Emergence of Three Myelin Proteins in Oligodendrocytes Cultured without Neurons

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Abstract. Oligodendrocytes, the myelin-forming cells of the central nervous system, were cultured from newborn rat brain and optic nerve to allow us to analyze whether two transmembranous myelin proteins, myelin-associated glycoprotein (MAG) and proteolipid protein (PLP), were expressed together with myelin basic protein (MBP) in defined medium with low serum and in the absence of neurons. Using double label immunofluorescence, we investigated when and where these three myelin proteins appeared in cells expressing galactocerebroside (GC), a specific marker for the oligodendrocyte membrane. We found that a proportion of oligodendrocytes derived from brain and optic nerve invariably express MBP, MAG, and PLP about a week after the emergence of GC, which occurs around birth. In brain-derived oligodendrocytes, MBP and MAG first emerge between the fifth and the seventh day after birth, followed by PLP 1 to 2 d later. All three proteins were confined to the cell body at that time, although an extensive network of GC positive processes had already developed. Each protein shows a specific cytoplasmic localization: diffuse for MBP, mostly perinuclear for MAG, and particulate for PLP. Interestingly, MAG, which may be involved in glial-axon interactions, is the first myelin protein detected in the processes at ~10 d after birth. MBP and PLP are only seen in these locations after 15 d. All GC-positive cells express the three myelin proteins by day 19. Simultaneously, numerous membrane and myelin whorls accumulate along the oligodendrocyte surface. The sequential emergence, cytoplasmic location, and peak of expression of these three myelin proteins in vitro follow a pattern similar to that described in vivo and, therefore, are independent of continuous neuronal influences. Such cultures provide a convenient system to study factors regulating expression of myelin proteins.

The central nervous system (CNS)[1] myelin membrane, which allows fast saltatory conduction to occur in nerve fibers (reviewed in reference 53) is made by oligodendrocytes. Myelin is very rich in lipids (~70% dry weight) (35), among which GC has been identified as a specific marker for the oligodendrocyte (49). In addition to lipids, rodent CNS myelin contains ~30% proteins (reviewed in references 26 and 34). These consist mainly of proteolipid protein (PLP; 50% of total protein), myelin basic protein (MBP; 30–35% of total protein), 2′,3′-cyclic nucleotide-3′-phosphohydrolase (5% of total protein), myelin-associated glycoprotein (MAG; <1% of total protein), and several enzymes. Other minor components of myelin have not been fully characterized yet.

There are four forms of MBPs in the rodent (6), which are synthesized on free polysomes in the oligodendrocyte cytoplasm and processes in vivo as well as in vitro (5, 7, 8, 12, 13, 15, 32, 55). These four forms of MBP are recognized immunologically by a monoclonal antibody produced against small mouse MBP (18). MBP has been localized immunocytochemically in myelinating oligodendrocytes (21, 22, 54, 58, 59). MBP is diffusely distributed in the cytoplasm and is present in the major dense line of myelin (37). PLP is a chloroform-methanol soluble integral protein of CNS myelin that has three putative transmembrane domains and may have domains exposed on each side of the bilayer (25, 61). PLP is also present in myelinating oligodendrocytes (3, 21, 33) and is synthesized on membrane-bound ribosomes mostly confined to the oligodendrocyte cell body. It is acylated at a step following passage through the Golgi complex and probably preceding incorporation in the myelin membrane (15, 27, 65). MAG is a minor transmembrane glycoprotein of CNS myelin that is confined to the periaxonal and non-compacted areas of the myelin sheath (reviewed in references 45 and 67).

We are interested in the factors and signals regulating oligodendrocyte differentiation and the expression of CNS myelin genes. Although in vivo immunocytochemical studies have revealed that certain myelin proteins emerge before the start of myelination (58–60) the dynamics of these events

[1] *Abbreviations used in this paper:* CNS, central nervous system; GC, galactocerebroside; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; PLP, proteolipid protein.

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cannot be studied easily in the intact animal. As described earlier (24, 32) oligodendrocytes cultured without neurons (30, 48) develop a phenotype closely resembling their in vivo counterpart and express GC on their surface (1, 2). Since in vivo studies have demonstrated a strict correlation between the structural and biochemical events of myelination (63), it is possible that most myelin protein genes are also expressed in vitro. We have recently shown that large amounts of MBP mRNA accumulate in cultured oligodendrocytes at a time corresponding to the fifth or sixth day after birth (72). This event is followed closely by MBP expression. Thus, MBP appears in a timely manner 5 to 7 d after the appearance of GC and in the absence of neuronal influence as described in optic nerve cells (32). Are the transmembrane myelin proteins, PLP and MAG, also expressed in oligodendrocytes cultured without neurons? If so, do MBP, PLP, and MAG appear in a definable sequence or simultaneously? Is that sequence of events different from that seen in vivo where myelination can proceed normally?

Our results show that cultured oligodendrocytes express the myelin proteins MBP, MAG, and PLP in a sequence and at sites very similar to those observed in vivo. Thus, the oligodendrocyte can express on schedule a set of myelin specific genes without continuous neuronal influence. Moreover, these results demonstrate that cultured oligodendrocytes provide a suitable, convenient system for future studies of the factors regulating expression of myelin proteins.

