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The reconstruction of an astrocytic environment in glia-deficient areas of white matter

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Summary

Injection of ethidium bromide into X-irradiated spinal cord white matter produces a lesion in which demyelinated axons reside in an environment that is permanently depleted of glial cells. By transplanting defined populations of glial cells into this lesion it is possible to recreate normal or novel glial environments. In this study we have transplanted cultures of astrocytes into the X-irradiated ethidium bromide lesion in order to (1) assess the ability of these cells to relate to components within the lesion environment and thereby contribute to tissue reconstruction and (2) establish an astrocytic environment around demyelinated axons that resembles pathological states such as the chronic demyelinated plaques of multiple sclerosis. In order to focus attention on the interactions between astrocytes and demyelinated axons we developed a protocol for depleting astrocyte cultures of oligodendrocyte lineage cells and Schwann cells based on complement-mediated immunocytolysis and *in vitro* X-irradiation. In addition to establishing the ability of transplanted astrocytes to form an astrocytic matrix around demyelinated axons, this study has also revealed the diversity of cell types present within neonatal forebrain cultures.

Introduction

Transplantation of cultured glial cells into areas of the adult CNS from which the resident glial population have been removed provides the opportunity to reconstruct specific glial environments around CNS axons. Such an approach enables one to study the cell-to-cell interactions that occur during CNS repair (Blakemore & Franklin, 1991) and may also be used to create cellular environments that serve as models for a variety of pathological states such as the astrocytic plaques of chronic multiple sclerosis lesions.

The direct injection of gliotoxins enables one to create a focal area of white matter from which the normal glial population has been removed. When ethidium bromide (EB) is injected into spinal cord white matter, both oligodendrocytes and astrocytes are destroyed leaving the *en passant* axons intact (Graça & Blakemore, 1986). The inherent tendency for such an area to be repopulated by host glia can be suppressed by exposing the spinal cord to 40 Grays of X-irradiation (Blakemore, 1977). The X-irradiated EB lesion therefore constitutes a glial *tabula rasa* in which a glial environment can only be recreated by extrinsic cell replacement (Crang *et al.*, 1992). By transplanting glial cells it has been possible to create either a PNS or CNS glial environment around demyelinated axons. Thus, transplanting Schwann cells either directly into (Blakemore & Crang, 1985) or at a distance from (Blakemore, 1977) an irradiated glia-free lesion results in widespread Schwann cell remyelination of demyelinated CNS axons. Alternatively, by transplanting mixed CNS glial cultures into X-irradiated lesions it is possible to create a CNS glial environment consisting of astrocytes, oligodendrocytes and axons remyelinated by central-type myelin (Blakemore & Crang, 1988). In both these situations a complete glial environment is restored. More recently it has been possible to create an environment containing oligodendrocytes but deficient in astrocytes by transplanting purified populations of oligodendrocyte progenitors (Crang *et al.*, 1992).

A number of previous studies have examined the behaviour and survival of astrocytes following transplantation into the CNS (Goldberg & Bernstein, 1988; Ignacio *et al.*, 1989; Zhou *et al.*, 1990; Emmett *et al.*, 1991; Hatton *et al.*, 1992). However, none of these studies have involved a detailed morphological analysis of the ability of transplanted astrocytes to integrate within an area of damaged CNS. The manner in which

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astrocytes interact with the host tissue is an important issue in the context of reconstructing glial environments in glia-deficient areas. At one end of the spectrum, the cells may form self-contained groups of cells that remain separate from the host tissue. At the other end of the spectrum, the cells may move into surrounding host tissue and relate to the host cells in a manner that recapitulates a naturally occurring situation. We have termed the ability of transplanted cells to relate to host tissue as their ability to integrate. Earlier studies involving transplantation of mixed glial cultures have indicated that transplanted astrocytes and oligodendrocyte lineage cells can reconstruct a complete glial environment (Blakemore & Crang, 1988; Franklin *et al.*, 1991). However, when astrocytes are transplanted together with even a small number of Schwann cells, their ability to reconstruct an astrocytic environment around demyelinated axons appears limited. The astrocytes form self-contained, monotypic clusters of cells, while the Schwann cells associate with the demyelinated axons which they subsequently remyelinate (Franklin *et al.*, 1992). One interpretation of these observations is that the integration capacity of transplanted astrocytes is dependent on the availability of oligodendrocyte lineage cells. An alternative explanation is that the integrative ability of astrocytes is masked or suppressed by the exuberant behaviour of the Schwann cells. Thus, the intrinsic ability of astrocytes to integrate in an environment of demyelinated axons remains unclear.

In order to resolve this issue, and thereby determine the feasibility of creating an astrocytic environment around demyelinated axons by transplantation, it was necessary to develop strategies for removing both oligodendrocyte lineage cells and Schwann cells from the astrocyte cultures. This required an extension of an earlier strategy to remove oligodendrocyte lineage cells based on complement-mediated immunocytolysis and cytosine arabinoside treatment (Franklin *et al.*, 1991), and involved exposing cultures to different doses of X-irradiation in an attempt to deplete them of immature cell types (Barbarese & Barry, 1989; Van der Maazen *et al.*, 1990). In so doing, not only have we been able to demonstrate that astrocytes can integrate into a glial free area of demyelination following transplantation, but have also highlighted the complex and diverse composition of CNS glial cultures obtained from neonatal forebrain.

Materials and methods

PREPARATION OF ASTROCYTE CULTURES

Astrocyte cultures were prepared from postnatal 1–5 day old PVG/Ola rat pups (for detailed description see Blakemore & Crang, 1992). The forebrain was removed and the meninges stripped off. Cells were dissociated by chopping with a scalpel blade, incubating in 0.25% trypsin in MEM-HEPES at 37° C for 15 min, and finally triturating in L15 medium by passage through 21- and 23-gauge needles. The resulting cell suspension was filtered through a 80 μm nylon mesh and the cells collected by centrifugation. The cells were resuspended in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and plated onto poly-L-lysine coated Nunc flasks at a density of 3–4 × 10^4 cells per cm^2. The culture medium was changed every 3–4 days *in vitro*.

REMOVAL OF OLIGODENDROCYTE LINEAGE CELLS

Four preparations of oligodendrocyte-lineage-depleted astrocyte cultures were prepared for transplantation based on different purification strategies involving combinations of complement-mediated immunocytolysis (CMI), cytosine arabinoside treatment, and X-irradiation (see Table 1). The following antibodies were used for CMI: monoclonal A2B5 (Eisenbarth *et al.*, 1979) and tetanus toxin/anti tetanus toxin to remove O-2A progenitors (Raff *et al.*, 1983a), and the monoclonal O4 (Sommer & Schachner, 1981) to remove immature and mature oligodendrocytes (Gard & Pfeiffer, 1989) and minimize Schwann cell contamination (Mirsky *et al.*, 1990).

Preparation 1

This preparation was prepared from five-day-old rat pups. The oligodendrocyte-lineage cells were removed from the cultures by complement-mediated immunocytolysis and

Table 1. This table summarises the cell preparation protocols.

Cell preparation	*Age of animals from which cultures were prepared (days)*	*Removal of OL-lineage-rich top-dwelling layer by shaking (DIV)*	*Exposure to cytosine arabinoside (DIV)*	*Immunocytolysis protocol (DIV)*	*X-irradiation (12 DIV) dose (Grays)*	*Transplantation (DIV)*
Prep 1	5	–	11	14	–	14
Prep 2	1	10	–	11*	10	13
Prep 3	3	10	–	11*	20	13
Prep 4	3	10	–	11*	30	12

* anti-tetanus toxin/tetanus toxin not included in immunocytolysis protocol.

DIV = days in vitro; OL-lineage = oligodendrocyte lineage.

cytosine arabinoside treatment using previously published protocols (Franklin *et al.*, 1991, 1992). In brief, after 11 days *in vitro*, cytosine arabinoside was added to the culture medium to a final concentration of 10^{-5} M. After 72 h the cytosine arabinoside-containing medium was removed, the cell layer washed with tissue culture medium and then incubated with 20 μg ml^{-1} tetanus toxin (Wellcome Biotech) for 30 min at 37° C. After washing off the tetanus toxin, a concoction of antibodies (anti-tetanus toxin, O4 and A2B5, 100 μl of each antibody solution was added to 5 ml tissue culture medium) and guinea pig complement (diluted 1 + 4 with tissue culture medium) was added and the culture and incubated for 2 h at 37° C. Finally, medium containing antibodies and complement was removed, the cell layer washed and prepared for transplantation as described below.

