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Myelination by oligodendrocytes isolated from 4–6-week-old rat central nervous system and transplanted into newborn shiverer brain

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SUMMARY

Oligodendrocytes isolated from 4–6-week-old rat brains were transplanted into newborn shiverer brains. Cells were identified as mature oligodendrocytes both by immunocytological and ultrastructural criteria. Normal myelin was detected using immunolocalisation (with an anti-MBP antiserum) and electron microscopy (presence of the major dense line). Patches of normal myelin (made by transplanted oligodendrocytes), widely spread throughout the host brains, were detected between 20 and 130 days after grafting. No sign of acute rejection was observed, but the graft became progressively delimited by astrocytic processes forming a continuous basal lamina.

Key words: Myelination; Matured oligodendrocytes; Shiverer transplantation model

INTRODUCTION

Remyelination in the mammalian central nervous system (CNS) has been shown to occur under various experimental conditions, such as chemically-induced (Hall 1972; Ludwin 1978; Blakemore 1981; Harrison 1983) or virus-induced demyelination (Herndon et al. 1977; Nagashima et al. 1979; Weiner et al. 1980; DalCanto et al. 1982).

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In human demyelinating diseases like multiple sclerosis (MS), remyelination also takes place, although to a limited extent (Perier and Gregoire 1965; Feigin and Popoff 1966; Ghatak et al. 1973; Prineas and Connel 1979; Harrison 1983). Schwann cells can participate in CNS remyelination (Blakemore 1983), but it is now clear that oligodendrocytes are responsible for most of the remyelination in the brain. The question as to whether these are differentiated, adult oligodendrocytes or undifferentiated stem cells is still open. On the one hand, Raff et al. (1983, 1985) have recently demonstrated in the rat that oligodendrocytes and type II astrocytes derive from a common progenitor cell (O2A), stained by A2B5 antibody. These progenitor cells are still present in the optic nerve of adult rats (Ffrench-Constant and Raff 1986). On the other hand, the ability of differentiated adult oligodendrocytes to divide and/or synthesize myelin again is suggested by several recent works (Ludwin 1984; Aranella et al. 1984; Szuchet et al. 1986; Wood and Bunge 1986).

The aim of this study was to investigate whether oligodendrocytes isolated from 4–6-week-old rat brain and characterized as matured cells by immunocytochemistry and electron microscopy (EM) can differentiate and synthesize myelin. The shiverer transplantation model (Lachapelle et al. 1983/84; Gumpel et al. 1985; Baulac et al. 1987) has been used to differentiate myelin formed by transplanted oligodendrocytes from that of the shiverer host. The mouse shiverer mutation consists of a large deletion in the gene coding for myelin basic protein (MBP) (Roach et al. 1985) located on chromosome 18 (Sidman et al. 1985). This deletion prevents the expression of MBP, leading to a defect in myelin compaction and an absence of the major dense line (MDL) (Jacque et al. 1978; Privat et al. 1979). Thus, myelin formed by normal oligodendrocytes can be easily detected, either by immunohistochemistry (using an anti-MBP antiserum) and/or by EM (showing the presence of MDL). In the present study, we transplanted oligodendrocytes isolated from young adult rat brains into the brains of newborn shiverer mice. Since myelination is postnatal in the mouse (Folch-Pi 1955; Monge et al. 1986), the transplanted cells were thus present in the brain during the normal process of myelination, when factors responsible for myelin differentiation are fully expressed.

MATERIALS AND METHODS

Isolation of oligodendrocytes

Oligodendrocytes were isolated from 4–6-week-old rat brains, according to Lisak et al. (1981). Briefly, rat forebrains were dissociated first mechanically and then by trypsin digestion. Oligodendrocytes were purified by centrifugation on a Percoll density gradient. The oligodendrocyte-rich fraction was then washed twice by centrifugation; the pellet was allowed to reaggregate in 50 μ l of DMEM supplemented with 10% fetal calf serum (Flow Lab.) for 1 h before transplantation.

Indirect immunofluorescence (IIF) on isolated cells

Immunological reagents

Details on immunological reagents and their use (mouse monoclonal antigalactosylceramide (GalC) antibody, rabbit polyclonal affinity purified anti-GalC antibody, rabbit anti human myelin basic protein (MBP) polyclonal antibody, rabbit anti-human gliofibrillary acidic protein (GFAP) polyclonal antibody, anti-neuron-specific enolase polyclonal antibody) have been presented elsewhere (Lubetzki et al. 1986). A2B5 monoclonal antibody (Eisenbarth et al. 1979) was used diluted 1 : 2.