Materials and Methods

Primary Rat Brain Cultures (5, 30)

16- to 17-d-old Sprague-Dawley rat fetuses or newborns 0 to 4 d of age were decapitated, and their brain hemispheres were removed. The hemispheres, freed of meninges, were soaked in minimal essential medium with 25 mM Hepes, gently dissociated mechanically by pipetting, and filtered through 130- and 33-μm nitex. The resulting dispersed cells were seeded at a density of 4×10^5 cells per cm^2, on 35-mm petri dishes or 13-mm wide glass coverslips which had been precoated with 0.1 mg/ml of poly-L-lysine, as described (47). The medium consisted of Dulbecco modified Eagle's medium with gentamycin 25 μg/ml and glucose 4.5 g/liter enriched in glutamine 2 mM. To this basic medium we added 50 μg/ml of transferrin, 5 μg/ml of insulin, 30 nM of selenium, and 30 nM of triiodothyronine as described by Eccleston and Silberberg (17). This medium was then enriched with 5% "hybridoma selected" fetal calf serum (Microbiological Associates, Walkersville, MD), since such serum concentration improved the early adhesion and growth of primary brain cultures without inhibiting galactocerebroside (GC) expression.

Enriched Oligodendrocyte Cultures

These cultures were obtained in two different ways (30, 48, 50).

In the first method, we seeded brain cells of 2-d-old rat (dissociated as described above) in Falcon (Falcon Labware, Oxnard, CA) or Costar (Costar, Cambridge, MA) 75-cm^2 plastic flasks and grew them as described above. After 8 to 10 d in vitro, when the process-bearing cells had positioned themselves over a continuous layer of astrocytes, the cells were put on a rotary shaker overnight at 37°C, at 250 rpm. The cells, which detached by the shaking, were washed, spun down, and filtered through a 33-μm nitex. They were then allowed to adhere to plastic 150-cm^2 flasks at 20°C for 10 min. During that time, most of the remaining flat cells adhered to the plastic, whereas the small round cells did not. These cells were decanted, washed, and seeded at a density of 5×10^4 to $1.5 \times 10^5/cm^2$ on poly-L-lysine-coated glass coverslips or 35-mm petri dishes. The defined medium, as described above, was used to grow these enriched cultures, but only 1% fetal calf serum was added to the enriched cultures, since recent reports have described an inhibitory effect of fetal calf serum on oligodendrocyte differentiation (17, 48). Cultures done in these conditions consistently yielded 60 to 80% GC positive cells. As discussed before (72), the shaking method is a simple way to reseed oligodendrocytes in higher relative numbers and gives them more space to continue their differentiation process.

The second method used to obtain enriched oligodendrocyte cultures is that described by Raff et al. (48, 50). In brief, optic nerves of 7-d-old rat were excised and dissociated as described. In cultures grown in the defined medium enriched with 1% fetal calf serum (see above) for 3 d, 60% of the cells were found to be GC positive. Video microscopy of living cells in differential interference contrast was performed with a 63 oil objective on an inverted Zeiss microscope (ICM 405).

Throughout this paper, the ages mentioned will not be the in vitro age (except if specifically noted) but the postnatal age. The age of the cells in both primary and enriched cultures will be calculated from the theoretical date of birth of the rat, whether the CNS tissue was dissociated and cultured before or after birth. We have assumed that the rats would be born 21 d after fecundation.

Antibodies

Mouse monoclonal antibodies to alpha- and beta-subunits of tubulin were obtained from Amersham Corp. (Arlington Heights, IL). The mouse anti-GC was a hybridoma IgG characterized by Ranscht et al. (51). The MBP antibody was a mouse hybridoma IgG developed against a small mouse MBP by Fritz and Chou (18). The mouse anti-MAG hybridoma was raised against human MAG and cross-reacted with rat MAG (16). This antibody was purified on protein A-Sepharose before use. All these antibodies were generously given to us by the scientists who had characterized them. The rabbit anti-GC prepared following Fry et al. (19) was a gift of Donald Silberberg.

For the staining of PLP protein, antibodies to a synthetic PLP peptide (from position 109-128 in the amino acid sequence of Laursen et al. [25]), coupled to keyhole limpet hemocyanin, were raised in rabbits. The peptide was synthesized by the Merrifield solid phase technique with a Beckman 990 automated peptide synthesizer (Beckman Instruments Inc., Palo Alto, CA) as previously described (52), desalted on a Sephadex G-10 column, and analyzed by high-pressure liquid chromatography. Antiserum was purified first by passage through a protein A-Sepharose column equilibrated with phosphate-buffered saline (PBS). The immunoglobulin fraction was eluted in a pH 2.4 citrate phosphate buffer, dialyzed, and concentrated. The antibody was next applied to an affinity column prepared by coupling the PLP peptide to an Affigel 10 support as described by the manufacturer (Bio-Rad Laboratories, Rockville Centre, NY). After an extensive PBS wash, the PLP antibody was eluted as described above by dropping the pH of the column buffer to 2.4. The PLP antibody was titered by a dot immunobinding assay in which PLP peptide diluted in rinse buffer (10 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 1 mM EDTA, 0.15 M NaCl) was spotted onto nitrocellulose filters. The filters were air dried, then preincubated with rinse buffer containing 10% goat serum to block nonspecific binding. Various dilutions of antisera were added, and incubations were carried out at room temperature for up to 2 h before filters were rinsed in the same buffer. Iodinated protein A or goat anti-rabbit immunoglobulin ($\sim 10^6$ cpm in 10 ml rinse buffer with 10% goat sera) was added for an additional 2 h before blots were rinsed, dried, and filmed. Affinity purified PLP antibody typically yielded titers at $\sim 10^{-6}$ at a protein concentration of 1 mg/ml and reacted with PLP isolated from myelin on dot and immunoblots (Hudson, L., manuscript in preparation).