Preparation 2

This preparation was prepared from one-day-old rat pups. The initial step involved shaking off the top-dwelling layer of cells, a component of the culture rich in oligodendrocyte lineage cells, after ten days *in vitro*. The cultures were then treated at 11 days *in vitro* as described for preparation 1 with the following alterations. Firstly, the tetanus toxin/anti-tetanus toxin component of the immunocytolysis procedure was omitted. Secondly, the cultures were not treated with cytosine arabinoside to kill dividing cells but instead were exposed to a total dose of 10 Grays of X-irradiation using a radiotherapy machine (Marconi Deep Therapy Apparatus type TF 1554) at 12 days *in vitro*. The cells were prepared for transplantation on the following day.

Preparation 3

This preparation was prepared from three-day-old rat pups. Preparation 3 was prepared in a similar fashion to preparation 2. The cells were shaken at ten days *in vitro*, subjected to immunocytolysis and 20 Grays X-irradiation at 11 days *in vitro*, and transplanted on the following day.

Preparation 4

Preparation 4 was prepared as for preparation 3 except that the cultures received a dose of 30 Grays of X-irradiation.

CHARACTERISATION OF CULTURES

The transplanted cell suspension was characterised by replating cells remaining following transplantation. These cultures were grown on and fixed with 4% paraformaldehyde 28 days after transplantation. Cells were characterised by immunofluorescent labelling with A2B5, O4, O1, anti-GFAP (Dako), anti-L1 and S100 (Dako). $GFAP^+/A2B5^-$ cells were identified as type 1-like astrocytes (Raff *et al.*, 1983a), $A2B5^+$, $O4^+$ and $O1^+$ cells as cells of the O-2A lineage (Raff *et al.*, 1983b; Sommer & Schachner, 1981), and $L1^+/S100^+$ cells as Schwann cells (Mirsky *et al.*, 1986).

PREPARATION OF X-IRRADIATED ETHIDIUM BROMIDE LESION

In all experiments adult PVG/Ola rats were used as recipients that were syngeneic with the donor tissue. The preparation of X-irradiated EB lesions has been described in detail elsewhere (Blakemore & Crang, 1992). Briefly, the animals were anaesthetised with a fentanyl citrate/fluanisone combination ('Hypnorm', Janssen Pharmaceuticals), placed in lateral recumbency and the spine between T13 and L4 was exposed to 40 Grays of X-irradiation using a radiotherapy machine (Marconi Deep Therapy Apparatus type TF 1554). Three days after X-irradiation a laminectomy was performed on the first lumbar vertebra under halothane anaesthesia. Ethidium bromide (1 μl of a 0.1% solution in normal saline) was injected into the exposed dorsal funiculus using a glass micropipette attached to a 10 μl Hamilton syringe. In eight animals, X-irradiated EB lesions were made but no cells were subsequently transplanted. These were killed at 14 and 28 days after lesion induction, and served as controls.

TRANSPLANTATION OF CELL SUSPENSIONS

Three days after lesion induction, the animals were re-anaesthetised and 1 μl of a cell suspension injected into the centre of the EB lesion through the same laminectomy site. Suspensions of cultured cells were prepared by detaching the cultured cells with trypsin/EDTA and suspending in MEM-HEPES at a cell density of 4–5 × 10^4 cells per μl.

ANALYSIS OF LESIONS

After the appropriate survival time the animals were killed by aortic perfusion with 4% glutaraldehyde in phosphate buffer under pentobarbitone anaesthesia. The lesion-containing length of spinal cord was cut into 1 mm coronal blocks which were post-fixed in OsO_4, dehydrated in ascending alcohols and embedded in TAAB resin. Sections (1 μm) were cut from each block, stained with Toluidine Blue, and examined by light microscopy. Selected areas from some blocks were trimmed for thin-sectioning and examined by electron microscopy. The cranio-caudal sequence of the blocks was preserved throughout processing.

Table 2. Analysis of antigen expression by samples of transplanted cell preparations maintained for a further 28 days *in vitro*.

Transplanted cell preparation	*A2B5*	*O4*	*O1*	*S100/L1*	*GFAP*
Prep 1	0	0	0	<1%	>95%
Prep 2	0	<1%	0	n.d.	>95%
Prep 3	0	0	0	n.d.	>95%
Prep 4	0	0	0	n.d.	>95%

n.d. – not determined

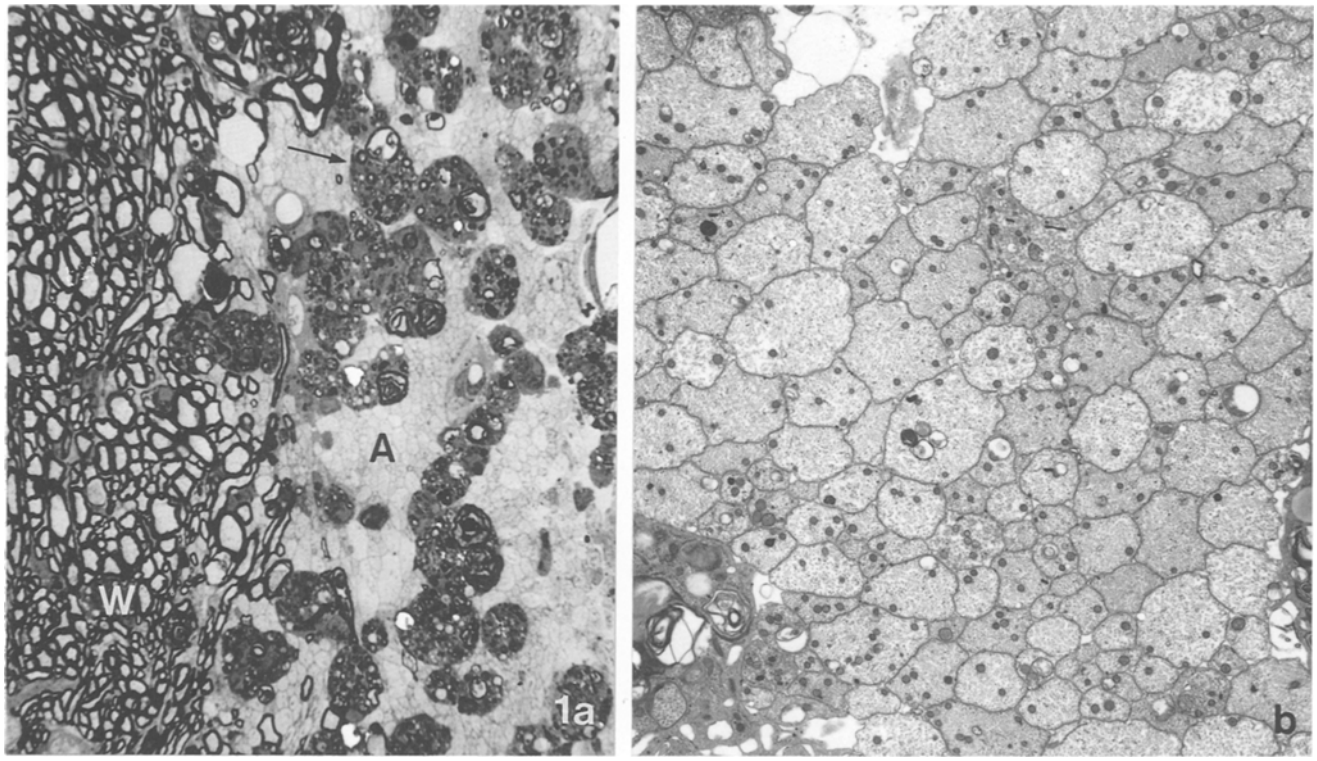


Fig. 1. Lesions from control animals at 28 DPT. (a) The lesion, adjacent to normal white matter (W), consists solely of demyelinated axons (A) and myelin-debris-filled macrophages (arrow). Toluidine Blue-stained resin section. × 400. (b) The axons are packed together tightly, and no glial cells are evident. × 5100.