Indirect immunofluorescence

IIF staining was performed on isolated cells to control the purity of the preparation on an aliquot of cells isolated during the same experiment, but not on the pellet of cells to be transplanted. In order to allow recovery of the cells after trypsinisation, the IIF procedure was performed on cells maintained in vitro for 18–24 h on poly-L-lysine-coated (PLL, Sigma) glass coverslips (Yavin and Yavin 1974). The technique of IIF has been described in detail elsewhere (Lubetzki et al. 1986). The specificity of immunostaining was controlled by incubating some of the cell preparations with non-immune rabbit IgG (100 µg/ml), preimmune rabbit sera (diluted 1:100), or MEM containing 10% fetal calf serum in place of the first antibody. These controls were consistently negative.

Standard electron microscopy (Transmission electron microscopy (TEM))

Fragments of the pellets of reaggregated cells were examined by TEM to determine the composition of the preparation and the state of the cells before transplantation. The other fragments were grafted. The samples were processed as usual for standard TEM. They were fixed at 4 °C in 0.1 M phosphate buffer, pH 7.4, containing 2.5% glutaraldehyde and washed overnight in the same buffer. After post-fixation in 2% osmic acid in water for 1 h, the samples were dehydrated in graded ethanol and propylene oxide and then embedded in Epon. Semithin sections were examined first after staining with toluidine blue. Ultrathin sections were then studied on a JEOL 100 CX electron microscope operating at 80 kV, after contrasting with uranyl acetate and lead citrate.

This technique was also used to study host-graft interactions after implantation. After intracardial perfusion of the fixative described above, the host brain was dissected out, and the implantation site, indicated by the presence of charcoal (see transplantation technique) was removed, post-fixed and processed for TEM.

Transplantation technique

After 1 h reaggregation in an incubator at 37 °C, the preparation of oligodendrocytes formed a solid, elastic pellet which could be fragmented with scissors. As the cells adhere strongly to each other, the fragments immediately formed spheres. The fragments

were marked with charcoal, and transplanted with a thin pipette connected to a peristaltic pump as described previously (Lachapelle et al. 1983/84). The pellet fragment (around 200 000 cells) was implanted into a newborn shiverer brain at various levels caudorostrally and at various distances from surface of the brain. Most of the grafts were placed in the striatum, hippocampus and rostral thalamus.

Immunolocalisation of the MBP-positive myelin in the shiverer brain

The immunolocalisation was performed 20–130 days after implantation. The technique used was previously described (Lachapelle et al. 1983/84). Briefly, after anesthesia, the host animals were perfused intracardially with a 4% PFA solution in 0.1 M sodium phosphate buffer, pH 7.4. After excision, the brains were immersed in the same fixative for 6 h and rinsed in the same buffer containing 5% sucrose for 48 h. After embedding in OCT (Miles) and freezing, the brains were sectioned sagittally on a cryostat. Sections were collected only at the level of the graft, identified by the presence of charcoal. Immunohistological localisation using an anti-MBP antiserum with either indirect immunofluorescence or PAP technique (Sternberger et al. 1970) was performed as previously described.

Immunohistological prelocalisation before TEM examination

Since MBP positive patches of myelin can be found throughout the host brains (Baulac et al. 1987), it was necessary to detect the patches by light microscopy before studying the myelin by TEM. The technique of immunological prelocalisation followed by TEM study has been reported previously (Gansmüller et al. 1986). Briefly, the host was perfused through heart with a solution of 4% PFA and 1% glutaraldehyde in sodium phosphate buffer. Alternating 10 and 60 μm sections were cut on a cryostat. The 10 μm sections were treated for immunohistological detection of MBP positive myelin patches. When MBP positive zones were detected, the corresponding regions were dissected on the preceding and following 60 μm sections, and processed for TEM observation (see below). Semithin sections stained by toluidine blue were first examined to determine the localisation of normal myelin. Ultrathin sections were then cut at the prelocalised sites and observed with TEM as previously described.

RESULTS

Immunocytochemical characterization of isolated cells

The cell population obtained after isolation was 95% oligodendrocytes, characterized as GalC positive cells (Raff et al. 1979) (Fig. 1). These oligodendrocytes were small round cells, with no visible processes. All the cells expressing GalC also stained with the anti-MBP antibody (Lubetzki et al. 1986). All our preparations were devoid of O2A progenitor cells, identified by the expression of A2B5, except one which