Fixation and Immunolabeling

Cells were fixed either in acid alcohol (5% acetic acid and 95% alcohol) or in formaldehyde 4% for 20 min followed by a short 0.1% Triton X-100 treatment. The immunofluorescence labeling methods have been described in previous studies (32, 48). We have made extensive use of double labeling immunofluorescence. More precisely, the staining for the three myelin proteins, MBP, MAG, and PLP, was frequently performed in cells that had been first labeled with GC. Since optimal staining with GC is obtained on living cells, the monolayer of living cells was first incubated in dilutions of the mouse monoclonal antibody and then in a goat anti-mouse rhodamine. Cells were subsequently fixed in acid alcohol for 10 to 15 min, rinsed in serum-containing medium, and then incubated with dilutions of rabbit and anti-PLP, followed by goat anti-rabbit fluorescein conjugate (Cappel Laboratories, Inc., Cochranville, PA). In the case of double labeling of GC with MAG or MBP, the GC labeling on living cells was performed with dilutions of the rabbit polyclonal anti-GC antibody, followed by incubation with a goat anti-rabbit fluorescein. Cells were then fixed with acid alcohol and incubated with dilutions of mouse monoclonal to MAG or MBP followed by goat anti-mouse rhodamine (Cappel Laboratories, Inc.). Additional double labeling experiments were done on fixed cells with acid alcohol followed by incubation with the mouse monoclonal anti-MBP and the rabbit polyclonal anti-PLP and their appropriate rhodamine and fluorescein conjugates. Similarly, double labeling for MAG and PLP, or PLP and tubulin, could also be performed on fixed cells. All preparations were examined on either a Zeiss photoscope III or ICM 405 microscopes, equipped

with epifluorescence and appropriate filters for rhodamine and fluorescein and differential interference contrast optics.

Transmission Electron Microscopy

Cells were fixed and processed in situ as described (24).

Results

Primary cultures from newborn rat brain did not contain any neurons (30) as assayed by the presence of neuron-specific enolase and neurofilaments (24, 72). Occasional neuron-specific enolase positive cells were found in cultures started at 16 to 17 d of fetal life and decreased in number with time. Oligodendrocytes were identified in these cultures by their staining with anti-GC antibody and were further examined for the expression of myelin proteins. In optic nerve cells derived from 7-d-old rat, GC is already present at the time of dissociation and seeding, but the three myelin proteins were detected only after a few days in culture, and all three were present at 5 d (equivalent to 12 d after birth).

A detailed analysis of the sequence of emergence and location of MBP, PLP, and MAG was done in cultured brain oligodendrocytes before and after enrichment by the shaking method (30). As discussed before (72), this method allows the reseeding of oligodendrocytes in higher relative numbers without interfering with their differentiation. All three proteins were detected 5 to 8 d after GC had first appeared in primary brain cultures. Myelin proteins were not detected in GC negative cells.

Time and Sequence of Emergence of Myelin Proteins

Table I shows the time at which three myelin proteins were first detected in representative sets of brain primary cultures. MBP and MAG were first detected between 5 and 7 d after birth, usually at the same time, in a fraction of GC-positive cells. A similar time of emergence (with respect to the theoretical time of birth) of myelin proteins was observed in brain cells cultured from 17-d-old fetuses (Table I, Exp. 3). The number of MBP positive cells quickly rose to 80 to 100% of GC-positive cells within the next 3 to 4 d, as observed earlier (32, 44, 70). The number of MAG-positive cells rose above 50% of GC positive cells over the same period. Whereas GC positive cells all expressed MBP by 9 to 12 d after birth (in both primary and enriched cultures), MAG was not found in all GC-positive cells before 15 d after birth. This may be due to the inability of the fluorescent antibody techniques to detect small amounts of MAG protein.

PLP was consistently detected 1 or 2 d later and/or in much fewer GC-positive cells than MBP and MAG (Table I). Very often PLP was first observed in some clusters of GC-positive cells, whereas other clusters were completely negative. A cluster of oligodendrocytes in culture may have evolved from a specific bundle of fiber tract in the brain, and therefore may be determined to express myelin proteins at a certain time, as happens in vivo. Only by day 19 were all MBP and GC positive cells also PLP positive.

Table I. Postnatal Time at which Three Myelin Proteins Were First Detected in GC-positive Cells

	Exp. 1	Exp. 2	Exp. 3*
MBP	Day 5	Day 6	Day 7
MAG	Day 7	Day 6	Day 7
PLP	Day 9	Day 9	Day 7‡

Two coverslips containing primary brain cell cultures were double labeled daily (after a few days in culture) with anti-GC antibody and an antibody to the myelin protein under study. An average of 300 GC positive cells per coverslip were examined for the presence of myelin protein staining.