Results

CHARACTERISATION OF TRANSPLANT CULTURES

The antigen expression of the cells that were grown in tissue culture for 28 days after the date of transplantation is summarised in Table 2. In brief, all four purification protocols yielded cultures that contained $GFAP^+$ type 1-like astrocytes but did not contain $A2B5^+$ or $O1^+$ cells of the oligodendrocyte lineage, and only rarely were $S100^+/L1^+$ Schwann cells identified. Preparation 2 contained a very small number of $O4^+$ cells (<1%).

CONTROL ANIMALS AND LESION SIZE

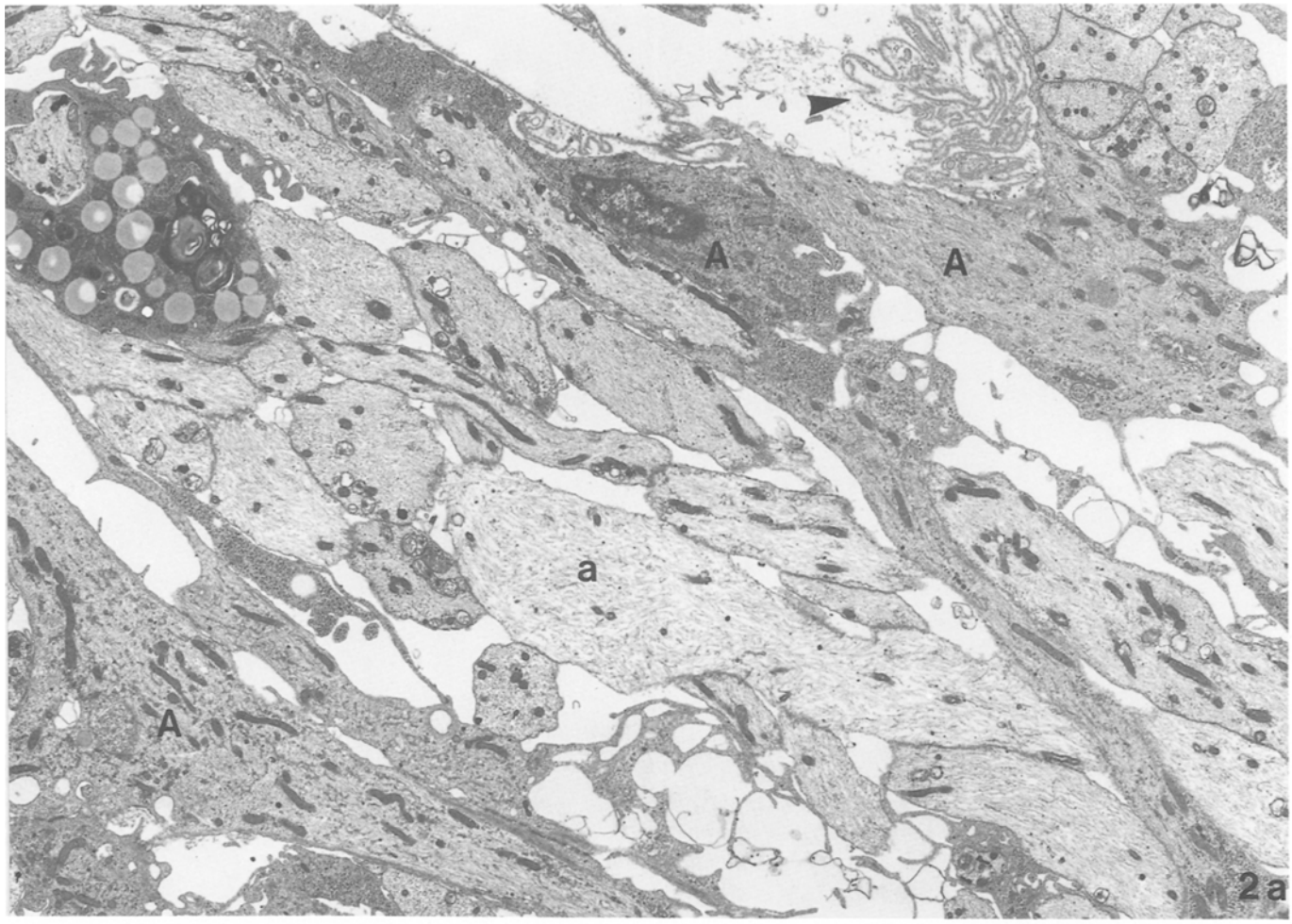
The X-irradiated EB lesions that did not receive cell transplants were examined 14 and 28 days after lesion-induction. At both survival times the lesions had similar appearances and consisted of demyelinated axons clumped together in an environment which contained no glial cells (Fig. 1). Large, myelin-filled macrophages were also seen within the lesion. The borders of the lesion were distinct with a sharp transition between areas of white matter containing a normal glial environment and the lesion where no glia were present (Fig. 1). In all animals the lesions were consistent with previously reported X-irradiated EB lesions (eg. Blakemore & Crang, 1985), and with similar lesions which had received a second sham injection of cell suspension medium alone (unpublished data).

The lesion size was similar in the control groups and in the transplanted groups. The lesions typically occupied between 30% and 50% of the cross-sectional area of the dorsal funiculus at their central point where both the lesion and transplant injections were made, and extended for between 4 and 6 mm along the longitudinal axis of the cord, tapering at both the cranial and caudal ends.

TRANSPLANTATION OF ASTROCYTE CULTURES PURIFIED BY IMMUNOCYTOLYSIS AND CYTOSINE ARABINOSIDE ONLY (PREPARATION 1)