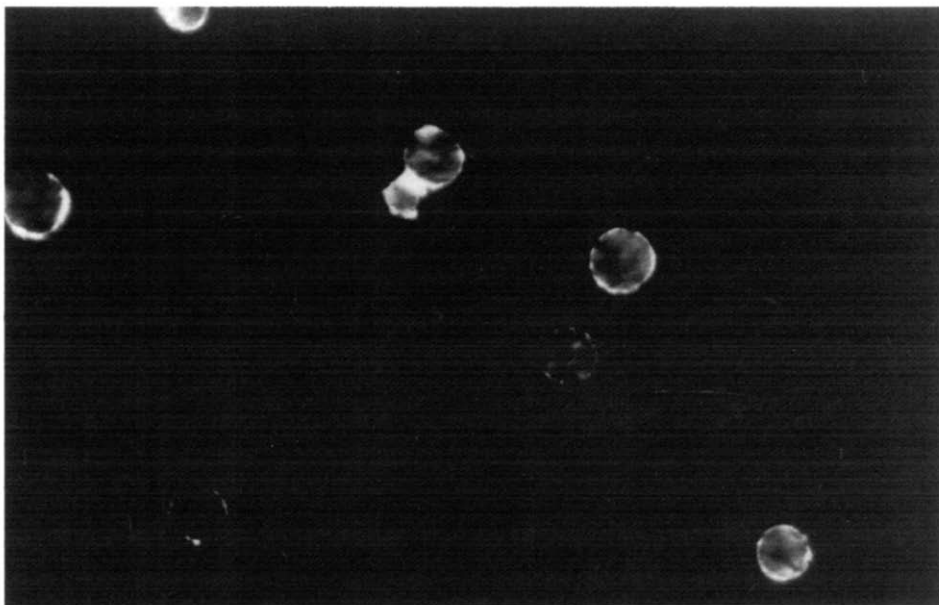


Fig. 1. Rat oligodendrocytes 18 h after isolation stained with an anti-GalC monoclonal antibody ($\times 400$).

contained 0.2% A2B5 positive cells. In preparations of newborn oligodendrocytes, however, we detected numerous A2B5 positive cells with the same technique (unpublished results). Furthermore, no cells positive for either neuron specific enolase or GFAP were ever observed, suggesting that our preparations were virtually devoid of neurons and immunodetectable astrocytic contamination. Cell debris was always observed.

Ultrastructural observation of cells after isolation

Examination of ultrathin sections of the pellets showed them to contain around 90% oligodendrocytes, identified by their chromatin, the electron density of the cytoplasm which contains an important rough endoplasmic reticulum, the presence of intense membrane activity (Fig. 2) and the formation of thin cytoplasmic processes. In a few cells, we observed some lamellar myelin-like structures. According to the criteria defined by Mori and Leblond (1970), most of the oligodendrocytes appeared as "dark" (mature) cells. However, a low percentage of cells looked like medium oligodendrocytes, with a larger nucleus and a lower cytoplasmic density. About 10% of the living cells were not oligodendroglial. Based on ultrastructural criteria, they could be identified as lymphocytes, ependymal cells, astrocytes and red blood cells. A variable amount of naked nuclei (which in certain preparations could reach 50% of the elements in the pellet) were always present. They were identified as neuronal nuclei by their characteristic double nuclear membrane, but no intact neuron was ever observed.

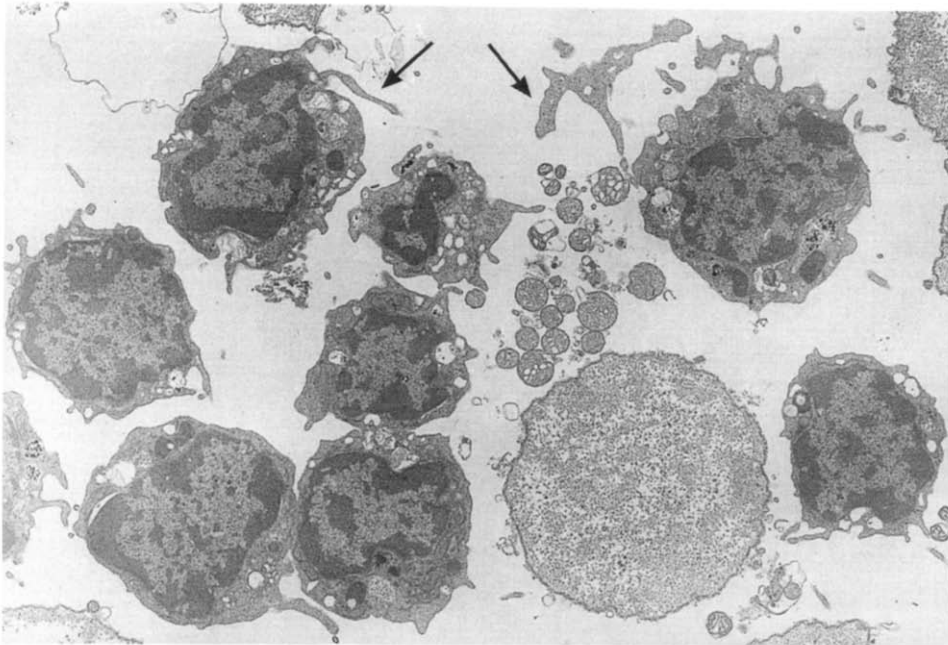


Fig. 2. Fragment of the pellet obtained just before transplantation. Observation using standard TEM. Note the presence of oligodendroglial processes (arrows) and the presence of one nude neuron nucleus ($\times 5000$).

