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Isolation and characterization of defective jimpy oligodendrocytes in culture

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Summary

This study characterizes jimpy oligodendrocyte-enriched secondary cultures isolated from 10–12 days *in vitro* primary glial cell cultures derived from 1–2-day-old jimpy mouse brains. Proliferation of defective oligodendrocytes was carefully investigated with regard to the expression of myelin basic protein and proteolipid protein and their respective mRNAs. Less than 5% of contaminating astrocytes ($GFAP^+$ cells) were usually present. The identity of jimpy oligodendrocytes was confirmed using an antibody directed against a peptide from the wild type proteolipid protein C-terminal sequence for immunocytochemistry and an oligonucleotide complementary to mRNA derived from exon 5 of the proteolipid protein gene for *in situ* hybridization. Both the antibody and the probe recognize only normal oligondendrocytes while jimpy oligodendrocytes always remain unstained. Proteolipid protein in normal and jimpy oligodendrocytes was detected with antibody recognizing normal and mutated forms. Between 80 and 95% of the cells in normal and jimpy cultures at 2 and 4 days *in vitro* in secondary cultures express myelin basic protein and proteolipid protein and their respective mRNAs. The percentage of oligodendrocytes (PLP^+ or MBP^+) in S phase of the cell cycle was 7–10% for both normal and jimpy oligodendrocytes. This contrasts with the *in vivo* situation where the proliferation rate of oligodendrocytes in jimpy brains is higher than in normal brains. In addition, jimpy oligodendrocytes remain unresponsive to basic fibroblast growth factor treatment while a similar treatment stimulates the proliferation of normal oligodendrocytes.

Introduction

The jimpy mutation is a recessive X-linked lethal mutation (Eicher & Hoppe, 1973). Jimpy mice are neurological mutants characterized by a severe deficiency of myelin in the CNS (Sidman *et al.*, 1964). All myelin components in jimpy CNS are expressed at reduced levels (Nussbaum *et al.*, 1969; Matthieu *et al.*, 1973; Barbarese *et al.*, 1979; Campagnoni *et al.*, 1984; Yanagisawa & Quarles, 1986; and for a review see Campagnoni & Macklin, 1988). The myelin basic protein (MBP) gene has been mapped to the jimpy locus on chromosome X (Willard & Riordan, 1985; Dautigny *et al.*, 1986). The genetic alteration in jimpy mice was established as a point mutation in exon 5 of proteolipid protein (PLP) gene (Hudson *et al.*, 1987; Macklin *et al.*, 1987; Nave *et al.*, 1987).

Despite the high proliferative rate of jimpy oligodendrocytes *in vivo* (Privat *et al.*, 1982; Skoff, 1982), their number remains similar to that observed in the normal brain during the first 2 weeks after birth (Ghandour & Skoff, 1988). The normal number of defective oligodendrocytes is probably maintained by the high cell death rate found in jimpy brain (Knapp *et al.*, 1986). These oligodendrocytes remain immature (Ghandour & Skoff, 1988) with a cell cycle defect (Knapp & Skoff, 1987). In addition to oligodendrocyte alteration, astrocyte abnormalities have been observed in jimpy nervous tissue (Keen *et al.*, 1976; Skoff, 1976; Hertz *et al.*, 1980; Omlin & Anders, 1983).

The relationship between the PLP gene or its abnormal product and both hypomyelination and alterations in oligodendrocytes, such as the under-expression of all the other myelin components (for review see Campagnani & Macklin, 1988) has not yet been clearly established. Further and extensive investigations are needed *in vivo* and *in vitro* to elucidate the signals leading to the hypomyelination in the CNS of jimpy mice.

The aims of the present study are first, to establish highly enriched oligodendrocyte cultures from jimpy mouse brain for detailed specific studies on altered

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oligodendrocytes and, second, to shed light on the relationship between the proliferation of jimpy oligodendrocytes and the expression of PLP, MBP and their respective mRNAs with or without basic fibroblast growth factor (bFGF) treatment. All previous *in vitro* studies on mutant jimpy oligodendrocytes were performed on mixed primary glial cell cultures where the number of oligondendrocytes was small compared to astrocytes (Bologa-Sandru *et al.*, 1981; Hertz *et al.*, 1980; Bartlett *et al.*, 1988; Knapp *et al.*, 1993) or in organotypic cultures (Billings-Gagliardi *et al.*, 1980, 1983).

Materials and methods

ANIMALS

Tabby and jimpy male (Tajp/Y) mutants and control mice were produced in our laboratory from breeding pairs of 1n (X) 1H Ta^+ jp^+/Ta jp strain (provided by Drs F. Lachapelle and N. Baumann, INSERM U 134, Paris). This strain was obtained by introducing the metacentric inversion 1n (X) 1H of the X chromosome including the Ta and jp alleles in B6CBA/$A^{W/J}$ Ta jp stock originally obtained from Jackson Laboratory (Bar Harbor, Maine). The main advantage of this strain is the suppression of viable recombinations between jimpy and Tabby loci (Lachapelle *et al.*, 1990a).

CELL CULTURES

Mixed primary glial cell cultures were prepared from newborn mouse brain hemispheres as previously described (Labourdette *et al.*, 1979) with slight modifications. Each brain was processed and cultured separately. Briefly, meninges were removed and cerebral hemispheres were mechanically dissociated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% calf serum (Gibco-BRL, France), Penicillin (50 U ml^{-1}) and Streptomycin (50 μg ml^{-1}). The suspension from each brain was plated into a 100-mm diameter Petri dish (Falcon) or into a 24-well plate containing 14-mm diameter glass coverslips coated with 20 μg ml^{-1} poly-L-lysine (Sigma). Cultures were then incubated in 5% CO_2 and 95% air in a humidified atmosphere (90%) at 37° C.

Oligodendrocytes in secondary cultures were prepared from 10–12 days *in vitro* (DIV) primary cultures according to a published procedure (Besnard *et al.*, 1989) with a slight modification. Oligodendrocytes growing on the layer of astrocytes were collected by syringing the medium over the cells using a 10 ml syringe (Terumo, Belgium). The cell suspension was preplated in Petri dishes for 15 min in the same incubator as described above and then centrifuged for 10 min at 900 rpm. The pellet was resuspended in DMEM containing 500 μg ml^{-1} bovine serum albumin (BSA), glucose (4.5 mg ml^{-1}) transferrin (10 μg ml^{-1}) and insulin (5 μg ml^{-1}). Cells were then plated on poly-L-lysine coated coverslips at 8×10^4 cells per coverslip into 24-well plates. Cultures were incubated under the same conditions as described above.

To insure that the mitotic activity of oligodendrocytes in culture was not due to the technique used to obtain oligodendrocytes, another established preparation of cell cultures was used. This different preparation of oligodendrocytes was obtained from glial cells isolated on Percoll gradient from newborn Wistar rat brains as described (Lubetzki *et al.*, 1991). Glial cell fraction, after washing in Hank's balanced salt solution (HBSS), was plated on glass coverslips coated with poly-L-lysine in Minimum Essential Medium (MEM) supplemented with 1% foetal calf serum (BRL-GIBCO), insulin (10 μg ml^{-1}), glucose (3 mg ml^{-1}), transferrin (50 μg ml^{-1}) and PDGF (5 ng ml^{-1}) (UBI, New York) at 3×10^5 cells per coverslip.

Oligodendrocytes in secondary cultures were treated with 10 ng ml^{-1} of purified bovin bFGF (a generous gift from Dr G. Labourdette) at 2 h after plating and at 2 DIV (Besnard *et al.*, 1989). Cultures at 2 and 4 DIV were then fixed with paraformaldehyde (see below).