Seven days post-transplantation

Lesions were examined in two animals seven days post-transplantation (DPT). A clear distinction existed between areas of the lesion containing injected cells and areas of the lesion that resembled the non-transplanted X-irradiated EB lesions. In neither animal were cells other than debris-filled macrophages observed amongst the demyelinated axons away from the point of injection. Cells were observed adopting various morphologies according to their location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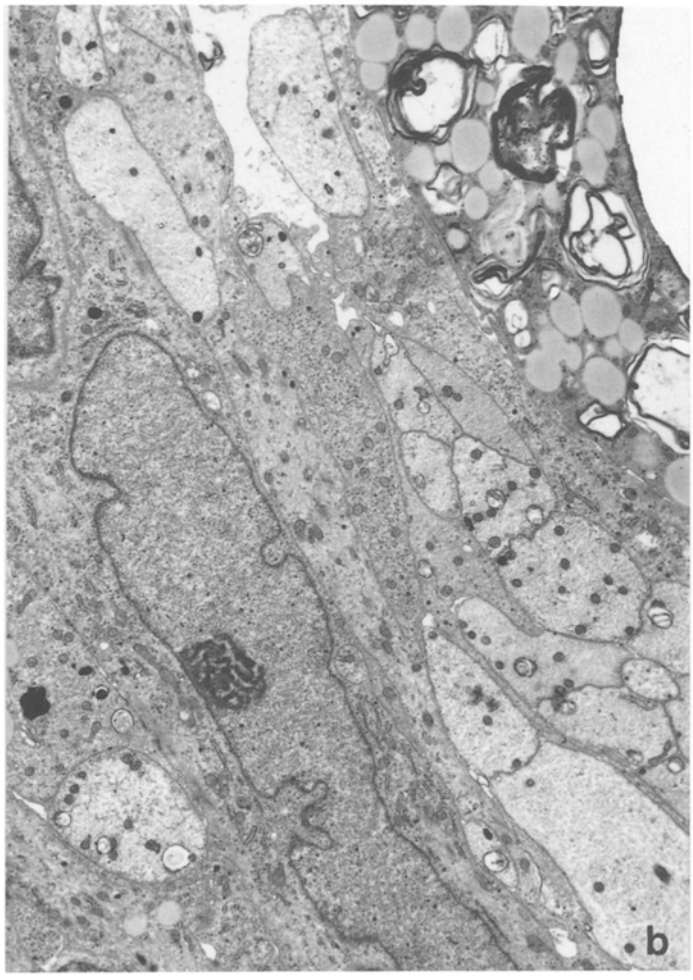
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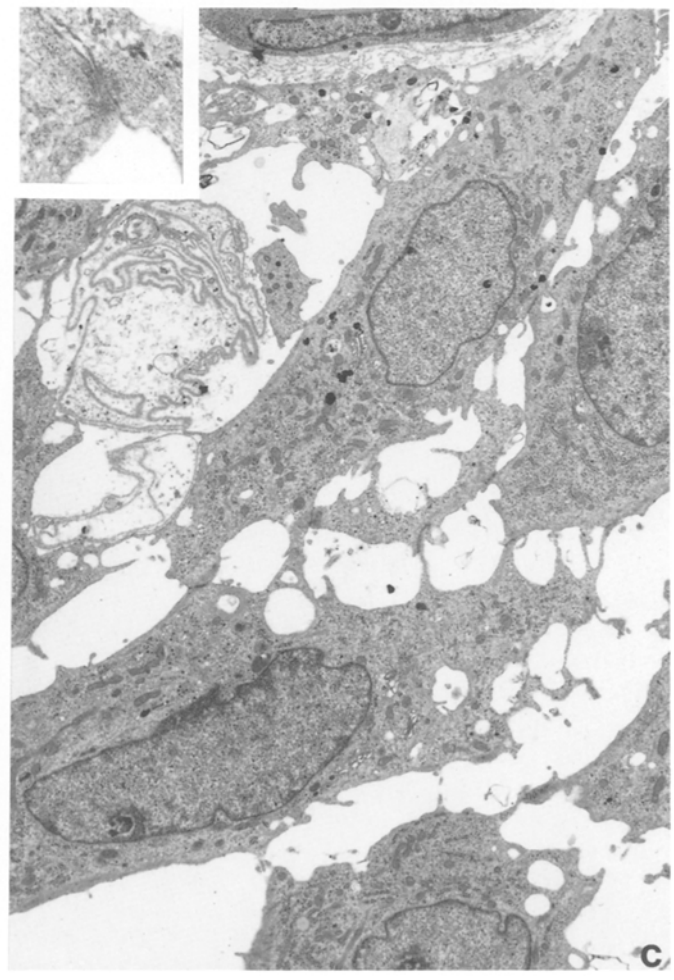
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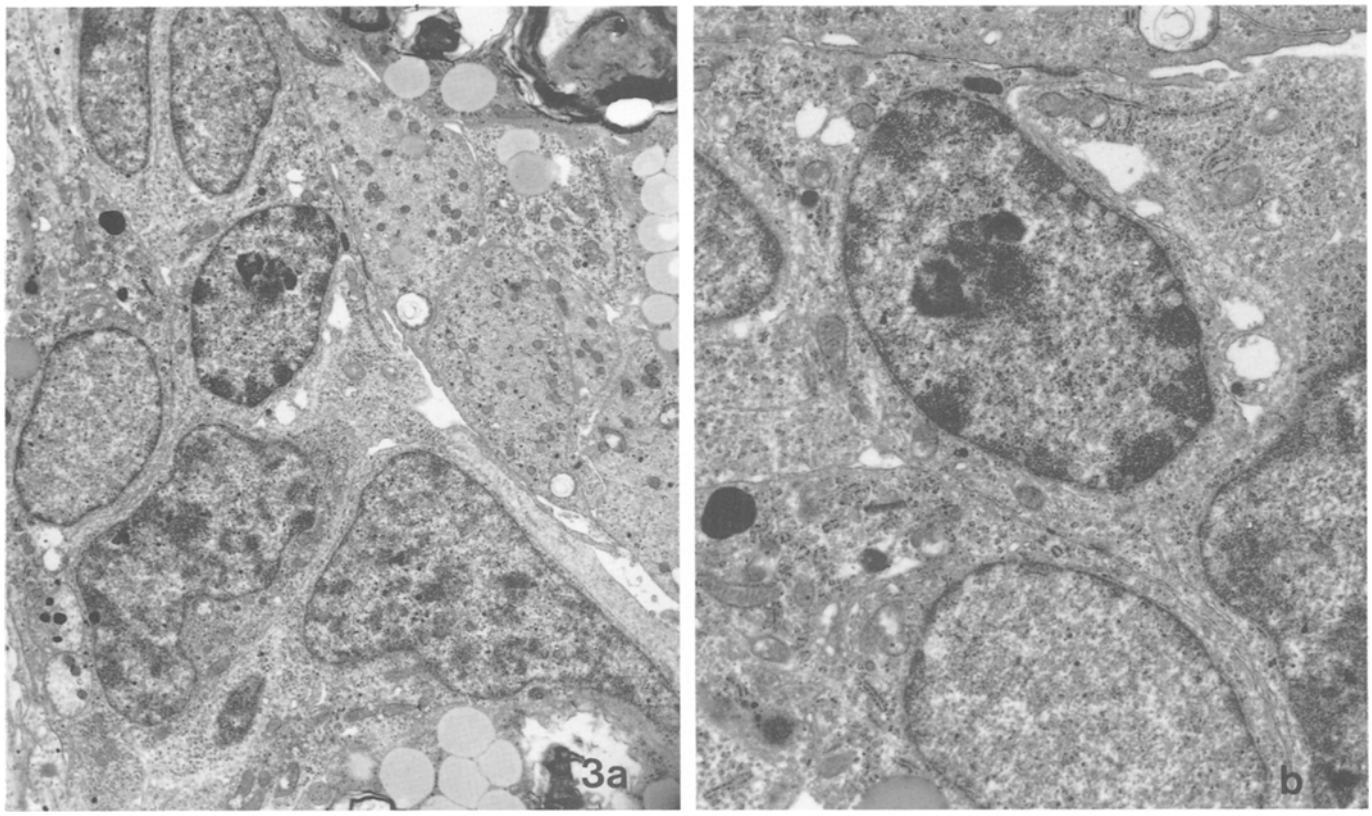


Fig. 3. Cluster of sub-ependymal-plate-like cells. These small cells have a high nucleus-to-cytoplasm ratio, form tightly packed clusters and are distinct from the large astrocytes (eg. Fig. 2a,c). × 6500. (b) Higher magnification of cells from cluster in (a). These cells contain free ribosomes, rER, Golgi complexes and sparse amounts of intermediate filaments. × 14 400.

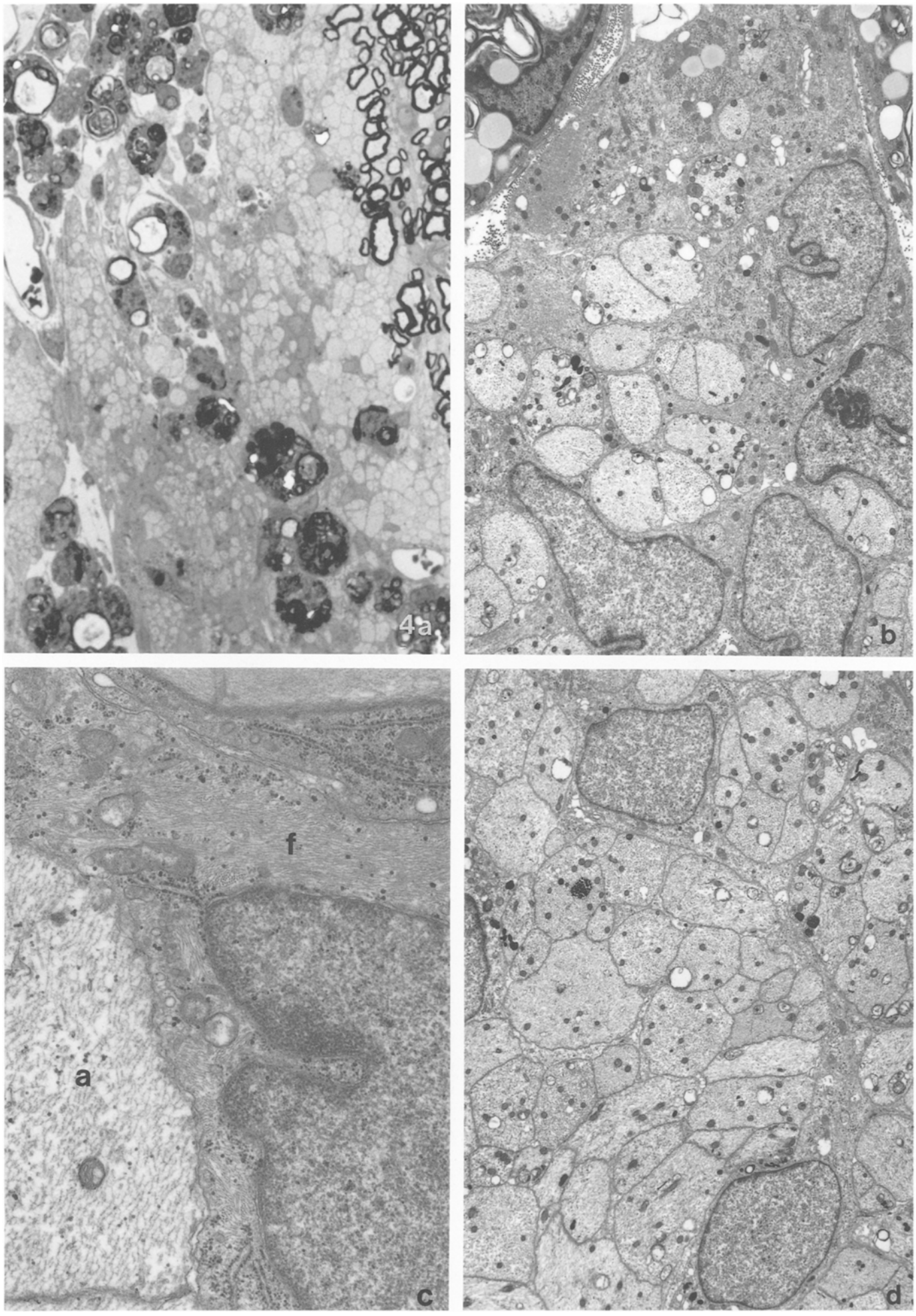
within the lesion. In non-cystic areas where the tissue density was high, the cells were present as long strands of longitudinally arranged cells, oriented in the direction of the injection needle tract (Fig. 2a). Ultrastructurally, these cells possessed abundant cytoplasm rich in stacks of intermediate filament and were readily identifiable as astrocytes. At the interface of the cords of injected cells and the demyelinated axons, a few astrocytes were observed with processes that extended between individual demyelinated axons (Fig. 2b). In more open, cystic areas of the lesion the cells adopted a more diffuse arrangement, but were connected with one another by short processes (Fig. 2c). Desmosome-like junctions were frequently seen at the points of contact of these processes.