Immunohistological studies of myelination by oligodendrocytes isolated from adult rats

Thirty-two shiverer host brains have been fixed for immunohistochemical observation 20–130 days after transplantation. Seven animals were killed 20 days after grafting, 21 between 40 and 90 days and 4 after 130 days. The sham control experiment in which shiverer brain fragments were grafted into newborn shiverer brain, always negative in previous studies (Lachapelle et al. 1983/84), were not repeated in these experiments. After fixation and dissection of the brain, the graft was localised by the presence of charcoal. The grafted host brains were sectioned sagittally on a cryostat. The sections ($8\ \mu\text{m}$) were collected only where charcoal was visible and were treated for immunohistochemical detection of MBP (IIF or PAP technique). In each series of experiments, sagittal cryostat sections of shiverer and normal brains of the same age were used as negative and positive controls for the immunohistological reaction.

Seven animals were studied 20 days after transplantation. In 5 cases, no MBP-positive myelin could be detected although the graft was present and appeared healthy. In one case, a few MBP positive fibers were detected, but only around the graft. In another case, where the graft was located in the midbrain, numerous MBP positive patches were seen not only around the graft but also in the hypothalamus, cerebellum and brainstem. This suggested that myelination by transplanted matured oligodendrocytes could start earlier than 20 days after transplantation.