IMMUNOCYTOCHEMISTRY

Cultures were fixed in 4% paraformaldehyde for 15 min at room temperature (RT) and then washed in 50 mM phosphate buffered saline (PBS) (pH 7.8). When the immunoperoxidase procedure was used, endogeneous peroxidase activity was abolished by incubating the cultures with 3% H_2O_2 in methanol or in PBS. Cultures were always preincubated in 10% normal goat serum (NGS) diluted in PBS.

Bromodeoxyuridine immunocytochemistry

Cultures were incubated in the presence of bromodeoxyuridine (BrdU) at 50–100 μM for 1–2 h in DMEM. Cultures were washed with DMEM, then with PBS and fixed in paraformaldehyde as described above. Fixed cultures were treated with 2N HCl for 20 min at RT and then with 1% Triton X-100 in PBS for 10 min. After extensive washes in PBS, the cells were incubated overnight at RT with 10% NGS and 1:20 dilution of a mouse monoclonal antibody to BrdU (Monosan). Cells were rinsed in PBS and then incubated with fluorescein (FITC), rhodamine (TRITC), peroxidase (PO) or alkaline phosphatase (AP)-conjugated goat anti-mouse IgG at 1:100 dilution (Jackson Immuno Research Laboratories).

Glial fibrillary acidic protein, MBP and PLP immunostaining

Paraformaldehyde fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min and then preincubated with 10% NGS for 30 min. After washed in PBS, cells were incubated for 2 h with rabbit antisera directed against either GFAP (Dako), bovine MBP (prepared in our laboratory) or a tridecapeptide corresponding to the sequence 117-129 of PLP (Trifilieff *et al.*, 1986) diluted 1:100 for MBP and PLP or 1:300 for GFAP. An antiserum to the sequence 269-276 of PLP C-terminal (a generous gift from Drs R. P. Skoff and J. Benjamins) was also used at a 1:50 dilution. Cells were then incubated with FITC, TRITC, PO or AP-conjugated second antibodies as described above. Peroxidase activity was revealed using 3,3'-diaminobenzidine -4HCl (DAB) at 0.02% in PBS and 0.003% hydrogen peroxide (H_2O_2) for 10–20 min. Alkaline phosphatase activity was developed in the presence of 5-bromo-4-chloro-3- indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT). A mixture of 35 μl BCIP (45 mg ml^{-1}) and 45 μl NBT (75 mg ml^{-1}) in 10 ml

Tris saline buffer (TBS) (pH 9.5) was used. All incubations were performed at RT.

IN SITU HYBRIDIZATION

Probes

Oligonucleotides complementary to MBP mRNA and PLP mRNA were used for *in situ* hybridization. The probes MBP AS1 and MBP AS2 are 21 and 27 bases long respectively, both from exon 1, PLP AS1 is 21 bases long from exon 3, PLP AS2 is 21 bases long from exon 5, PLP AS3 is 24 bases long from exon 2; sense oligonucleotides were used for controls.

Probe labelling

Oligonucleotides (100–150 pmoles per reaction) were labelled with digoxigenin (DIG)-dUTP by tailing (Boehringer Mannheim) in the presence of dATP and terminal transferase (DIG Oligonucleotide Tailing Kit; Boehringer Mannheim). Labelled oligonucleotides were purified by ethanol precipitation. The labelling efficiency was checked by dot blots using a series of dilutions of each oligonucleotide on nitro-cellulose strips.

In situ hybridization

Cell cultures on coverslips in 24-well plates were fixed as described above and then dehydrated in alcohol, rehydrated, treated with 0.5% Triton X-100 for 10 min and then washed in standard saline citrate buffer (SSC). Hybridization buffer contained 50% formamide, 5% dextran sulfate, 250 μg ml^{-1} salmon sperm DNA, 2 × Denhardt and 2 × SSC. Cultures were hybridized in the presence of 15–30 pmoles DIG-labelled probes per 250 μl hybridization buffer at 45–50° C overnight. After washes in 4 × SSC, 2 × SSC and 1 × SSC, cells were incubated with affinity-purified alkaline phosphatase conjugated sheep antibody directed against DIG (Boehringer Mannheim) at 1:200 dilution in TBS (pH 7.5) for 2 h at RT. Alkaline phosphatase activity was detected in TBS (pH 9.5) in the presence of NBT and BCIP for 2–4 h as described above.

Results

Identification of jimpy oligodendrocyte cultures

To identify oligodendrocytes in cultures derived from Ta males, antibodies directed against the wild type PLP C-terminal sequence and oligonucleotide probes corresponding to the deleted sequence from exon 5 of the PLP gene were used for immunocytochemistry and *in situ* hybridization, respectively. Oligodendrocytes at 12 DIV primary cultures from control mice showed strong staining for the presence of wild type PLP (not shown) or for its mRNA (Fig. 1) while oligodendrocytes in jimpy cultures were negative (Fig. 2). Proteolipid protein and its transcripts were not detectable in the astrocyte bed layer in normal and jimpy cultures (Figs 1, 2).

GFAP$^+$ cells in secondary cultures of oligodendrocytes

The presence of contaminating astrocytes in normal and jimpy oligodendrocyte secondary cultures was checked by immunostaining with an antibody to GFAP. Most cultures showed a low percentage of astrocytes (2–5%) (Table 1). The percentage of contaminating astrocytes in some cultures, occasionally reached 15% but such cultures were not used for the present study.

Table 1. Average number of contaminating astrocytes detected with anti-GFAP in oligodendrocyte secondary cultures at 2 DIV.

Oligodendrocyte cultures	*Control*	*Jimpy*
Total number of cells	89.1 ± 19.9	107.2 ± 7
GFAP$^+$ cells	1.6 ± 1	2.3 ± 1.5
Percentage of GFAP$^+$ cells	1.8%	2.2%

The total number of cells was determined by counting the cells phase-contrast optics. Values ± SDs for 60 fields (15 fields per coverslip) from four different cultures (objective × 10).

Expression of MBP, PLP and related mRNAs in normal and jimpy oligodendrocytes in secondary cultures

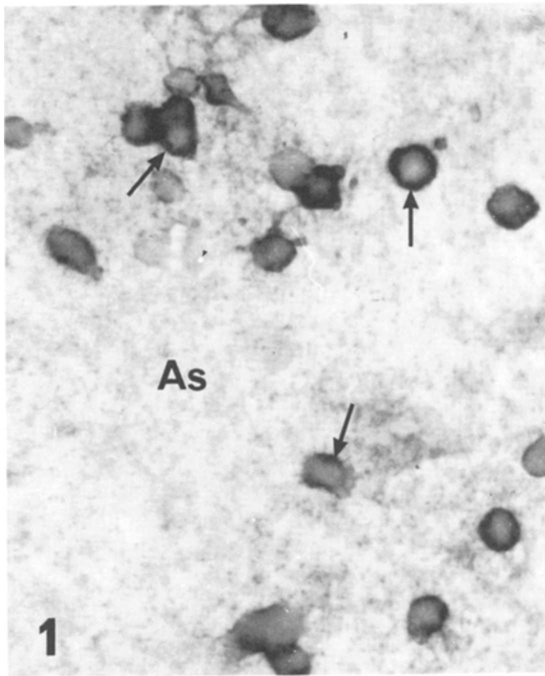
Oligodendrocytes in secondary cultures from both control and jimpy brains were immunoreactive for the antibody to the PLP tridecapeptide but with a difference in staining intensity (Figs 3, 4). In contrast to the heavy uniformly-distributed staining in cell bodies and processes of normal oligodendrocytes (Fig. 3), a weak and punctate staining, was observed in jimpy oligodendrocytes (Fig. 4). The antibody to the wild type PLP C-terminal sequence detected only normal oligodendrocytes (Fig. 7) while jimpy oligodendrocytes remained unstained (Fig. 8) as described above for oligodendrocytes in primary cultures. Myelin basic protein was also detected in normal and jimpy oligodendrocytes (Figs 5, 6).