In addition to the large, filament containing astrocytes, a second, much less abundant, cell type could be identified. These cells existed in tightly packed nests or clusters of cells (Figs 3a,7). In contrast to the astrocytes, these cells were much smaller with a high nucleus to cytoplasm ratio. Their nuclei were of various shapes with slight chromatin clumping around the nuclear rim. The small amount of cytoplasm contained abundant free ribosomes and ribosomal rosettes. Endoplasmic reticulum and Golgi bodies were scarce and the mitochondria were small and few in number (Fig. 3b). These cells bore many ultrastructural features of immature glial cells (Levine & Goldman, 1989) and, particularly in terms of their tendency to form tight clusters of cells, resembled cells from the sub-ependymal plate (SEP) (Blakemore, 1969; Privat & Leblond, 1972; McDermott & Lantos, 1991).

Fourteen days post-transplantation

Lesions were examined in five animals at 14 DPT. At this survival time it was still possible to observe cores

Fig. 2. Lesions following transplantation of preparation 1 at 7 DPT. (a) Large astrocytes (A) were present, although there was little interaction with the surrounding demyelinated axons (a). Whorls of basal lamina were often seen in association with these large astrocytes (arrowhead). × 5000. (b) At the interface between injected astrocytes and demyelinated axons occasional astrocyte processes were seen which extended between demyelinated axons. × 6300. (c) In cystic areas of the lesion, where no demyelinated axons occurred, injected astrocytes formed a loose network of cells linked by short, thin processes. × 5000. (Inset) These processes were often linked by desmosome-like junctions. × 40 000.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of large astrocytes near the point of injection as well as occasional nests of small SEP-like cells. However, in contrast to the lesions at 7 DPT, many more cells were present amongst the demyelinated axons (Fig. 4a). In areas close to the point of injection, large astrocytes could be seen with processes that separated demyelinated axons from one another creating an environment which consisted of demyelinated axons surrounded by filament-containing astrocyte processes (Fig. 4b). In other areas astrocytes with less abundant cytoplasm could be seen with fine processes that had insinuated their way between demyelinated axons (Fig. 4d). Often these smaller astrocytes contained nuclei with a contorted outline, somewhat similar to the immature or 'beta' astrocyte described by Reyners and co-workers (Reyners *et al.*, 1986). Further away from the point of injection, single SEP-like cells were observed within areas consisting of demyelinated axons (Fig. 5a,b). A further type of cell that could be identified in such areas possessed ultrastructural characteristics intermediate between astrocytes and undifferentiated cell types (Fig. 5c). These cells possessed areas of cytoplasm remote from the nucleus which contained abundant stacked glial filament. However, closer to the nucleus the density of glial filament was less and instead was rich in free ribosomes, rER and Golgi complexes (Fig. 5d). This perinuclear morphology contrasted with that seen in astrocytes close to the point of injection (eg Fig. 4b,c).

No oligodendrocyte remyelination was observed; however, a very small number of Schwann cells were seen when sections were examined by electron microscopy.

At the cranial end of the lesion there was extensive Wallerian degeneration and numerous myelin-debris-filled macrophages. In the cranial-most parts of the lesion astrocytes were only observed surrounding blood vessels. This appearance of the cranial end of the lesion was a consistent observation for all experimental groups.

Twenty-eight days post-transplantation

Lesions were examined in three animals at 28 DPT. The extent of astrocyte integration with the demyelinated axons was greater than that observed at 14 DPT, with larger areas of the lesion consisting of demyelinated axons separated from one another by a dense matrix of astrocyte processes (Fig. 6). However, the most striking difference at 28 DPT compared with 7 and 14 DPT was the presence of small number of axons that were remyelinated by central myelin, implying the presence of differentiated oligodendrocytes within the lesion (Figs 7,8). Frequently, these patches of oligodendrocyte remyelination occurred in close proximity to clusters of SEP-like cells (Fig. 7). Occasional Schwann cell-remyelinated axons were also observed (Fig. 8).

TRANSPLANTATION OF ASTROCYTE CULTURES PURIFIED BY IMMUNOCYTOLYSIS AND EXPOSURE TO 10 GRAYS OF X-IRRADIATION (PREPARATION 2)

Fourteen days post-transplantation

Lesions were examined from three animals at 14 DPT. The lesions were similar in appearance to the 14 DPT lesions obtained following transplantation of preparation 1 cultures, and it was not possible to identify clear differences between the two groups. There was no evidence of oligodendrocyte remyelination or Schwann cell remyelination.

Twenty-eight days post-transplantation

Lesions from three animals were examined at 28 DPT. In all three animals central-type (Fig. 8) and peripheral-type (Fig. 9) remyelination was observed. In this

Fig. 4. Lesions following transplantation of preparation 1 at 14 DPT. (a) Cells were present throughout the lesion and many of the axons are separated by fine cell processes. Toluidine Blue-stained resin section. × 500. (b) Large astrocytes close to the point of injection. The demyelinated axons in this field have been separated into small groups by astrocyte processes. × 6400. (c) Astrocyte, with an abundance of perinuclear intermediate filament (f), closely apposed to a demyelinated axon (a). × 28 300. (d) Further away from the high cell density areas of the lesion at the point of injection, astrocytes were observed with little perinuclear cytoplasm and long thin processes separating groups of demyelinated axons (compare with Fig. 4b). × 5250.