* Midbrain cultures started in fetal day 17.

‡ Only 7% of the GC positive cells contained PLP at that time.

Differential Localization of Myelin Proteins

When MAG was first detected in the cytoplasm of GC positive cells at 6 d after birth, there was a higher intensity of staining close to the nucleus (Fig. 1 *a*). Although long cell processes labeled with GC antibodies had already developed, MAG was confined to the cytoplasm at that stage (compare Fig. 1, *a* with *b*). 3 to 6 d later (9 d after birth or later), MAG had spread to the cell processes (Fig. 1 *c*). In these processes, a fine punctate pattern was sometimes detected along the membrane (Fig. 1 *c*) at a time when neither MBP nor PLP had spread centrifugally (see below).

When MBP was first detected in GC-positive cells, the staining was weak but definitely present throughout the cytoplasm. It became more intense with time (Fig. 1 *d*), whereas only a few granules, mostly perinuclear, of PLP-specific fluorescence were detected in the same cells (Fig. 1 *e*). At 14 d after birth, the number of stained PLP granules had increased (against a diffuse background) and were detected throughout the cytoplasm in some MBP-positive cells (Fig. 1, *f* and *g*). Other MBP-positive cells did not express PLP, as mentioned in Time and Sequence of Emergence of Myelin Proteins above (Fig. 1, *f* and *g*). Both MBP and PLP stayed confined to the cytoplasm for several days (Fig. 1, *d*–*g* and Fig. 2, *a* and *b*). However, at 14 to 15 d after birth, several oligodendrocytes contained MBP and PLP in their processes where these proteins were co-localized in some spots (Fig. 1, *h* and *i*). Thus, when acid alcohol fixation is used, the first myelin protein detected by immunofluorescence in the processes is MAG, followed by MBP and PLP 5 to 7 d later.

When PLP localization was compared with GC staining in young primary cultures, PLP was strikingly confined to the cell body of cells with numerous GC-positive processes (Fig. 2, *a* and *b*). At 14 to 15 d after birth, PLP was found to be co-localized with GC in some spots in the processes (Fig. 2, *c* and *d*, arrowheads). Processes of oligodendrocytes are very rich in tubulin (24) (Fig. 2 *e*), and PLP was also found in these processes at 14 d (Fig. 2 *f*). Myelin proteins were virtually never seen throughout the GC-positive network, especially not in the very fine filopodia (24).

Oligodendrocytes Cultured in the Absence of Neurons Produce Some Myelin

The normal destination of these myelin proteins would be the sites on the cell membrane where myelin is being assembled. Several studies have reported the presence of myelin-like membranes in oligodendrocyte cultures (9, 29, 36, 41, 43, 56). Differential interference contrast analysis of the living brain oligodendrocytes cultured for 2 to 3 wk (15 to 22 d after birth), and the optic nerve oligodendrocytes cultured for 5 d in vitro (12 d postnatal), revealed a number of membrane-bound extracellular vesicles along the cell body and processes (Fig. 3 *a*). Such blebs could be labeled with anti-GC, MBP (Fig. 3 *b*) (32); or PLP antibodies. Blebs were not seen along living astrocytes or flat cells in the culture. Electron micro-