The specificity of the PLP staining in jimpy oligodendrocytes was confirmed by the detection of the corresponding mRNA by *in situ* hybridization with related probes. The transcript coded by exon 5 of the PLP gene is absent in jimpy oligodendrocytes (Fig. 10) while it is present in healthy oligodendrocytes (Fig. 9). In contrast, the transcripts recognized by the probes from exon 3 of PLP gene are expressed in both normal and jimpy oligodendrocytes (Figs 11, 12).

At 2 and 4 DIV in secondary oligodendrocyte cultures the vast majority of the cells (80–95%) express both PLP (recognized with the antibody to the tridecapeptide) and MBP and their corresponding mRNAs.

Oligodendrocyte proliferation

Oligodendrocytes in S phase of the cell cycle were detected using the immunoperoxidase procedure after a 1 h pulse of BrdU. BrdU$^+$ cells were double labelled for MBP or PLP (recognized with the antibody to the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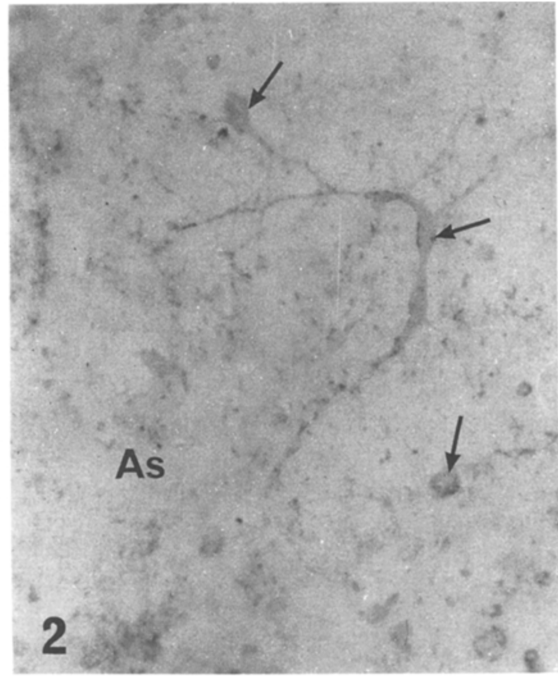
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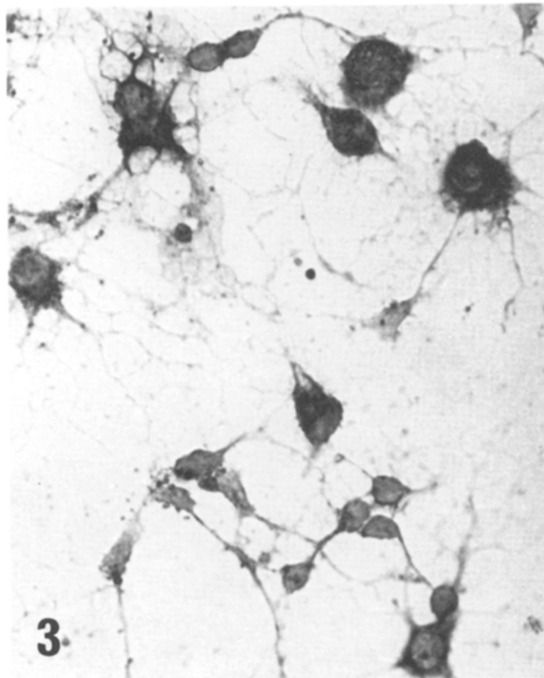
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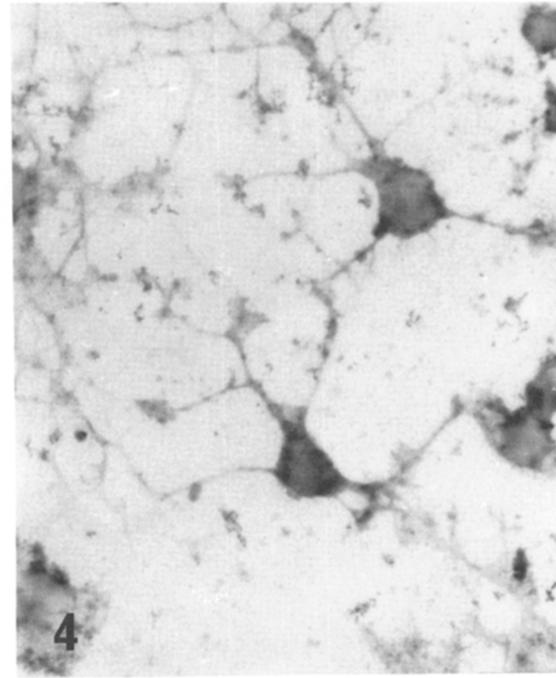
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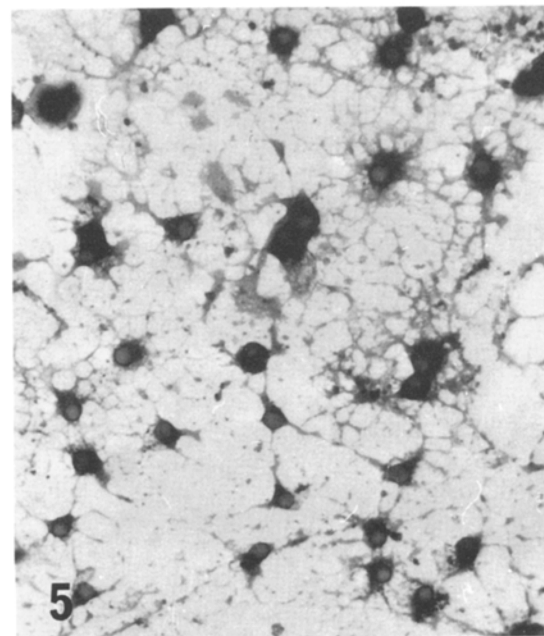
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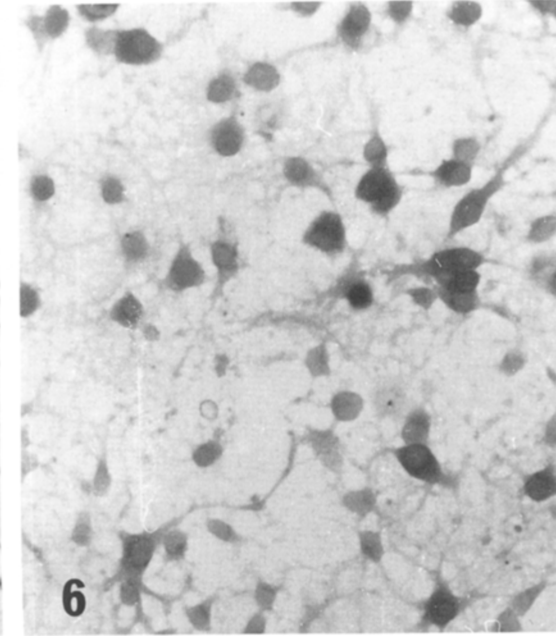
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Table 2. Average number of MBP$^+$, MBP$^+$BrdU$^+$, MBP mRNA$^+$ and MBP mRNA$^+$BrdU$^+$ cells in oligodendrocyte secondary cultures at 2 DIV.

Oligodendrocyte cultures	*Control*	*Jimpy*
MBP$^+$ cells	45.8 ± 14.2	53.0 ± 16.4
MBP$^+$ BrdU$^+$ cells	4.2 ± 1.8	4.1 ± 1.8
Percentage of dividing MBP$^+$ cells	9%	7.7%
MBP mRNA$^+$ cells	39 ± 10	32 ± 16
MBP mRNA$^+$BrdU$^+$cells	3.5 ± 1.1	2.9 ± 1.8
Percentage of dividing MBP mRNA$^+$ cells	8.9%	9.1%

Values ± SDs from 90 fields (15 fields per coverslip) from three different cultures (objective ×20).

