflammation provides the stimulus that initiates remyelination (reviewed, Franklin, 2002) and provides proof of principle for the ‘temporal mismatch’ hypothesis as an explanation for remyelination failure in multiple sclerosis (Chari and Blakemore, 2002b). This hypothesis proposed that if OPCs were destroyed in large areas of demyelination, remyelination would fail because the slow rate of repopulation shown by endogenous OPCs in old individuals would result in a temporal mismatch between the acute inflammatory environment associated with myelin sheath breakdown and the interaction of OPCs with demyelinated axons; that is, in the absence of an acute inflammatory environment to provide the signals required to drive remyelination, the process will fail. In a previous study, we obtained some support for this concept by delaying the interaction of OPCs with demyelinated axons by transplanting cells at a distance from a focus of acute demyelination induced in OPC-depleted tissue, and showing that the extent of remyelination was significantly reduced compared with the situation when OPCs were present at the time of, or soon after, demyelination was initiated (Blakemore et al., 2002). Having established that OPC repopulation rates are extremely slow in old animals (Chari et al., 2003; Foote and Blakemore, 2005), our current results strengthen the temporal mismatch hypothesis by demonstrating for the first time in an experimental model the three further situations required for this hypothesis to be sustainable: (i) OPCs can repopulate OPC-depleted chronically demyelinated tissue; (ii) in the absence of acute inflammation, these repopulating cells persist without remyelination occurring; and (iii) when an acute inflammatory environment is created, remyelination occurs efficiently.

The finding that areas of chronic demyelination can contain both axons that can be remyelinated and a normal density of remyelination competent OPCs without evidence of remyelination would indicate that, unlike in myelination during development, the axon does not provide the stimulus that initiates OPC proliferation (Barres and Raff, 1999). It is well established that OPCs become activated, increase in number and change their morphology in association with acute damage to the central nervous system (Levine et al., 2001). It is not known whether this activation occurs as a consequence of changes in properties of the environment that regulate the activation status of OPCs or are a consequence of direct stimulation of the OPC by factors associated with acute inflammation. In this context it is known that certain molecules, such as II-1β, associated with acute inflammation can stimulate OPC division directly (Vela et al., 2002) and could influence OPC division and survival via their effects on astrocytes (Aloisi et al., 1995; Silberstein et al., 1996; Mason et al., 2001). In adult rats, demyelination is associated with rapid activation of OPCs, as indicated by up-regulation of the transcription factor Nkx2.2 and olig2 (Fancy et al., 2004), and, in terms of remyelination, the two consequences of OPC activation are cell division and differentiation into remyelinating oligodendrocytes. The OPCs which repopulated the OPC-depleted chronically demyelinated taipep spinal cord established themselves at a normal density and showed a normal morphology and no evidence of oligodendrocyte differentiation, suggesting that they are unstimulated and under the control of the factors that regulate OPC behaviour in normal tissue. However, once activated by acute inflammation they proliferate and differentiate into remyelinating oligodendrocytes. Investigation is needed of whether inflammation also induces changes on the axon that would facilitate myelination, such as removal of the polysialylated neural cell adhesion molecule shown to inhibit myelination (Charles et al., 2000).

In the present experiments two forms of inflammation were associated with stimulation of remyelination. The saline/charcoal injections stimulated a predominantly macrophage (monocyte/macrophage/microglial) response, whereas lymphocyte infiltration was a feature of the more widespread remyelination found in the animals with meningitis. Macrophages have been shown to have a significant role in facilitating remyelination (Kotter et al., 2001), as have both lymphocytes (Bieber et al., 2003) and the inflammatory responses associated with rejection of xenogeneic cells (Blakemore et al., 1995). It was not possible to establish the cause of the inflammatory reaction in the animals with meningitis and extensive remyelination around the periphery of the spinal cord. The most probable explanation is that it represents an immune response to the transplanted cells. This group of animals was not immunosuppressed, the
transplanted cells were allografts and expressed a foreign protein and, moreover, the tissue had been subjected to a high dose of X-irradiation. The dose of X-irradiation used in our studies is known to render tissue more susceptible to immune-mediated inflammation (Love et al., 1987) and GFP can be a powerful immunogen (Stripecke et al., 1999; Follenzi et al., 2004). Although it is clear that acute inflammation provides a stimulus that initiates remyelination and that macrophages and acutely reactive astrocytes have a positive effect on the success of remyelination (Franklin et al., 1991; Hinks and Franklin, 1999), there is also evidence that activated microglia and astrocytes can have a negative effect. The reactive microglia present in the Long Evans rat (Zhang et al., 2003), which are also present in the taiep rat (Goetz et al., 2000), have been shown to have a negative effect on remyelination and, unlike during the acute situation, astrocytes have been implicated as having a deleterious effect on remyelination when the inflammatory response initiated by demyelination is waning (Blakemore et al., 2002; 2003). One therefore has to consider that astrocytes and microglia/macrophages can have both positive and negative effects on remyelination. The duality of influence of a single cell type is also apparent in vitro, where macrophages can either enhance or impede remyelination in co-culture systems (Diemel et al., 2004). Similarly, astrocytes impede OPC migration under some culture conditions but can be altered by exposure to cytokines to negate this effect (Fok-Seang et al., 1998). It is likely, therefore, that it is the nature of the response of these cells to acute inflammation that creates an environment conducive to remyelination. In this context it is possible that the immune modulating therapies currently in use for multiple sclerosis could be having a deleterious effect on remyelination. However, OPC repopulation was not affected by the immunosuppressant regimen used in the present study, indicating once again that OPC repopulation of OPC-depleted tissue and remyelination are distinct processes.

In conclusion, our experiments have relevance for both interpreting the pathology present in areas of chronic demyelination in multiple sclerosis and developing OPC transplantation as a therapy for chronic demyelinating diseases. They demonstrate that (i) the failure of remyelination observed in OPC-containing areas of chronic demyelination cannot be taken as evidence that chronically demyelinated axons have undergone changes that preclude their remyelination; (ii) as with normal tissue, the presence of endogenous OPCs in areas of chronic demyelination prevents the establishment of transplanted OPCs and (iii) acute inflammation provides the stimulus for remyelination.

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