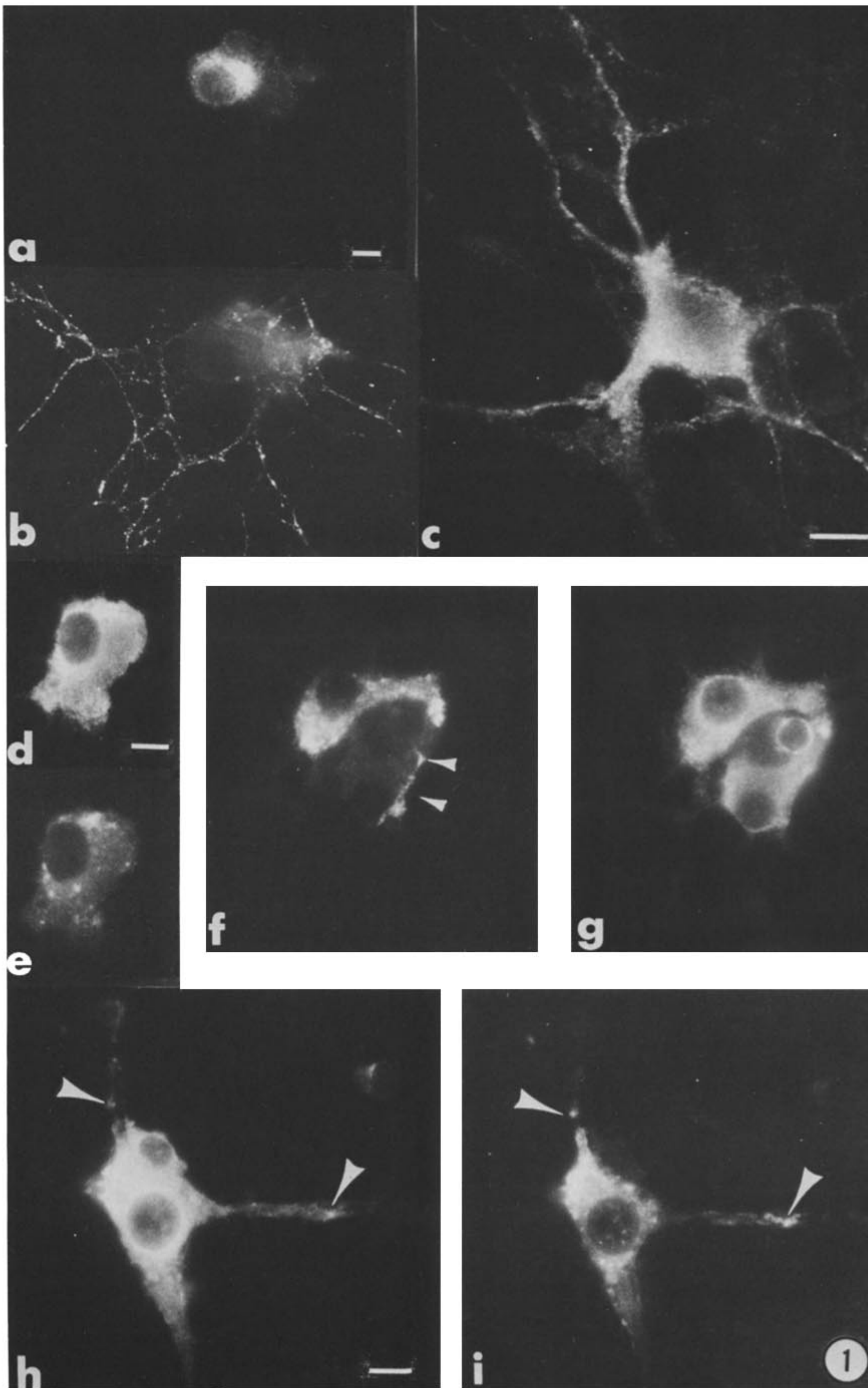

Figure 1. Detection of three myelin proteins in cultured brain oligodendrocytes. *a* and *b* show an oligodendrocyte at 6 d after birth double labeled with MAG antibodies in *a* and with GC antibodies in *b*. MAG staining is mostly perinuclear (note the excentric nucleus), whereas Gc covers the processes. At 13 d after birth MAG is present throughout the cytoplasm and shows a punctate pattern of staining along the processes (*c*). *d* and *e* show an oligodendrocyte double labeled for MBP (*d*) and PLP (*e*) at 9 d after birth. MBP is diffuse throughout the cytoplasm, and PLP forms small perinuclear granules. In *f* and *g*, a group of cells at 14 d after birth were stained for PLP (*f*) and MBP (*g*). PLP is present in only one of the MBP-positive cells and forms patches throughout the cell cytoplasm and, probably, along a thin process apposed to the second PLP-negative MBP-positive cell (arrowheads). *h* and *i* show two other cells, also at 14 d after birth, where MBP (*h*) and PLP (*i*) are detected in the cytoplasm as well as in three main processes of one of them. MBP and PLP are co-localized in the processes at arrowheads. Bars, 10 μm.