Table 3. Average number of PLP$^+$, PLP$^+$BrdU$^+$, PLP mRNA$^+$ and PLP mRNA$^+$BrdU$^+$ cells in oligodendrocyte secondary cultures at 2 DIV.

Oligodendrocyte cultures	*Control*	*Jimpy*
PLP$^+$ cells	43.0 ± 16.2	52.6 ± 26.4
PLP$^+$BrdU$^+$ cells	4.2 ± 1.7	4.6 ± 2.0
Percentage of dividing PLP$^+$ cells	9.8%	8.7%
PLP mRNA$^+$ cells	42 ± 13	36 ± 12
PLP mRNA$^+$BrdU$^+$ cells	3.9 ± 1.8	3.8 ± 1.6
Percentage of dividing PLP mRNA$^+$ cells	9.4%	10.5%

Values ± SDs from 90 fields (15 fields per coverslip) from three different cultures (objective ×20).

tridecapeptide) (Figs 13, 14) or for their respective mRNAs (Figs 15–18).

A quantitative study was performed in primary and secondary cultures. The percentage of MBP$^+$ BrdU$^+$ cells and MBPmRNA$^+$ BrdU$^+$ cells for jimpy and control oligodendrocytes varied between 7.7% and 9.1% (Table 2) while the percentage of PLP$^+$ cells and PLPmRNA$^+$ cells in similar cultures varied between 8.7% and 10.5% (Table 3). The percentage of proliferating oligodendrocytes either PLP$^+$ or MBP$^+$ in the primary cultures was lower, between 2% and 4% (not shown). No significant difference was observed in the number of proliferating jimpy oligodendroctyes compared to the normal oligodendrocytes. MBP$^+$ oligodendrocytes with mitotic figures were also observed for normal oligodendrocytes in different cultures prepared by Percoll gradient (Figs 19–20). Control experiments for immunostaining were performed using normal rabbit serum after BrdU detection (Figs 21, 22).

Effect of bFGF on proliferating oligodendrocytes

Oligodendrocytes in secondary cultures were treated with bFGF 2 h after planting and at 2 DIV. Cells in S phase were double labelled for BrdU and either MBP, PLP or for their respective mRNAs at 2 DIV and 4 DIV and counted (Table 4). The percentages of normal oligodendrocytes in S phase after bFGF treatment were

Table 4. Average number of PLP$^+$, PLP$^+$BrdU$^+$ cells in jimpy and control oligodendrocyte secondary cultures at 2 and 4 DIV in the presence of bFGF.

Oligodendrocyte cultures	*Control*	*Jimpy*
2 DIV		
PLP$^+$ cells	30.3 ± 3.8	25.3 ± 5.6
PLP$^+$BrdU$^+$cells	5.9 ± 1.2	2.9 ± 0.9
Percentage of dividing PLP$^+$ cells	19.5%	11.6%
4 DIV		
PLP$^+$ cells	32.3 ± 4.2	25.5 ± 8.8
PLP$^+$BrdU$^+$ cells	5.7 ± 10	2.5 ± 0.9
Percentage of dividing PLP$^+$ cells	17.6%	9.9%

Values ± SDs from 15 and 17 coverslips (15 fields per coverslip) from four different jimpy or normal cultures respectively (objective ×25).

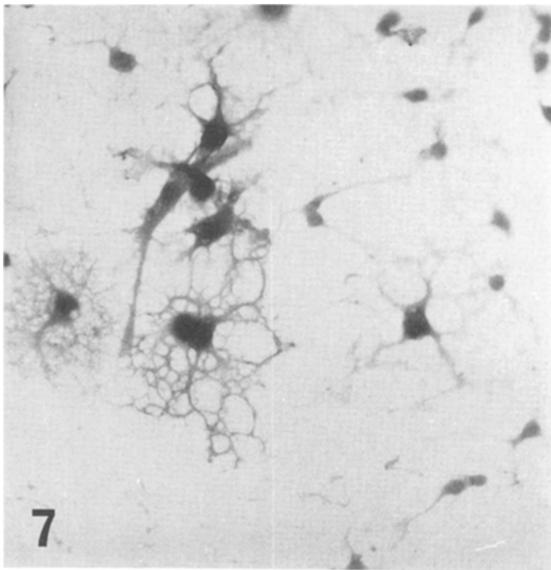
Figs 1, 2. Twelve DIV primary mixed glial cell cultures from normal (Fig. 1) and from jimpy mice (Fig. 2). Detection of PLP mRNA using *in situ* hybridization with exon 5 probe. Oligodendrocytes (arrows) from normal mice are heavily labelled while the bed layer of astrocytes (As) remains unstained (Fig. 1). Both oligodendrocytes and astrocytes from jimpy male are unstained (Fig. 2). ×280.

Figs 3, 4. Two DIV oligodendrocyte secondary cultures are stained with the antiserum to the PLP tridecapeptide. Oligodendrocytes from normal (Fig. 3) and from jimpy mice (Fig. 4) are labelled. The staining is less intense in jimpy oligodendrocytes compared to controls. ×400.

Figs 5, 6. Four DIV oligodendrocyte secondary cultures from normal (Fig. 5) and jimpy mice (Fig. 6) are stained with the antiserum to MBP. Both normal and jimpy oligodendrocytes are labelled for MBP. Intense staining is present in the perikarya and processes of normal oligodendrocytes. Weak labelling is observed in cell bodies and in some processes of jimpy oligodendrocytes. ×240.

Figs 7, 8. A 2 DIV oligodendrocyte secondary culture from a normal mouse (Fig. 7) is labelled with anti PLP C terminal antibody while a similar culture from a jimpy mouse (Fig. 8) is unstained. ×240.