Among the 25 host animals killed 40–130 days after transplantation, 6 were

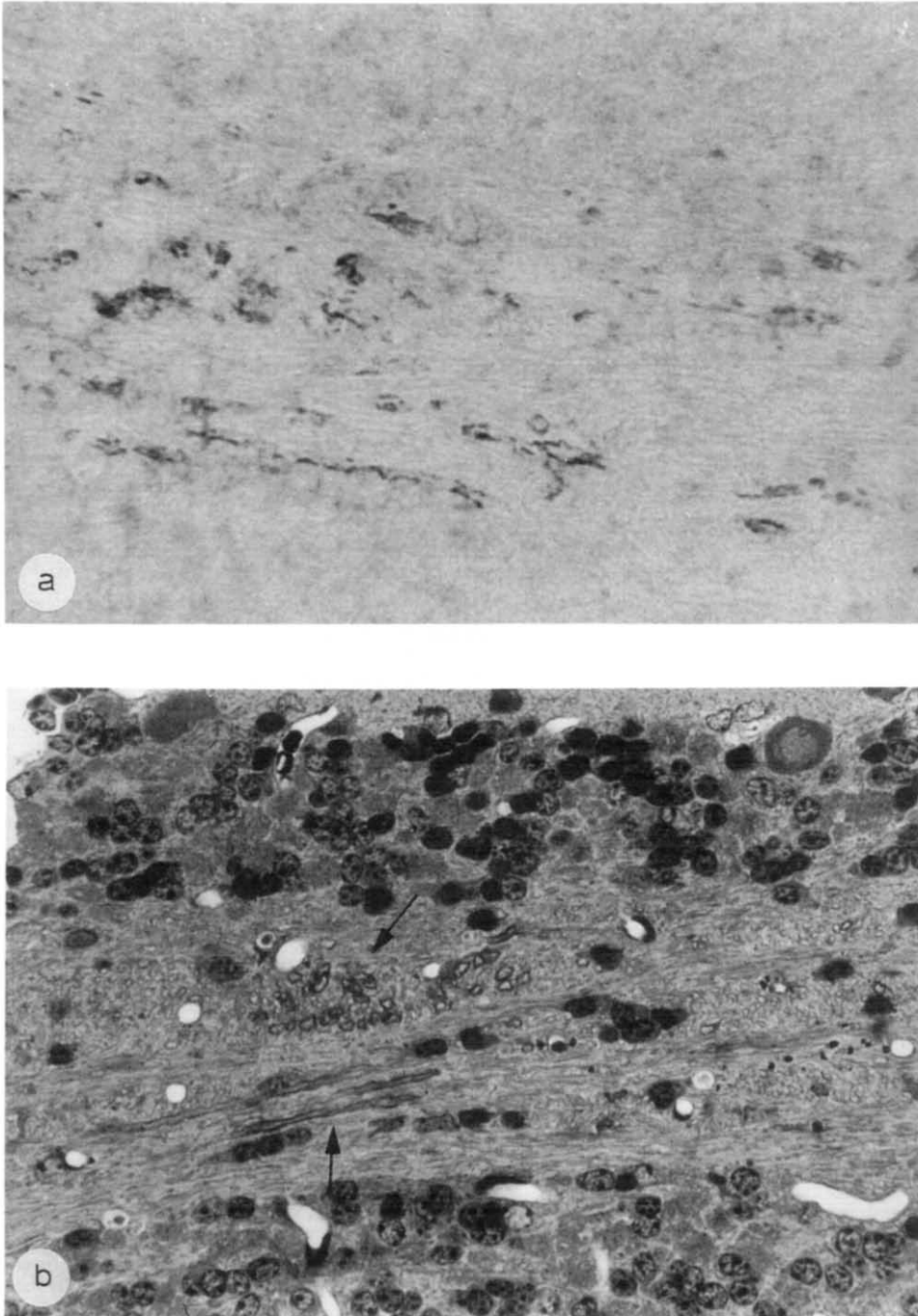


Fig. (3a-b). Immunoprelocalisation of myelin MBP positive patches followed by semithin sections. (a) $10\ \mu\text{m}$ section showing a patch of MBP positive myelin in the cerebellum, after a graft in the rostral thalamus. ($\times 400$). (b) Semithin section of the prelocalised patch. Toluidine blue staining. Even at this low magnification, it is possible to distinguish normal myelin (arrows) from shiverer myelin. ($\times 500$).

negative and 11 showed MBP positive fibers only around the graft (less than 1–2 mm). In the other 8 cases, MBP positive myelination was observed at a distance (more than 1 cm) (Fig. 3).

The distance of migration in the 4 cases examined 130 days after transplantation was comparable to that of MBP positive myelination in the animals killed 20 days after transplantation. Thus, no correlation could be found between extent of myelination and delay after grafting. Migration was most often caudal to the graft (to cerebellum, pons and spinal cord). However, at least in one case, rostral migration was observed.

TEM study of the myelination by transplanted oligodendrocytes

In 2 additional cases (J40 and J130) long distance migration was observed by immunoprelocalisation, from the rostral thalamus caudally to various levels of the brain including cerebellum, brainstem and anterior spinal cord. The presence of MDL in the myelin sheath in the immunopositive regions confirmed that this myelin was due to implanted oligodendrocytes; the fact that MDL positive myelin was still present at J130 indicated that implanted rat oligodendrocytes had not been rejected and were still viable and functional (Figs. 3 and 4).

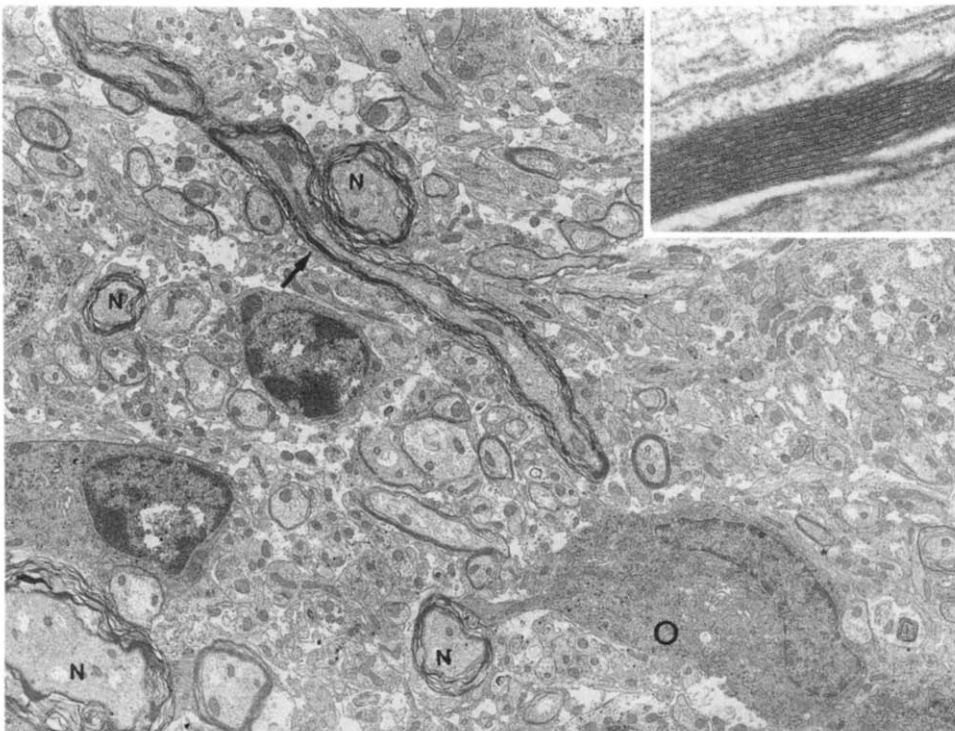


Fig. 4. Ultrathin section of the positive patch shown in Fig. 3. Myelin formed by transplanted oligodendrocytes is normal myelin (N) with MDL (see insert). Note that at lower magnification the myelin seems somewhat dislocated, due to immunoprelocalisation procedure. One oligodendrocyte (O) appears to be in relation with a normally myelinated axon. ($\times 6500$; insert, $\times 82500$).