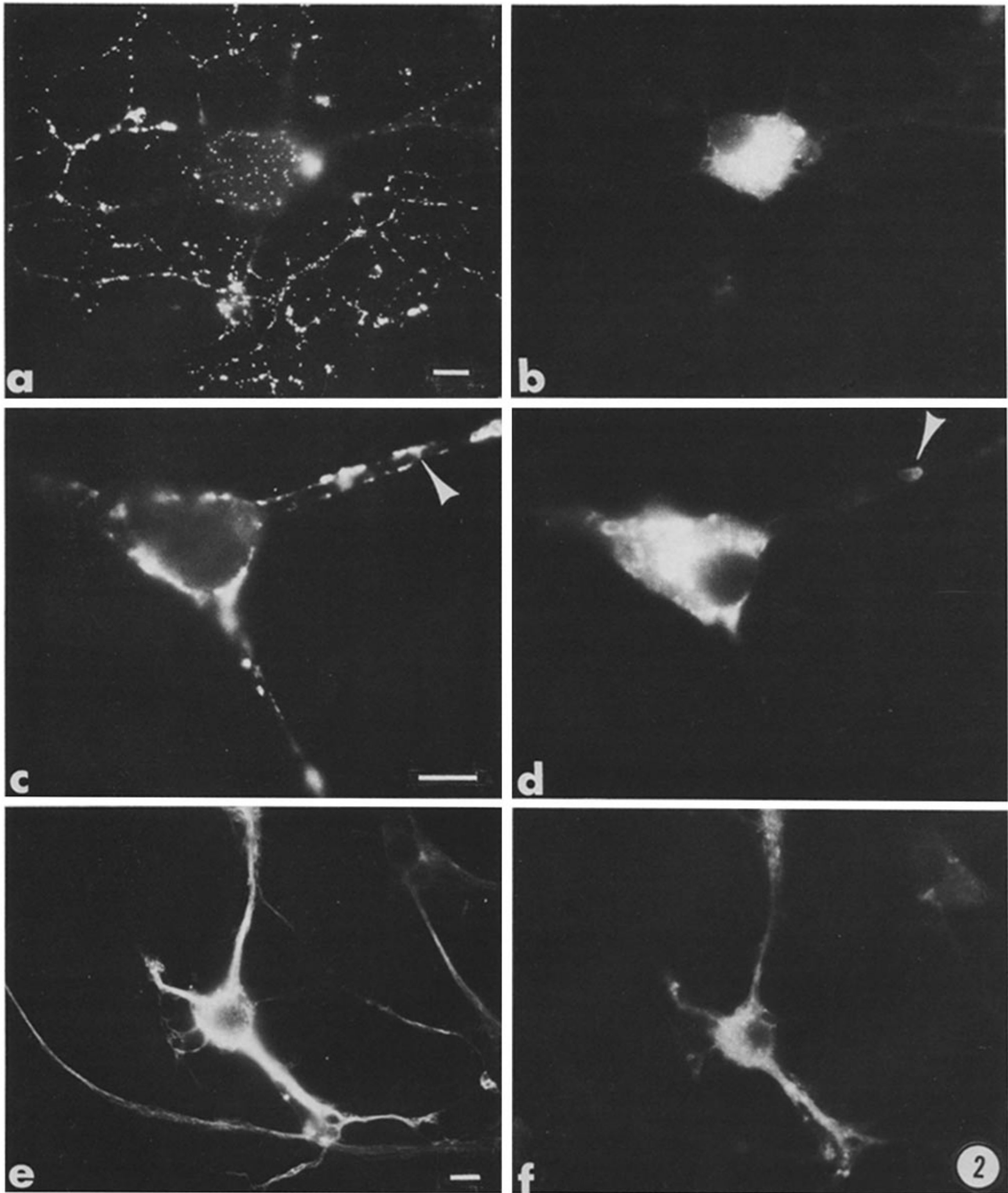

Figure 2. Double labeling for PLP and GC (*a–d*) and tubulin (*e* and *f*) in cultured brain oligodendrocytes. *a* and *b* show the same oligodendrocyte stained with antibodies to GC in *a* and antibodies to PLP in *b* at 9 d after birth. PLP forms bright dots only inside the cytoplasm, whereas GC is present on the entire network of processes. *c* and *d* show an oligodendrocyte at 15 d after birth, double labeled with antibodies to GC in *c* and to PLP in *d*. PLP staining is intense and forms bright dots in the cytoplasm and occasionally co-localized with GC in the processes (arrowhead). The other two processes do not appear to contain PLP, except at their emergence. *e* and *f* show an oligodendrocyte at 14 d after birth, with clear staining for beta-tubulin (*e*) and PLP (*f*) in the processes. Bars, 10 μm.

scopic studies of these cells demonstrated that these vesicles consisted of extracellular multilamellar structures (Fig. 3*c*). The membranes were formed of multiple dense layers, with a 7-nm periodicity in some cases, whereas, in others, alternate dense lines and intraperiodic lines of 12-nm periodicity were similar to that characteristic of myelin in vivo (Fig. 3*d*).