Figs 9–12. Proteolipid protein mRNA detection in oligodendrocyte secondary cultures at 2 DIV from normal (Figs 9 and 11) and jimpy mice (Figs 10 and 12). Probe from exon 5 of the PLP gene detects normal (Fig. 9) but not jimpy oligodendrocytes (Fig. 10). Probe from exon 3 of PLP gene reveals both normal (Fig. 11) and jimpy oligodendrocytes (Fig. 12). ×400.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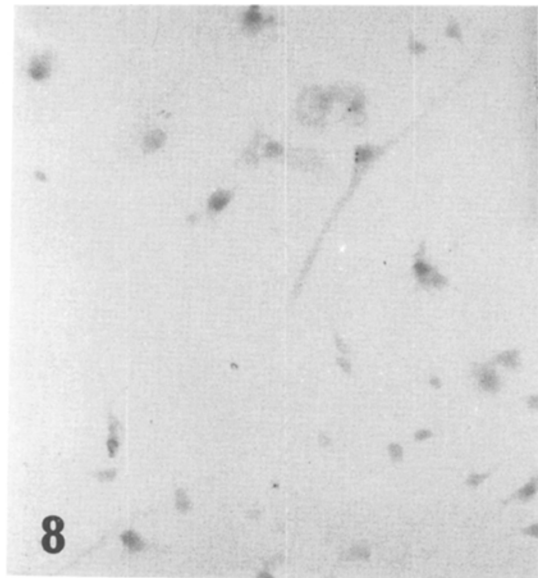