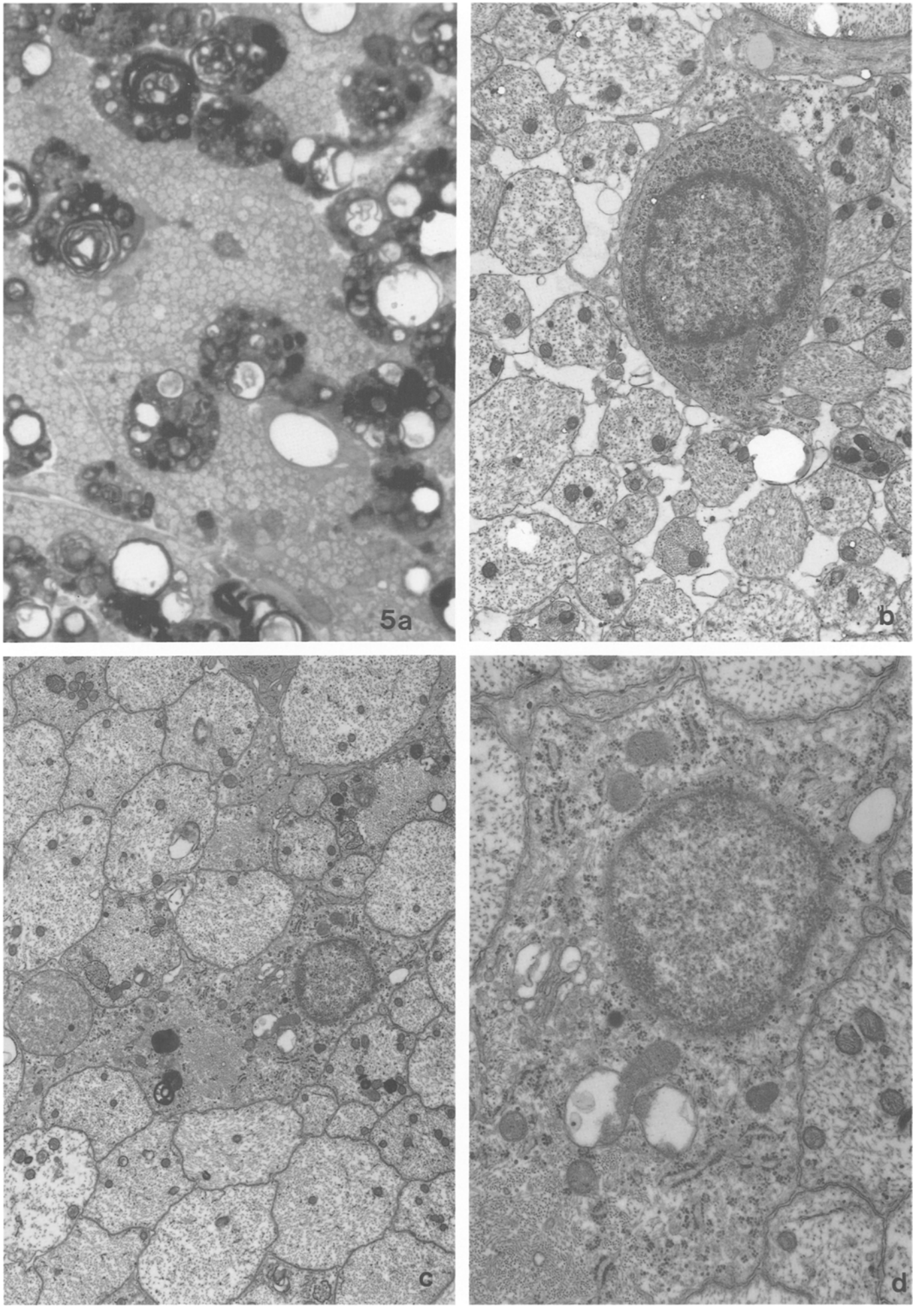
Fig. 5. (a) Area of the lesion remote from the injection point containing cells at low density among demyelinated axons. Toluidine Blue-stained resin section. × 800. (b) Glial progenitor-like cell. This cell has a high nucleus to cytoplasm ratio and a cytoplasm containing abundant free ribosomes. × 13 000. (c) Glial-filament-containing cell with perinuclear features of immature glial cell. × 8 700. (d) Higher magnification of cell in Fig. 5c. Compare with astrocyte in Fig. 4c. × 28 300.

Fig. 6. Extensive area of astrocyte integration with demyelinated axons. Many of the axons in this field have been isolated from other demyelinated axons by astrocyte processes. × 5700.

Fig. 7. Area of lesion following transplantation of preparation 1 at 28 DPT, containing SEP-like clusters of cells and axons remyelinated by oligodendrocytes. A group of remyelinated axons and associated oligodendrocyte cell body are indicated by the arrow. Toluidine Blue-stained resin section. × 570.

Fig. 8. Axons remyelinated by thin sheaths of central-myelin. × 6250.

Fig. 9. Schwann cell remyelinated axon. The Schwann cell is covered by a thin basal lamina (arrowhead). × 23 800.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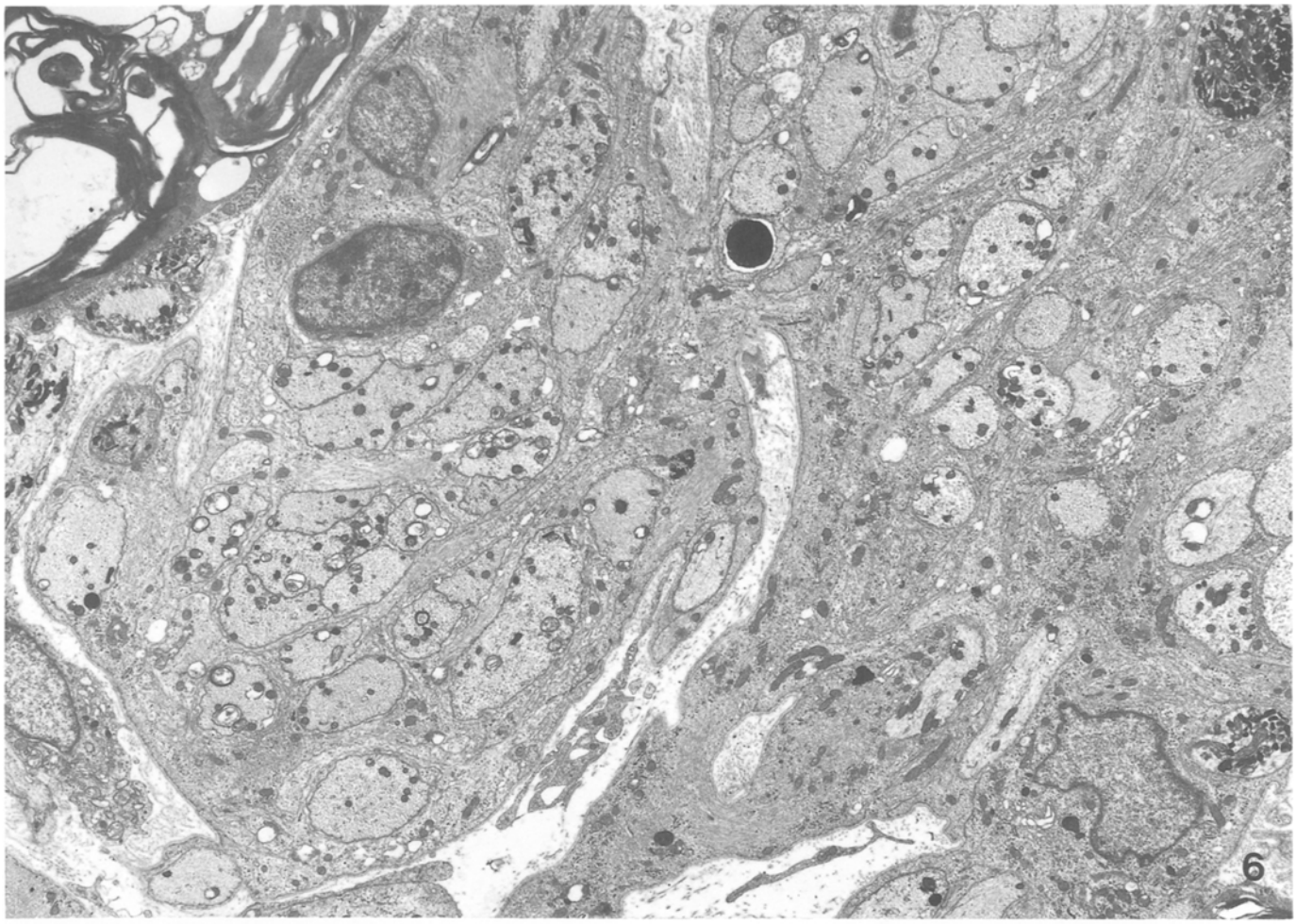
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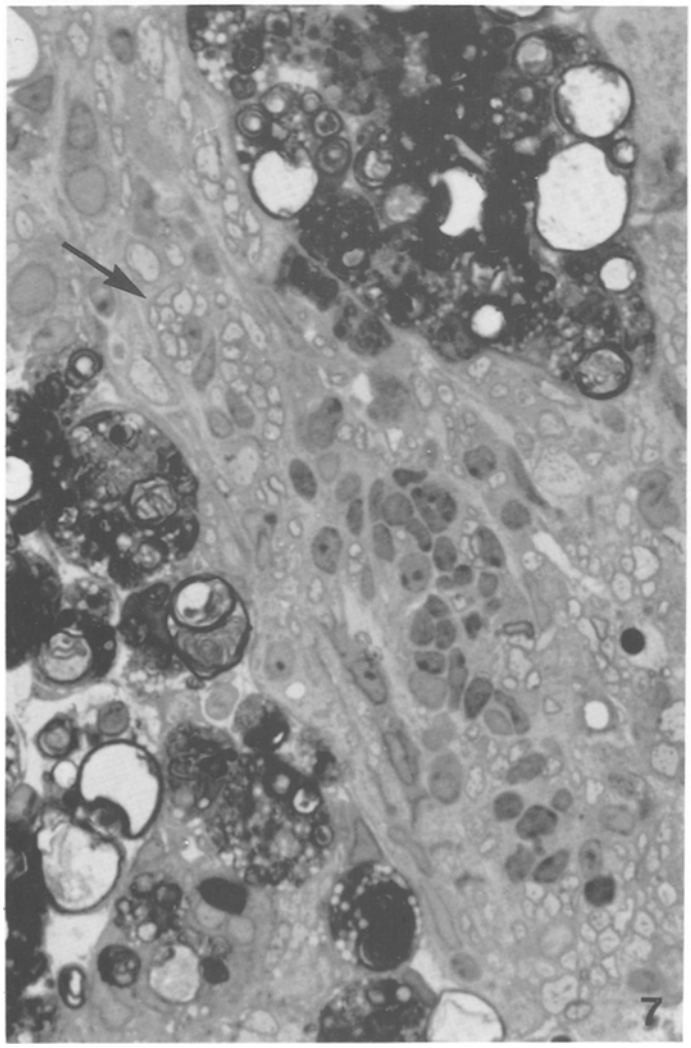
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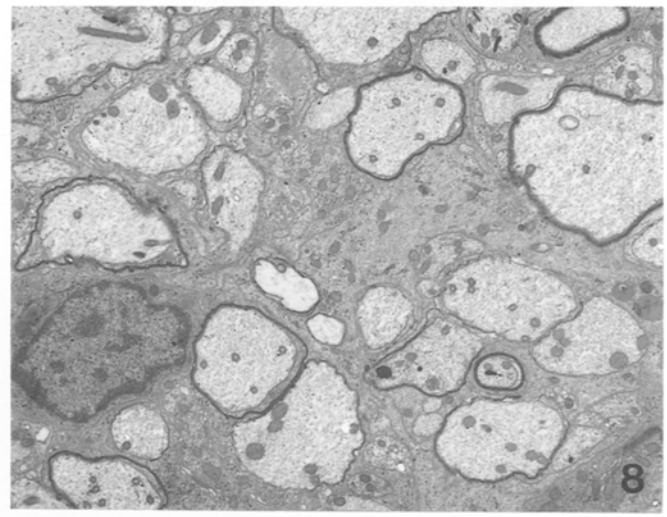
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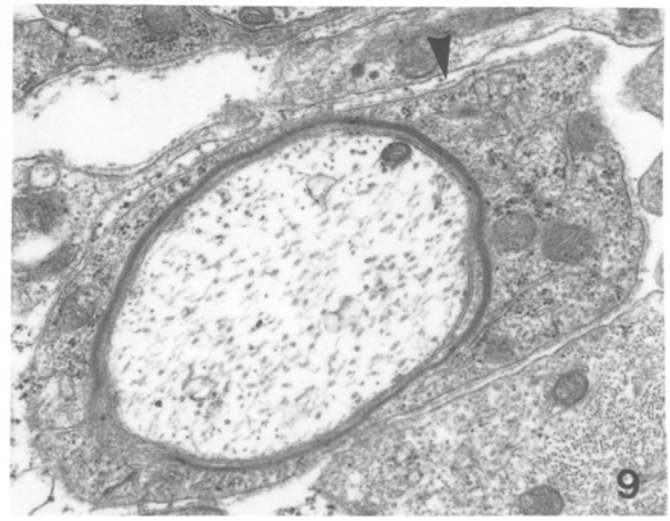
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respect the lesions resembled those receiving preparation 1 cultures at 28 DPT. However, the extent of remyelination was greater than that observed for preparation 1 transplants, and in one animal over 30% of the axons within the lesion had been remyelinated by oligodendrocytes. In all cases the lesions were rich in astrocytes and occasional clusters of SEP-like cells were also observed.