TEM studies of the host-graft interactions 7–130 days after grafting

Host-graft interactions at the site of implantation (marked with charcoal) were observed by standard TEM. Ten shiverer host brains were studied in this series (7, 20, 40, 70, 90 and 130 days after grafting).

Seven days after grafting, cell debris or neuronal nuclei were no longer visible at the implantation site. The tissue around the graft was very loose, forming a network of cell processes. Glial processes and axons could be observed. Since the graft is deprived of neurons and astrocytes are virtually absent, this indicates that the host participates

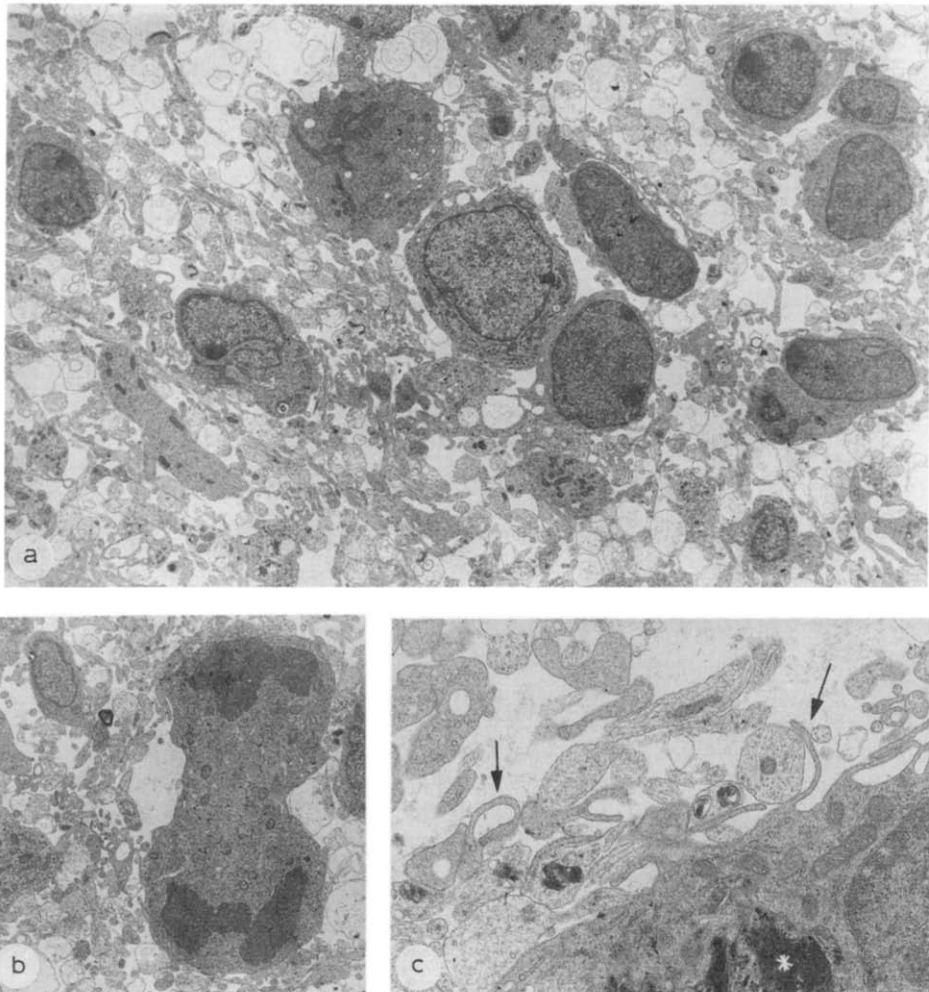


Fig. 5(a–c). Ultrastructural observation of the graft 7 days after transplantation. (a) The parenchyma appears very loose, forming a network of processes from all types of cells. Several oligodendrocytes are observed, with numerous processes ($\times 3200$). (b) Oligodendrocyte mitosis. ($\times 4300$). (c) Oligodendrocyte with a strong membrane activity and cell processes (arrows), some of which have begun to ensheath axons. Note that the oligodendrocyte have phagocytosed charcoal (*), an often observed phenomenon ($\times 11000$).

greatly in the formation of the cell process network. Within this loose parenchyma, we observed a high density of oligodendrocytes; it was not possible, however, to differentiate between donor and host oligodendrocytes although some of them had



Fig. 6. Ultrastructural observation of the graft 20 days after transplantation. Numerous astrocytic processes (Asp) are observed, identified by the presence of gliofilaments (see insert). Note the presence of fragments of basal lamina (arrows). ($\times 12500$; insert, $\times 20000$).