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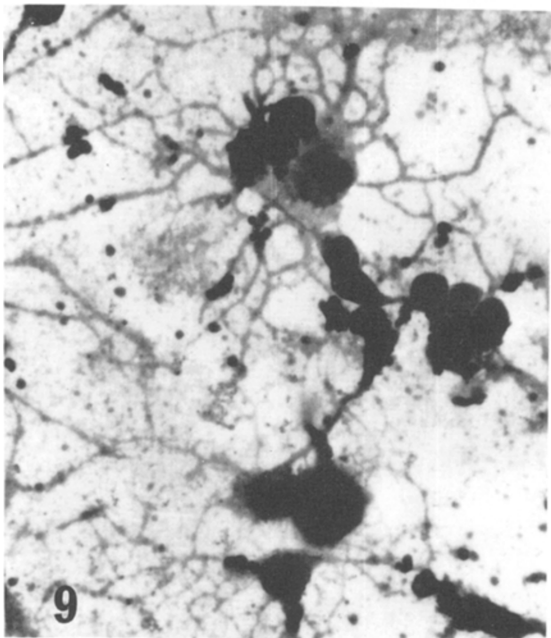
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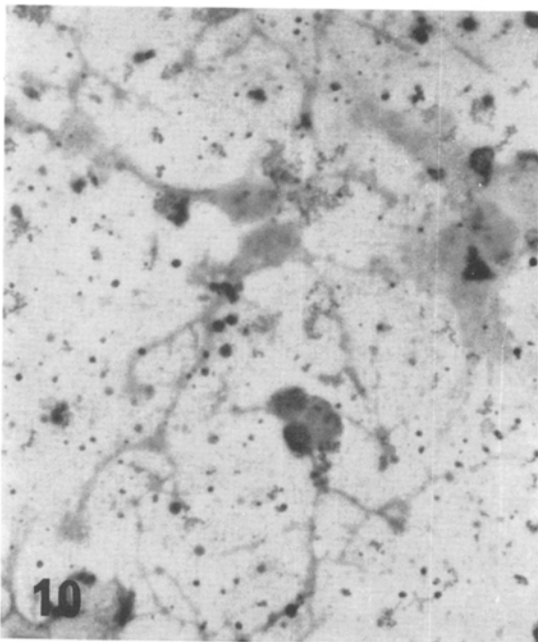
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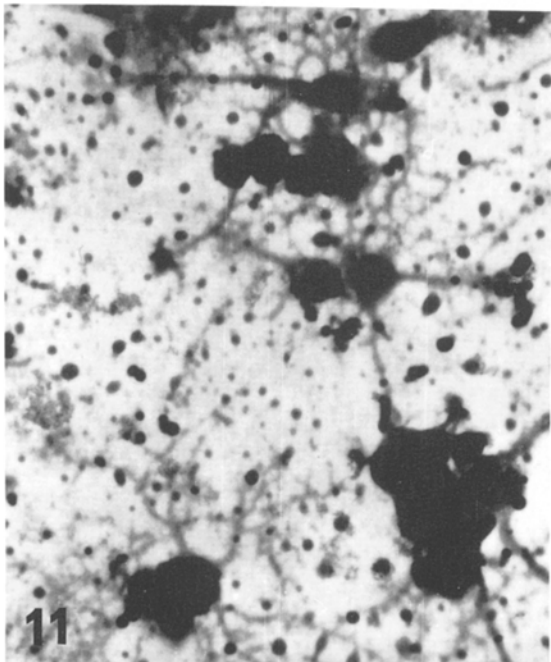
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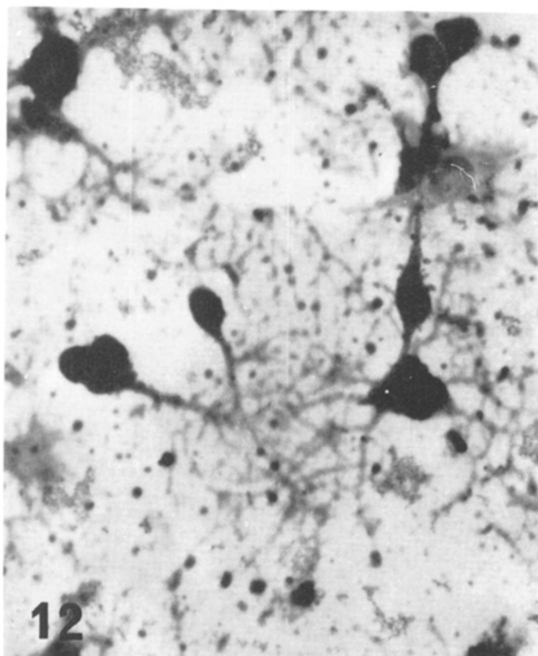
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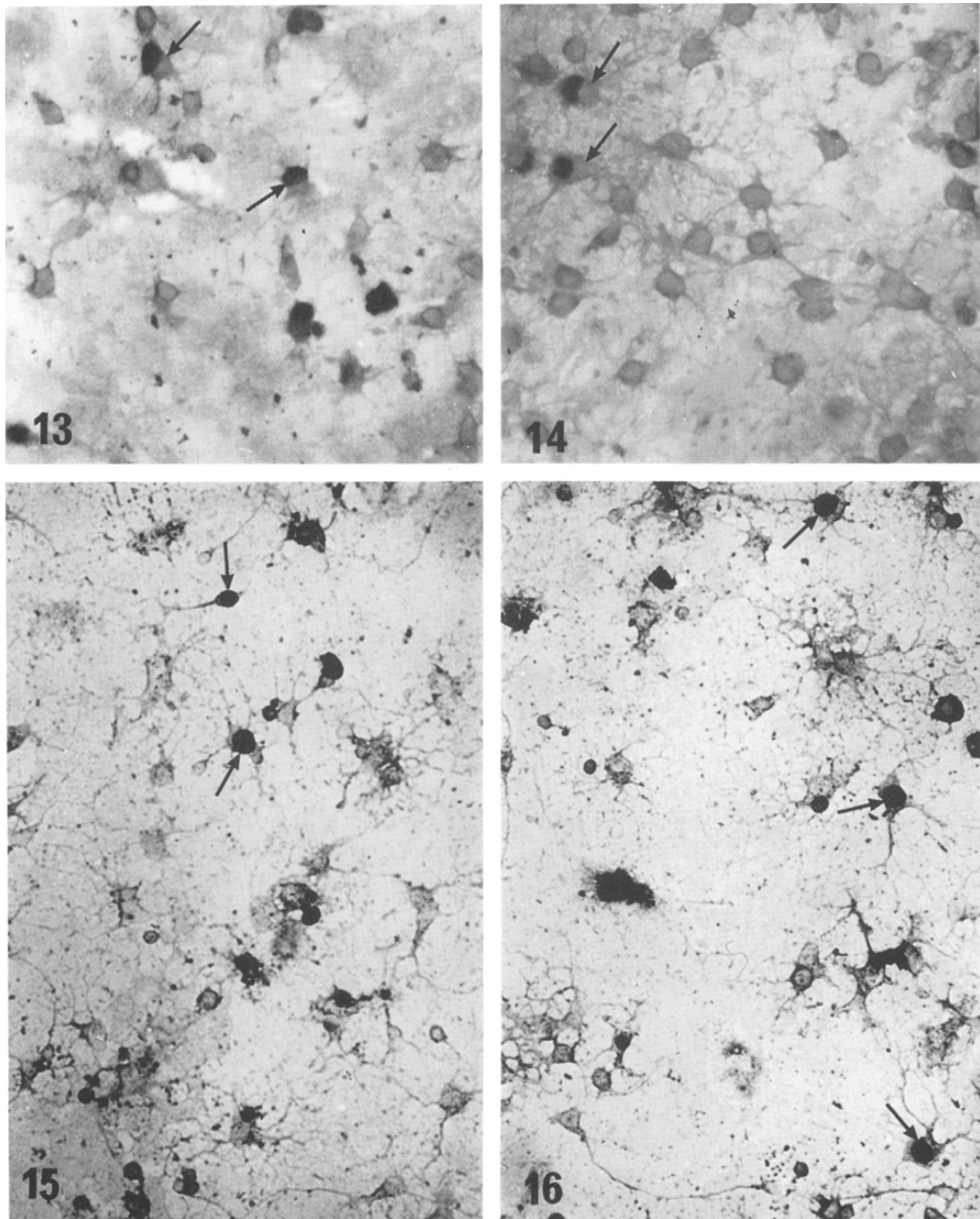
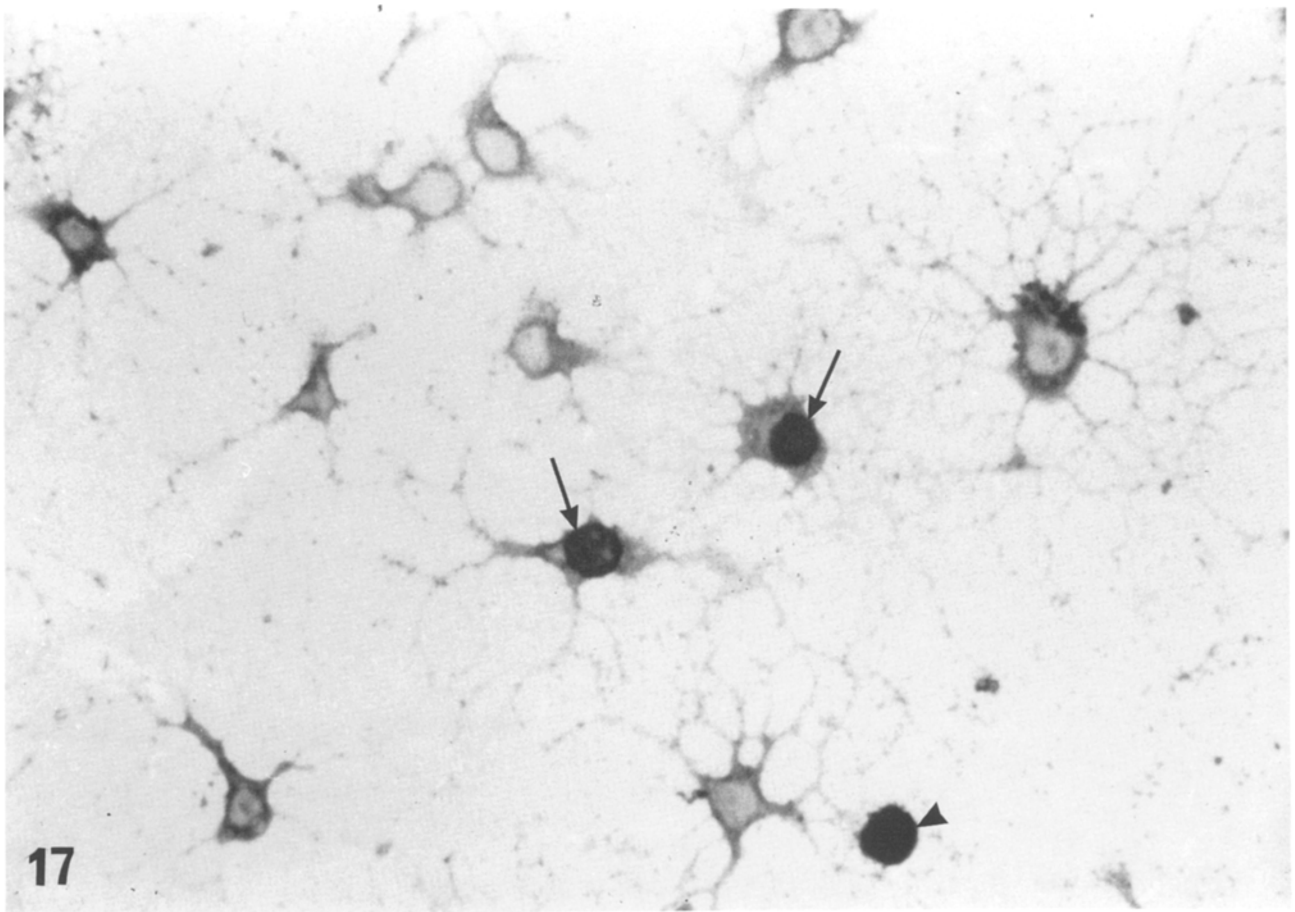