TRANSPLANTATION OF ASTROCYTE CULTURES PURIFIED BY IMMUNOCYTOLYSIS AND EXPOSURE TO 20 GRAYS OF X-IRRADIATION (PREPARATION 3)

Twenty-eight days post-transplantation

Lesions from three animals were examined at 28 DPT. Large astrocytes could be seen in association with the needle tract, and there was integration with demyelinated axons away from this area. Moreover, there were fewer small cells within the surrounding areas of demyelinated axons, although the cells present in these areas resembled those observed in similar locations in the previous preparations. There was no evidence of oligodendrocyte or Schwann cell remyelination within any of the three lesions, neither were clusters of SEP-like cells observed.

TRANSPLANTATION OF ASTROCYTE CULTURES PURIFIED BY IMMUNOCYTOLYSIS AND EXPOSURE TO 30 GRAYS OF X-IRRADIATION (PREPARATION 4)

Twenty-eight days post-transplantation

Lesions were examined in four animals at 28 DPT. The lesions were of a similar appearance to those described for transplantation of preparation 3, and it was not possible to identify clear differences between the two groups. Thus, these lesions contained large astrocytes with some degree of astrocyte integration with demyelinated axons close to the point of injection. There were small cells in the surrounding areas of demyelinated axons which had ultrastructural features of immature glial cells. No clusters of SEP-like cells were observed and there was no oligodendrocyte or Schwann cell remyelination.

The types of cells identified within lesions following transplantation of cell preparations 1–4 are summarized in Table 3.

Discussion

In this paper we have addressed the issue of whether it is possible to reconstruct an astrocytic environment around demyelinated CNS axons by means of cell transplantation. To do this we have transplanted cultures of astrocytes derived from neonatal forebrain into a permanently glia-free region of spinal cord white matter created by the local injection of ethidium bromide into X-irradiated tissue.

Generation of purified astrocyte cultures from neonatal forebrain

In order to focus attention on the ability of cultured astrocytes alone to establish within glia-free lesions and thereby create a purely astrocytic environment, it was necessary to remove oligodendrocyte lineage cells from our cultures and minimize the degree of Schwann cell contamination that invariably occurs in CNS cultures. Our initial strategy was to employ a purification protocol used in previous experiments which involved complement-mediated immunocytolysis with tetanus toxin antibody and A2B5 monoclonal to remove O-2A progenitors (Raff *et al.*, 1983a) and O4 monoclonal to remove immature and mature oligodendrocytes (Gard & Pfeiffer. 1989), in combination with cytosine arabinoside treatment. This strategy is an extension of that used by other workers to remove O-2A lineage cells (Raff *et al.*, 1983a; Grinspan *et al.*, 1990; Emmett *et al.*, 1991) and has been shown to deplete mixed glial cultures of O-2A progenitors and

Table 3. This table summarises the various cell types identified within the lesion following transplantation of cell preparations 1–4 and for the control animals. The criteria on which cell identification was based are outlined in the text.

Transplanted cell preparation	*Survival time (days)*	*Large astrocytes (mature?)*	*Small astrocytes (immature?)*	*SEP clusters*	*Glial progenitors*	*Schwann cells*	*Oligodendrocytes*
Prep 1	7d	+	−	+	−	−	−
	14d	+	+	+	+	+	−
	28d	+	+	+	+	+	+
Prep 2	14d	+	+	+	+	+	−
	28d	+	+	+	+	+	+
Prep 3	28d	+	+	−	+	−	−
Prep 4	28d	+	+	−	+	−	−
Control	28d	−	−	−	−	−	−

later stages in the O-2A lineage for 28 days (longest period examined) *in vitro* (Franklin *et al.*, 1990).