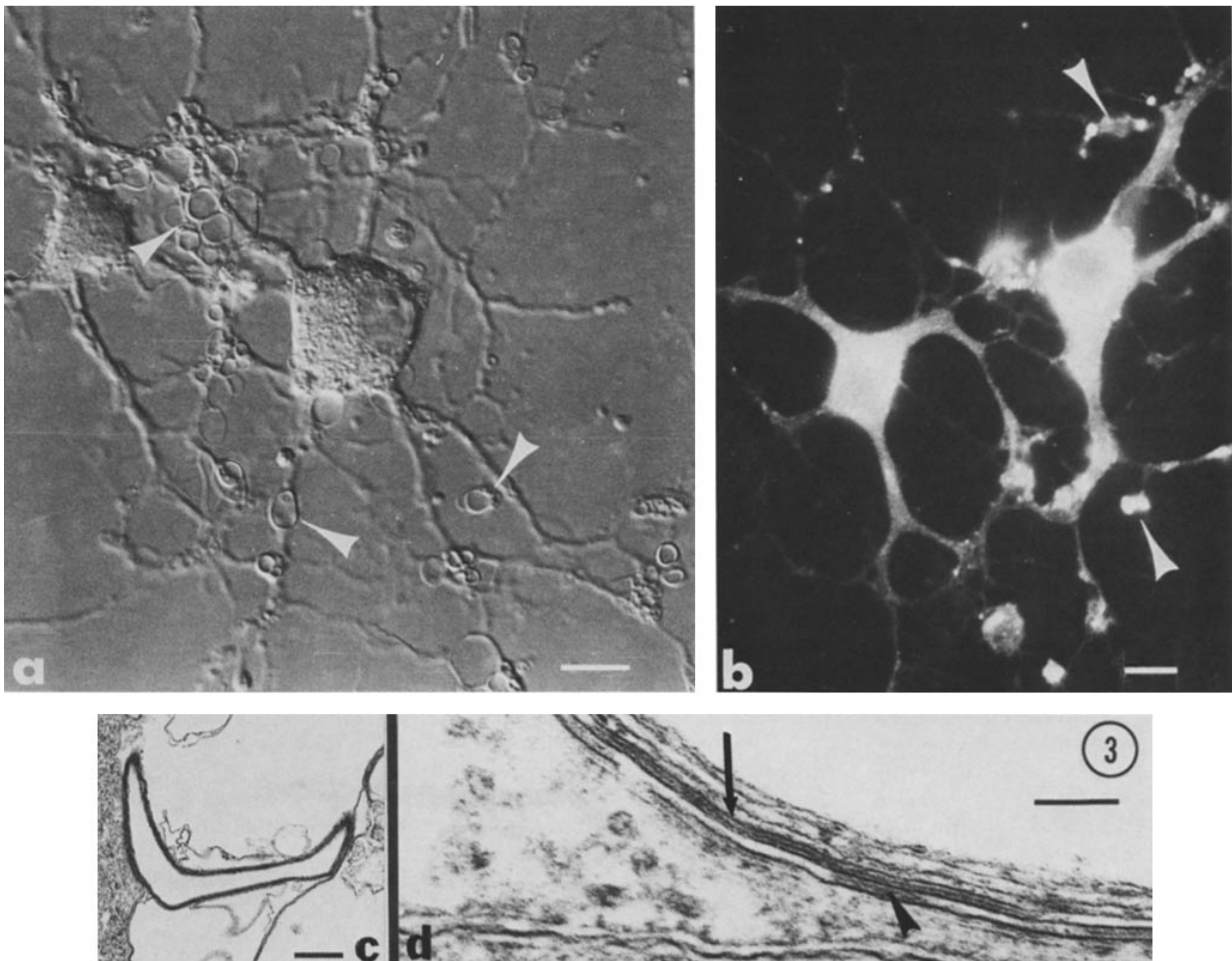

Figure 3. Myelin-like membranes in oligodendrocytes. *a* and *b* show optic nerve–derived oligodendrocytes after 5 d in culture (12 d after birth). In *a*, differential interference microscopy on the living cell reveals the presence of numerous membrane whorls along the cell body and processes (arrowheads). In *b*, such cells were fixed and immunolabeled for MBP. The staining is diffuse in the cytoplasm and the processes, and appears concentrated in membrane whorls (arrowheads). *c* shows the ultrastructural features of such membrane whorls in an oligodendrocyte culture at 33 d after birth. At low magnification (*c*), membrane layers of high electron density are apposed to the oligodendrocyte cytoplasm (on the left). High magnification on such membrane (*d*) reveals the dense line (black arrow) and intraperiodic line (arrowhead) characteristic of myelin. The intraperiodic line, however, is sometimes discontinuous or missing. Bars: *a* and *b*, 10 μm; *c*, 0.5 μm; *d*, 0.1 μm.

Discussion

We show here that rat oligodendrocytes, cultured in the absence of neurons, invariably express three myelin proteins, MBP, MAG, and PLP, about a week after the emergence of GC. These proteins emerge only after the oligodendrocyte has established its characteristic processes (24), which may participate in the vesicular transport of myelin proteins to the myelin membrane. In addition, these cultured oligodendrocytes actively assemble myelin proteins into structures that resemble myelin (9, 29, 36, 41, 43, 56), despite the absence of axons for enwrapping. Thus, despite the limitation of the culturing technique in which dissociated oligodendrocytes can only develop a two-dimensional network, these cultures promise to be useful in the study of factors regulating myelination.

MBP, MAG, and PLP are usually detected in vitro at 5 to 7 d after birth, regardless of when the cells are dissociated from the brain (Table I, Exp. 3). Similarly, GC always appears around birth (1). Most oligodendrocytes initially express MBP at 5 to 6 d after birth, followed very closely by MAG, and after 1–2-d lag, by PLP. However, subsets of oligodendrocytes, which probably correspond to different fiber tracts in the brain, appear to express the myelin proteins in the same order but in a more delayed fashion. Although these cells are finally MBP positive at ~10 d after birth, MAG was not found in 100% of the cells until day 15 and PLP until day 19. Thus, the synthesis of each of these proteins may be regulated by a different mechanism.

In vivo observations have shown that MBP is first detected in the cytoplasm of oligodendrocytes before myelination starts and is later seen in the processes of the cell and in myelin (58, 59). MBP is first seen in the brain at 5 d (54), and in the anterior commissure of the rat between 5 and 7 d of age (58). Thus, the timing of appearance of MBP in vitro closely resembles that observed in vivo. The diffuse pattern of cyto-

plasmic staining of MBP observed in vitro and in vivo is consistent with the fact that this protein is synthesized on free ribosomes in the cytoplasm. A study on the synthesis of MBP in 12–30-d old rat midbrain suggests that free polysomes synthesizing MBP might also be present in the myelinating processes in vivo (15). In cultured oligodendrocytes, MBP is detected in the processes at ~14 to 15 d after birth which is about a week after it was first detected in the cytoplasm. MBP mRNA has also been detected in the processes at that stage of differentiation (72).