Figs 13, 14. Double labelling immunocytochemistry for BrdU and PLP detected with antitridecapeptide in primary mixed glial cultures at 12 DIV from jimpy (Fig. 13) and normal mice (Fig. 14). Oligodendrocytes in S phase of the cell cycle (arrows) in both cultures are immunostained for PLP. ×240.

Figs 15, 16. Double labelling immunocytochemistry for BrdU and *in situ* hybridization for PLP mRNA with exon 3 probe in 2 DIV oligodendrocyte secondary cultures from jimpy (Fig. 15) and normal mice (Fig. 16). Double labelled oligodendrocytes (arrows) in both cultures are detected. ×240.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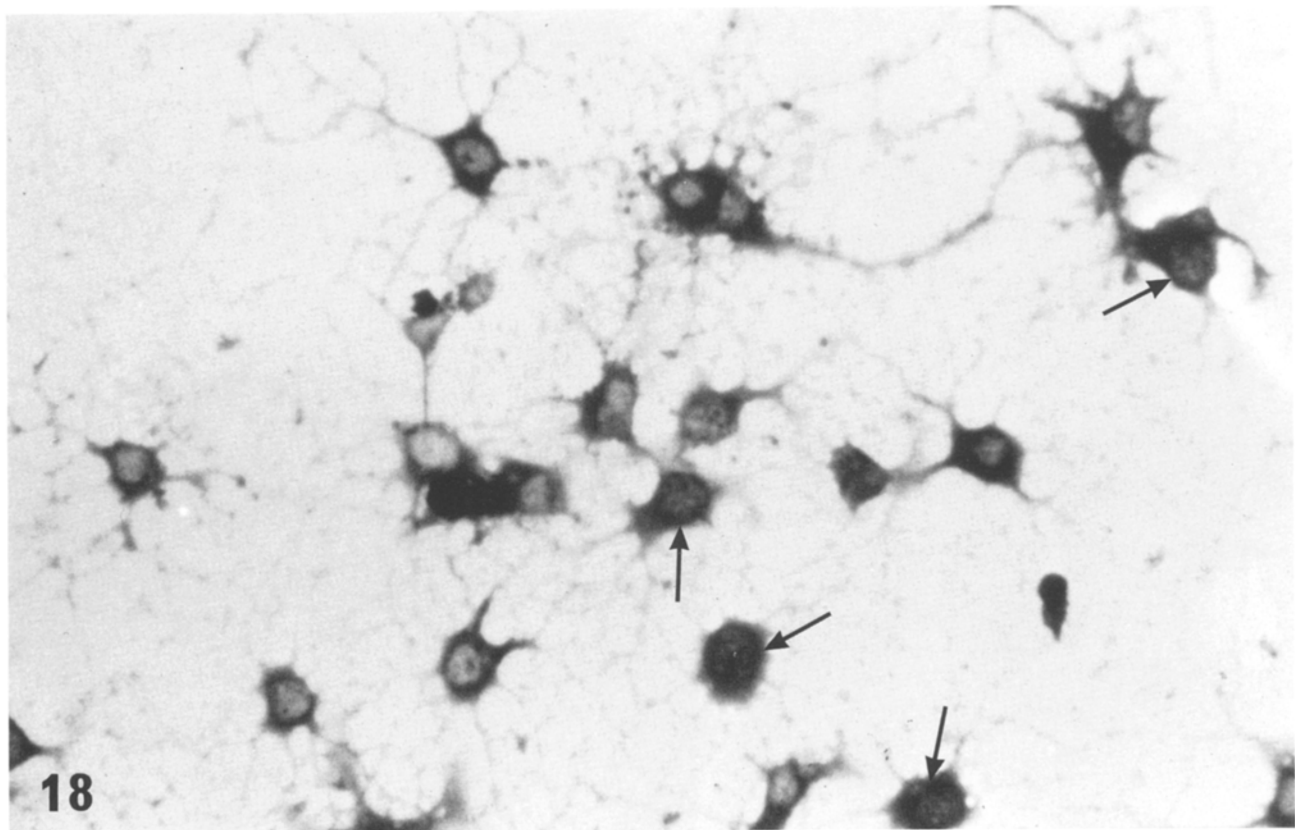
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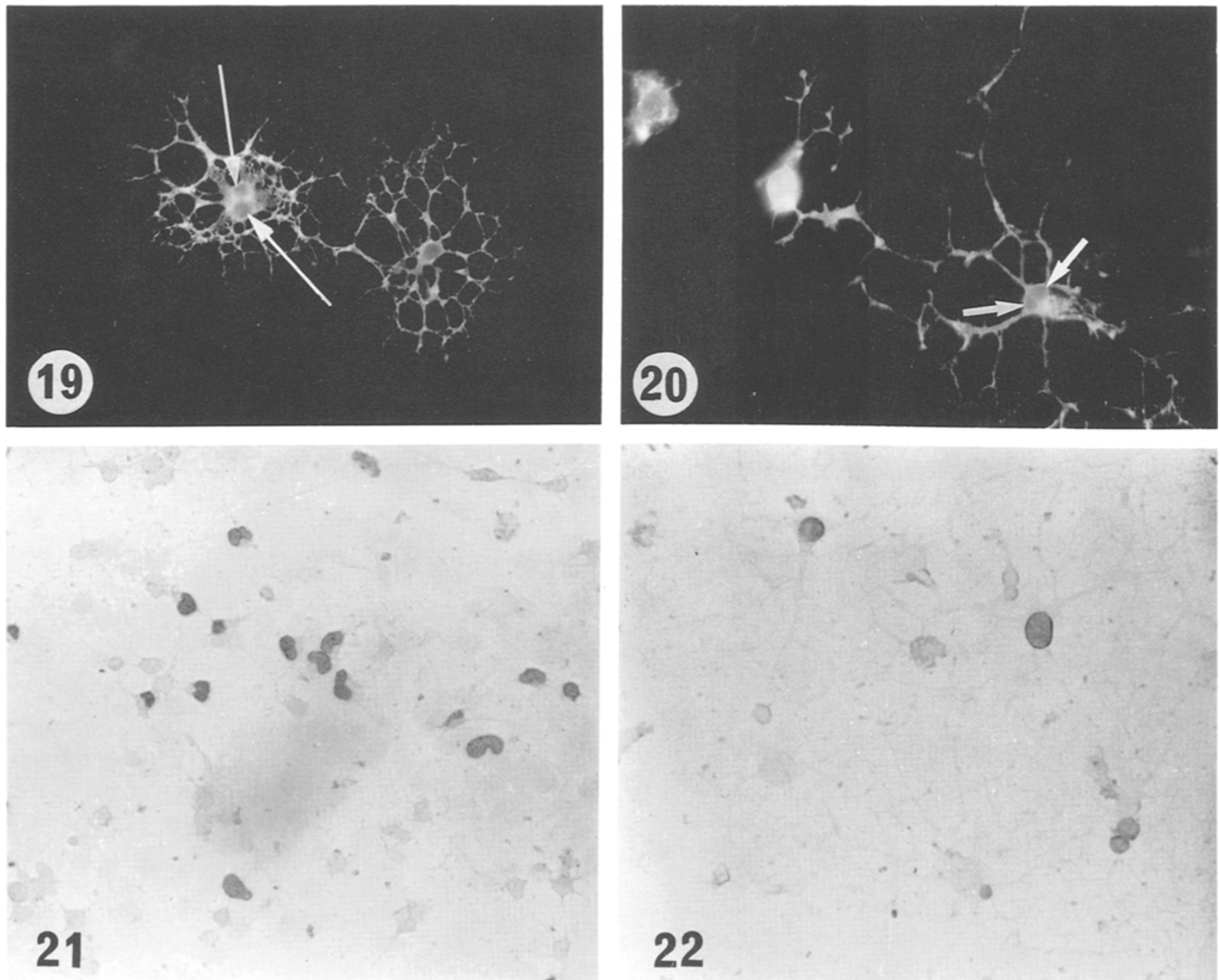

Figs 18, 19. Sixteen DIV normal oligodendrocyte primary culture, prepared from Percoll gradient. Note the mitotic aspect of fluorescein-labelled MBP^{+} oligodendrocytes (arrows). ×200.

Figs 20, 21. Control immunocytochemical experiments in 10 DIV primary mixed glial cell cultures (Fig. 20) and 2 DIV oligodendrocyte secondary culture (Fig. 21). After BrdU immunodetection with DAB, cultures were incubated with normal rabbit serum and then with secondary antibody PA-conjugated. Note the total absence of immunostaining in the cytoplasm. Only cell nuclei in S phase of cell cycle are labelled. ×300.

19.5% and 17.6% while for jimpy oligodendrocytes they were 11.6% and 9.9% at 2 and 4 DIV, respectively.

Discussion

A highly enriched jimpy oligodendrocyte culture was prepared and characterized to use as a tool for further functional studies on defective oligodendrocytes. All previous published *in vitro* studies on jimpy oligodendrocytes were based on organotypic cultures (Billing-Gagliardi *et al.*, 1980, 1983) or on mixed glial primary cultures, where astroctyes represent the majority of the cells (Bologa-Sandru *et al.*, 1981; Hertz *et al.*, 1980; Bartlett *et al.*, 1988; Knapp *et al.*, 1993). The culture described here contains less than 5% contaminating astrocytes while the majority of the cells (80–95%) are oligodendrocytes as judged by the presence of PLP, MBP and their respective mRNAs.

Jimpy oligodendrocytes and PLP

The relationship between the failure of oligodendrocytes

Figs 17, 18. Double labelling immunocytochemistry for BrdU and *in situ* hybridization for PLP mRNA with exon 3 probe in 2 DIV oligodendrocyte secondary cultures from jimpy (Fig. 17) and normal mice. Arrows show oligodendrocytes in S phase of the cell cycle which are PLP $mRNA^{+}$ in both jimpy and normal cultures. The staining intensity for PLP mRNA in normal oligodendrocytes is higher than in jimpy oligodendrocytes. Arrowhead in Fig. 17 shows nuclear staining for BrdU but PLP mRNA is completely absent from the cytoplasm. ×500.

to produce myelin in the CNS of jimpy mice and the point mutation on the PLP gene (Hudson *et al.*, 1987; Macklin *et al.*, 1987; Nave *et al.*, 1987) remains unclear. Investigations to establish the direct cause of hypomyelination in relation to the PLP mutation are currently under investigation by a number of research groups. Several approaches have been used to establish a system to analysing the role of PLP, including gene transfer to other cell types (Colman *et al.*, 1990; Nave & Lemke, 1991) and the production of transgenic lines (Mastronardi *et al.*, 1993; Wight *et al.*, 1993; Kagawa *et al.*, 1994; Readhead *et al.*, 1994). The preparation of jimpy oligodendrocyte-enriched cultures reported here, where the influence of other cell types is absent should reflect the influence of the mutated PLP on certain physiopathological aspects of jimpy oligodendrocytes.