The appearance of oligodendrocytes and central-type remyelination in the lesions examined 28 DPT indicates that, in spite of the apparent absence of O-2A progenitor or subsequent stages in the oligodendrocyte lineage *in vitro*, the transplant suspensions nevertheless contained cells capable of generating oligodendrocytes. A possible explanation is that our cultures carried cells earlier in the oligodendrocyte lineage than the A2B5$^+$ progenitor. Two recent tissue culture studies have described such a cell, termed the 'pre-O-2A progenitor' (Grinspan *et al.*, 1990; Hardy & Reynolds, 1991). A clear difference exists between the behaviour of the cells, the lesions and the cells grown in parallel *in vitro*, where the culture did not generate A2B5$^+$ cells or later phenotypes of the oligodendrocyte lineage. Clearly, the lesion environment contains factor(s) not present *in vitro* that promote the differentiation of the transplanted oligodendrocyte precursors. A notable difference between the *in vitro* and *in vivo* environments is the abundance of demyelinated axons in the latter situation. In this context it is of interest that axons are a potential source of platelet-derived growth factor (Yeh *et al.*, 1991), a growth factor that induces differentiation and division of pre-O-2A progenitors (Grinspan *et al.*, 1990). The precise morphological identity of the oligodendrocyte precursors that were presumably present at 14 days could not be ascertained since, in the absence of suitable markers, they could not be reliably distinguished from astrocyte precursors on ultrastructural grounds.

In an attempt to render our astrocyte cultures incapable of generating oligodendrocytes, we added the step of X-irradiating the cultures prior to transplantation to our purification protocol. Three doses of irradiation were used; 10 Grays, 20 Grays and 30 Grays. The dose of 10 Grays was insufficient to eliminate the oligodendrocyte precursors since oligodendrocyte remyelination was still observed at 28 days post-transplantation. In this context it is noteworthy that oligodendrocyte lineage cells from forebrain appear particularly refractory to X-irradiation-induced death *in vitro* (Van der Maazen *et al.*, 1991); moreover, the SEP is rapidly repopulated following 10 Grays of head X-irradiation (Cavanagh & Hopewell, 1972). In contrast, the cultures that were treated with 20 and 30 Grays of X-irradiation did not generate oligodendrocytes by 28 days post-transplantation, indicating that these high levels of X-irradiation were necessary to deplete the cultures of cells capable of generating oligodendrocytes. The absence of oligodendrocyte remyelination confirmed that the oligodendrocyte remyelination seen at 28 DPT following transplantation of preparations 1 and 2 was not due to recruitment of oligodendrocyte-lineage cells from host tissue and therefore also served to validate the X-irradiated EB lesion as a system where recruitment of host glia into the lesion does not occur. Moreover, levels of X-irradiation necessary to deplete oligodendrocyte lineage cells *in vitro* are not fatal to the cells contributing to the creation of an astrocytic matrix *in vivo*, although the degree to which their normal function is impaired is not known.

The presence of Schwann cells in culture subjected to an immunocytolysis/cytosine arabinoside based O-2A-lineage depletion protocol is variable. The presence of an antigen recognised by the O4 monoclonal antibody on Schwann cells *in vitro* (Mirsky *et al.*, 1990) would suggest that the inclusion of O4 in the immunocytolysis protocol would result in a depletion in Schwann cells. However, this has not been a consistent finding in our studies. In an earlier study, when similarly prepared astrocyte cultures were transplanted into an X-irradiated EB lesion, sufficient numbers of Schwann cells survived the immunocytolysis protocol to generate extensive Schwann cell remyelination (Franklin *et al.*, 1992). In this study, although small pockets of Schwann cell remyelination were evident in the lesions following transplantation of the non-irradiated cultures, these were clearly not present in large enough numbers to extensively remyelinate the lesion. This suggests that there may be a critical ratio of astrocytes to Schwann cells in the transplant suspension which if exceeded results in a situation where Schwann cells become the dominant cell contributing to the reconstruction of the lesion. The tendency for cultured Schwann cells to lose the O4 binding site when divorced from axonal contact (Mirsky *et al.*, 1990) may lead to a situation where the number of Schwann cells continuing to express this binding site, and hence remain amenable to O4-mediated immunocytolysis, varies from culture to culture.

A variety of morphologically distinct cell types can be identified following transplantation of astrocyte cultures into X-irradiated EB lesions

When preparation 1 cultures were transplanted into X-irradiated EB lesions two distinct populations of cells could be recognised after a survival period of 7 days. Large astrocytes with abundant cytoplasm containing stacks of intermediate glial filaments could be seen in association with the point of injection. These cells most likely corresponded to the large, heavily-labelling GFAP$^+$ type 1-like astrocytes identified in tissue culture. The second cell type were smaller cells that existed as tightly packed clusters. These cells resembled cells of the sub-ependymal plate in terms of both their ultrastructural morphology and their packing density (Blakemore, 1969, McDermott & Lantos, 1991). Since the cultures were prepared from the entire neonatal forebrain, it is almost certain that the sub-ependymal plate (SEP) (or subventricular zone) was

included within the culture and therefore within the transplant suspension. Thus, it is conceivable that the SEP-like clusters seen within the lesions were indeed of SEP origin. It is interesting that clusters of SEP-like cells are not seen within lesions that received transplants of cultures that had been exposed to 20 or 30 Grays of X-irradiation (preparations 3 and 4). These levels of X-irradiation are required to depopulate the sub-ependyma of adult rat (Cavanagh & Hopewell, 1972), and also appear necessary for efficient depletion of SEP cells *in vitro*.

In addition to the readily identifiable large astrocytes and SEP-like clusters, a number of other cell-types with distinctive morphologies became apparent at later survival times. Some of these small cells resembled immature glial cells, resembling neither oligodendrocytes nor astrocytes (Levine & Goldman, 1989), while others contained glial filament and were regarded as immature astrocytes. Thus, by reducing the number of oligodendrocyte lineage cells transplanted into non-repairing EB lesions, the heterogenous nature of the residual cells is revealed. While in tissue culture we have identified a single class of $GFAP^+/A2B5^-$ astrocytes, widely referred to as type 1 or type 1-like astrocytes (Raff, 1990), a variety of astrocytes morphologies are observed within the lesions in addition to mature astrocytes and SEP-plate like cells. It is possible that the diversity of astrocyte types observed within the lesions reflects a heterogeneity of the astrocyte population in tissue culture that is not revealed using a limited panel of antibodies. Such a conclusion is consistent with a number of recent tissue culture studies that have drawn attention to the diversity of astrocytes (Miller & Sziget, 1991; Vaysse & Goldman, 1992). It is also possible, however, that this variation may reflect different stages of maturation of essentially similar cells.

Transplanted astrocytes are able to form an integrated astrocytic matrix around demyelinated axons

This study has demonstrated that transplanted cultured astrocytes are able to establish an astrocytic environment around demyelinated axons. Large astrocytes appear to possess a limited capacity to extensively integrate in glia-free lesions since they are generally only found close to the point of injection. Away from the point of injection, immature glia and immature astrocytes could be seen amongst the demyelinated axons at early survival times. At longer survival times these remote areas of the lesion may contain an integrated astrocyte matrix consisting of demyelinated axons surrounded by robust astrocyte processes containing prominent glial filament (eg. Fig. 6). Thus the extent of astrocyte integration increases with time, with areas containing immature cells at early survival times becoming astrocyte-integrated areas at later survival times. We have interpreted these observations as indicating that immature cells migrate away from the injection point and subsequently differentiate into mature, matrix-generating astrocytes. A number of studies provide support for the concept of migrating immature astrocytes (Goldberg & Bernstein, 1988; Ignacio *et al.*, 1990), which appear to migrate both *in vivo* and *in vitro* more rapidly than mature astrocyte (Smith & Miller, 1991). By contrast the large filament containing type 1-like astrocyte possesses a limited capacity to migrate within an environment of demyelinated axons, although these cells were consistently observed in association with blood vessels in the cranial limits of the lesion. Such a pathway of migration of large 'epithelioid' astrocytes has been described following transplantation of cultures similar to those used in our experiments into non-lesioned adult brain (Emmett *et al.*, 1991). The relationship between the immature glial cells, the immature astrocytes and the large mature astrocytes is not clear from these studies, neither is the relationship of these immature glial cells to the cells within the SEP-like clusters, with which they share a morphological similarity. It is tempting to speculate that these clusters may be a source of the migratory immature glial cells. However, the clusters can not be the sole source of these cells since they are evident in the lesions containing 20 Gray and 30 Gray irradiated cultures, situations where clusters were not observed.

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