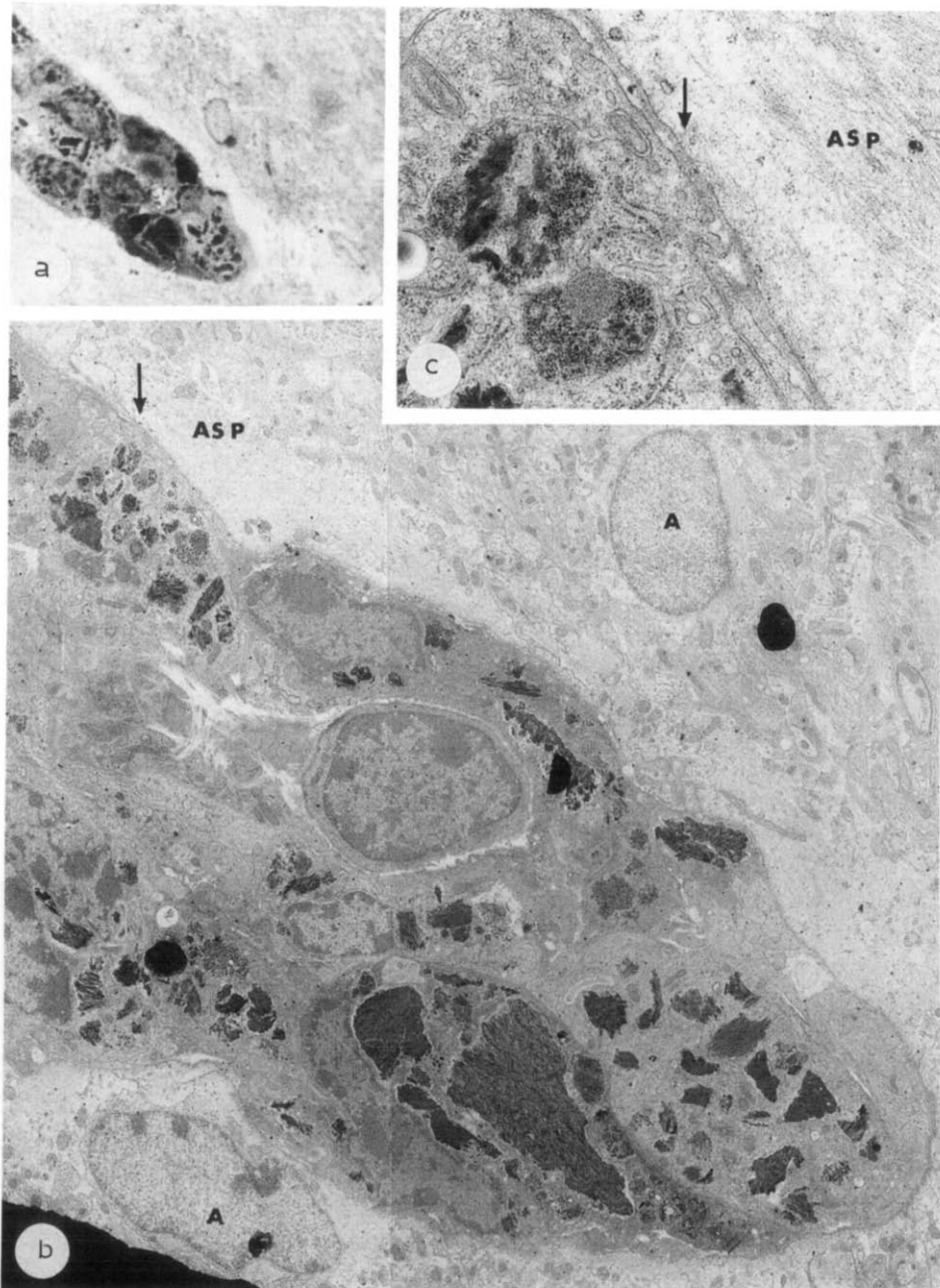


Fig. 7. Observation of the graft 70 days after transplantation. (a) Semithin section at the level of the graft. ($\times 625$). (b) Ultrastructural observation of the graft, showing the presence of astrocytes (A) and astrocytic processes (Asp) ($\times 4000$). (c) Higher magnification showing the presence of gliofilaments (Asp) and a continuous basal lamina (arrows) isolating the graft from the host parenchyma ($\times 25000$).

phagocytosed fragments of charcoal. The oligodendrocytes appeared healthy, with a high membrane activity, numerous long cell processes, presence of typical endoplasmic reticulum, Golgi apparatus, microtubules and filaments. Both dark and medium oligodendrocytes were still recognizable. Interestingly, we observed mitoses among oligodendrocytes with dark cytoplasm (Fig. 5). No myelination (either shiverer or normal) was observed around the graft at this age; the region appeared to be highly vascularized.