Oligodendrocytes from jimpy brain hemispheres isolated in secondary cultures are able to express detectable level of PLP and MBP. The detection of mutated PLP in jimpy oligodendrocytes in culture reported here is consistent with the isolation and the identification of PLP from jimpy brain (Fannon *et al.*, 1994). A patchy staining pattern was observed for PLP in jimpy oligodendrocytes. It is not yet clear if the jimpy PLP is blocked in the Golgi apparatus (Roussel *et al.*, 1987) or if such a staining pattern is related to the low level of PLP present in jimpy cells; the highest level of PLP was detected in Golgi apparatus. The PLP transcript localization within the cell is restricted to the cell body and to broader processes for both normal and jimpy oligodendrocytes. The use of probes from exon 5 of PLP gene for *in situ* hybridization, as well as the use of an antibody to PLP C-terminal was very helpful to distinguish normal from jimpy oligodendrocytes. This criterium was necessary to unambiguously identify jimpy oligodendrocytes and to eliminate doubt on the possible rearrangement of Tabby and jimpy genes in the X chromosome (Wolf & Holden, 1969; Skoff & Nowicki-Montgomery, 1981). However, in our jimpy colony (see Materials and Methods), this possibility is greatly reduced (Lachapelle *et al.*, 1990a).

Despite the deficit in the number of myelinated fibres in jimpy CNS (Robain & Mandel, 1974; Billings-Gagliardi *et al.*, 1980) and the decrease in the biochemical level of myelin components (for review see Campagnoni & Macklin, 1988), the presence of mutated PLP in oligodendrocytes in jimpy mice does not prevent a number of those cells making myelin in the cervical spinal cord (Duncan *et al.*, 1989).

Our observation in culture, together with data obtained from an *in vivo* study (Duncan *et al.*, 1989) or from intracerebral transplantation of jimpy oligodendrocytes into normal brain (Lachapelle *et al.*, 1990 a,b) might suggest that mutated PLP may not be toxic or if it is, it does not prevent jimpy oligodendrocytes in our culture from dividing or making myelin *in vivo* (Duncan *et al.*, 1989; Lachapelle *et al.*, 1990 a,b). In addition, the capacity of defective jimpy oligodendrocytes to express myelin proteins in culture can be enhanced by adding of conditioned medium from normal cultures (Bartlett *et al.*, 1988; Skoff & Knapp, 1990). Also, the production of myelin in jimpy cerebellum was increased by co-culture with normal optic nerve (Wolf *et al.*, 1981) and also the capacity of myelination was also enhanced by transplantation of jimpy oligodendrocytes into shiverer brain (Gumpel *et al.*, 1987; Lachapelle *et al.*, 1990 a,b). The expression of PLP or any related product of PLP gene products in other cell types cannot be excluded, since an abnormality of astrocytes in jimpy in early development has been noted (Skoff, 1976) and also a high level of PLP mRNA has been recently reported in astrocytoma cell lines (Campagnoni *et al.*, 1994).

Myelin markers and jimpy oligodendrocyte proliferation

The ability of differentiated oligodendrocytes to divide is poorly documented, particularly *in vitro*. However convincing light and electron micrographs showing mitotic differentiated oligodendrocytes have been published (Sturrock & McRae, 1980; Sturrock, 1981; Arnella & Herndon, 1984; Skoff *et al.*, 1994). This concept requires careful re-evaluation. In order to establish a relationship between the ability of differentiated oligodendrocytes to divide *in vitro* and the expression of myelin specific proteins, the proliferation of oligodendrocytes combined with the detection of MBP, PLP and their respective mRNAs was carefully investigated in jimpy and normal oligodendrocyte cultures in the present study.

Despite the presence of abnormal PLP in jimpy oligodendrocytes in culture, these cells proliferate at the same rate as healthy oligodendrocytes. In both jimpy and normal secondary cultures, 7–10% of the cells were in S phase of the cell cycle as detected 1 h after incorporation of BrdU. The identity of BrdU-labelled cells was confirmed by the presence of PLP, MBP and the related mRNAs as revealed by immunocytochemistry and *in situ* hybridization respectively. No significant differences were noted between the proliferative rates of jimpy and normal oligodendrocytes. The present results contrast with the highly increased proliferation of jimpy oligodendrocytes *in vivo* as measured by ^{3}H-thymidine incorporation (Privat *et al.*, 1982; Skoff, 1982). This would suggest that high proliferation rates of jimpy oligodendrocytes *in vivo* could result from stimulation by other cell types or from contact inhibition between oligodendrocytes and axons due to astrocyte hypertrophy (Skoff, 1976; Omlin & Anders, 1983) or by stimulation of proliferation by the debris of unformed myelin or by microglial cells or by other factors (for review, see Scolding *et al.*, 1994). However, stimulation

by strocytes seems unlikely since, in mixed primary cultures from jimpy and normal brain, the proliferation rate of oligodendrocytes is reduced (2–4%) compared to pure secondary cultures. An inhibitory effect in the presence of astrocytes has been observed on neuroblasts in culture (Yoshida *et al.*, 1986). The inhibitory effect on oligodendrocyte proliferation by astrocytes is probably due to the direct contact between these two cell types, since the conditioned medium from astrocyte cultures stimulates the proliferation of oligodendrocyte precursors (Raff *et al.*, 1988; Chan *et al.*, 1990) and also their differentiation (Dutly & Schwab, 1991). Stimulation of oligodendrocytes to proliferate by replating them was also noted in earlier studies (Pruss *et al.*, 1982; for review, see Wood & Bunge, 1984). The present study clearly shows that PLP^+ and PLP $mRNA^+$ oligodendrocytes are able to divide. Dividing MBP^+ and MBP $mRNA^+$ oligodendrocytes were also observed in both normal and defective jimpy cultures. The observations of early detection of PLP in transgenic mice (Wight *et al.*, 1993) and the expression of myelin genes in the embryonic ventricular zone (Yu *et al.*, 1994; Timsit *et al.*, 1995) also suggest that proliferation of those cells represents a potential source of oligodendrocyte cell line.

What is the primary cause of jimpy oligodendrocytes dysfunction?

A direct effect of mutated PLP or its isoform DM20 to induce abnormal proliferation of oligodendrocytes in jimpy brain seems unlikely, because the presence of the same defective PLP in cells in culture did not significantly affect their proliferation. The contrast between the abnormal proliferation and the defective cell cycle of jimpy oligodendrocytes observed *in vivo* (Privat *et al.*, 1982; Skoff, 1982; Knapp & Skoff, 1987) and the normal proliferative rate of jimpy oligodendrocytes in culture reported in the present study suggests that jimpy oligodendrocytes may receive abnormal signal responsible for their aberrant proliferation *in vivo*. Multiple signals are required from other cell types for oligodendrocyte proliferation (Barres & Raff, 1994). Recent studies on transgenic mice over expressing PLP indicate that these mice showed symptoms of demyelination, but unfortunately no further studies were performed to explore how the abnormal level of PLP affects oligodendrocyte proliferation (Mastronardi *et al.*, 1993; Kagawa *et al.*, 1994; Readhead *et al.*, 1994). The inability of PLP and DM20 transgene to enhance myelination in jimpy (Nadon *et al.*, 1994) demonstrates the complexity of PLP gene expression and function. The failure of bFGF to stimulate jimpy oligodendrocytes to divide in culture contrasts with the action of bFGF to regenerate the normal oligodendrocyte cell lineage and to enhance oligodendrocyte proliferation (Besnard *et al.*, 1989; Gard & Pfeiffer, 1990; Grinspan *et al.*, 1993). It is clear from the present study that the abnormal jimpy PLP message and proteins do not interfere significantly with oligodendrocyte proliferation and early stages of differentiation. Several questions arise from the absence of stimulation by bFGF on jimpy oligodendrocytes, while under the same conditions, the stimulation was optimum for normal oligodendrocytes at the concentration used. Are functional properties of bFGF receptors modified? Are jimpy oligodendrocytes only stimulated at earlier stages of oligodendrogenesis? Or is the presence of an additional signal necessary to stimulate jimpy oligodendrocytes? Further investigations along this line will be fruitful.

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