Twenty days after grafting, the loose appearance of the parenchyma diminished. The oligodendrocytes still extended long processes but their membrane activity was reduced. The main difference between post transplantation days 7 and 20 was the presence, at 20 days, of a large population of astrocytes. Numerous astrocytic processes could be identified in the area of the graft by the presence of gliofilaments (Fig. 6). At 40, 70 and 130 days after grafting, huge astrocytic processes envelopped and progressively isolated the grafted cells. The graft, still healthy, appeared to be completely surrounded by astrocytic processes and was delimited by a continuous basal lamina (Fig. 7).

DISCUSSION

We have demonstrated in these experiments that matured oligodendrocytes, isolated from 4–6-week-old rat brains, were able to survive, migrate and make normal myelin when transplanted into the brain of a newborn shiverer host. The transplanted cells were 95% oligodendrocytes, identified immunocytochemically by the presence of GalC and MBP, two specific markers of matured oligodendrocytes (Raff et al. 1979) but only 90% of the cells were identified as oligodendrocytes by TEM criteria. This discrepancy is probably due to the impossibility of performing a real cell count on TEM material. Furthermore, a few astrocytes were observed by TEM, although they were not detectable by immunocytochemistry. This can be explained by the difficulty of GFAP staining on newly isolated cells which are round with no extended processes.

Since O2A progenitor cells are still present in the adult rat optic nerve (Ffrench-Constant and Raff 1986), it might be argued that these immature cells are responsible for the myelination observed. However, in our experiments, very few cells were A2B5 positive, and it seems unlikely that they could account for all the myelination detected. Moreover, several studies suggest that mature differentiated oligodendrocytes can divide and/or synthesize myelin. Ludwin et al. (1984) and Aranella et al. (1984) demonstrated that adult oligodendrocytes are able to incorporate [³H]thymidine after experimental demyelination or CNS lesion. More recently, Wood and Bunge (1986) showed that adult oligodendrocytes can remyelinate dorsal root ganglia axons in vitro. In neuron-free cultures, Szuchet et al. (1986) demonstrated that adult oligodendrocytes retain the capacity to make myelin membranes, a phenomenon she called myelin palingenesis. As in these studies, our data also suggest that matured oligodendrocytes are still able to myelinate.

It cannot be excluded, however, that perineuronal (or satellite) oligodendrocytes

are responsible for myelination, although this is not normally the case, since there are no immunocytological or ultrastructural criteria to distinguish them from other oligodendrocytes.

Our experiments raise the question as to whether matured oligodendrocytes can migrate after transplantation into a newborn brain. Since the host brains were sectioned only at the level of the graft, a large proportion of the MBP-positive patches, present either laterally or in the contralateral hemisphere (Baulac et al. 1987), was missed. The extent of migration is therefore certainly underestimated. Nevertheless, in 10 cases (8 after immunohistochemical staining and 2 after immunoprelocalisation and EM) long distance migration (more than 1 cm from the graft) was observed. This long distance migration was recently studied on serial sections of the whole brain, after transplantation of either newborn mouse (Gansmüller et al. 1986) or embryonic human (Gumpel et al. 1987) CNS fragments. However, in the latter experiments, the grafted fragments contained a high percentage of progenitor cells. These progenitor cells are able to migrate, as demonstrated by Small et al. (1987) in the rat optic nerve. Our results suggest that matured oligodendrocytes, when present in the brain during the normal myelination process might reexpress those properties enabling them to migrate. However, passive transport along elongating axons (Gansmüller et al. 1986) cannot be totally ruled out. Finally, no sign of acute rejection was observed. The grafted cells at the site of implantation appeared healthy 5 months after transplantation; the MBP positive patches were still present, indicating the survival of the transplanted oligodendrocytes. However, the graft itself was progressively surrounded by numerous astrocytic processes and became completely isolated from the host parenchyma by a delimiting basal lamina. A similar observation was made after transplantation of embryonic human (Gumpel et al. 1987) or newborn mouse CNS (Gansmüller et al. 1986). This phenomenon seems peculiar to the CNS transplantation and remains to be elucidated. Interestingly, no such host reaction was ever observed around the MBP-positive myelin patches, suggesting a specific tolerance for grafted cells participating in CNS development. It should be noted that long term immunological rejection cannot be excluded since the survival of the shiverer mouse never exceeds 150 days.

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