In cultured oligodendrocytes, MAG is first seen in the perinuclear area, a location consistent with a Golgi stage, and soon after is found in the processes, where it might be associated with transport vesicles (57). MAG was also detected by radioimmunoassay in our enriched cultures at 12 d after birth (Quarles, R. H., unpublished observation). MAG has been detected shortly after MBP in oligodendrocytes between 7 and 12 d in the anterior commissure in vivo (60), and in CNS aggregates in vitro (68). In these studies, a diffuse cytoplasmic staining and a dotted, granular pattern were observed in the processes. Thus, the intracellular sites where MAG is detected in cultured oligodendrocytes are very similar to those seen in vivo. The fact that MAG is detected in the processes of cultured oligodendrocytes much earlier than MBP and PLP suggests that MAG is transported to the periphery in specific membrane vesicles where it may be needed for interactions with axons as proposed earlier (60, 66, 69). However, the peak time of staining of oligodendrocytes in vivo, as well as the peak number of positive cells in vitro, occurs later for MAG than MBP (57).

The MAG antibody we used in our study reacts with the polypeptide portion of MAG molecule (16) and therefore does not bind to other glycoconjugates but to MAG only (23). In another study where MAG was detected several days before MBP in canine oligodendrocytes (73), a monoclonal IgM of a patient with gammopathy was used as the detection system for MAG. Human IgMs of such patients have been shown to react with the carbohydrate residues of MAG as well as with those of other glycoproteins and glycolipids (16, 23, 46). Therefore, these IgMs may react with glycoconjugates that might be present on oligodendrocyte progenitor cells (48).

PLP, a major myelin protein probably involved in compaction and stabilization of myelin, was also detected in oligodendrocytes cultured without neurons, but it usually appeared later than MBP and MAG. Similarly, in vivo, the peak synthesis of PLP occurs several days later than that of MBPs (11). A similar delay in emergence of PLP versus MBP was detected in vivo by Hartman et al. (21), who observed a granular pattern of PLP staining as in our cultured oligodendrocytes. However, these authors observed MBP and PLP only in oligodendrocytes connected to myelin sheath, whereas we found these proteins in the absence of neurons and ensheathment. The striking concordance of our in vitro results with these in vivo observations contrasts with a short report of the induction of PLP 7 to 10 d before MBP in mixed cultures of fetal rat brain (28).

MBP and, to a lesser extent, PLP were seen in the processes of cultured oligodendrocytes only about a week (or later) after their emergence in the cytoplasm. These proteins were partially co-localized with GC along the processes in myelin-like membranes. In vivo studies have shown that in one specific tractus, it takes 6 to 12 d for MBP to move from the cytoplasm to the oligodendrocyte process and a myelin internode (22). The factors regulating the direction and rate of transport of MBP and PLP in the processes are unknown. Using microinjection and antibodies directed against the cytoplasmic domain of PLP or MAG, as well as high resolution optics on living cells, it might be possible to follow the path and rate of transport of these transmembrane proteins toward the periphery as was done with a viral glycoprotein in viral infected cells (4). Protein transport in oligodendrocytes cultured in the absence of neurons should be compared with that in oligodendrocytes seeded over pure neurons in a dissociated culture system (33, 70, 71). It is indeed possible that neuronal signals may be required for efficient transport of MBP and PLP (but not MAG) to the periphery.

Thus, in contrast to earlier observations (38, 39), we find a relatively well-coordinated regulation of myelin proteins in primary and enriched oligodendrocyte cultures derived from rat CNS, possibly because we used defined medium with low levels of serum. Earlier studies have shown that a fourth myelin protein, 2′3′-cyclic nucleotide-3′-phosphohydrolase can be induced by cAMP between 4 and 10 d in vitro (but not after 13 d), also indicating a potential for early expression of this enzyme in the absence of neurons (31). Oligodendrocytes in a two-dimensional network not only express all essential myelin proteins but can also assemble some myelin. Moreover, when astrocytes and oligodendrocytes are cultured without neurons in three-dimensional aggregates, well-compacted myelin is found in the absence of axons (20). This is in contrast to neonatal Schwann cells, the myelin-forming cells of the peripheral nervous system, which require neuronal influences for assembly of their basement membrane and the myelin sheath (10, 14, 42). When cultured in isolation, Schwann cells stop expressing GC on their surface and myelin proteins after a few days (32). However, the cells can be induced to re-express GC, as well as some myelin proteins, with dibutyryl cAMP (40; Shuman and Pleasure, personal communication) or axolemma fraction (64). This indicates an immediate dependence of Schwann cells on humoral and/ or neuronal factors to express myelin components.

In cultures derived from 7-d-old rat optic nerve, there is a progenitor cell that can differentiate into an oligodendrocyte or astrocyte type II, depending on signals provided by components in the culture medium (48, 50). These progenitor cells, however, do not require the presence of other cells such as astrocytes or neurons to differentiate along either pathway (62). It was proposed that signals in the medium probably substitute for signals from other cells (62) that may be operative in the optic nerve in vivo. The cellular and molecular mechanisms that control the differentiation of these progenitor cells are currently unknown. However, once the differentiation choice has been made and GC is expressed, oligodendrocytes appear regulated to go through a complete morphological and biochemical differentiation independent of continuous neuronal influences. Whether the astrocytes are necessary for the expression of myelin proteins remains to be determined.

After this paper was submitted for publication, a communication at the Tenth Meeting of the International Society for Neurochemistry by Jacques et al., entitled "Sequential expression of myelin markers in oligodendrocytes from mouse olfactory bulb," confirmed that MBP and PLP were expressed at 7 d, 5 d after galactocerebroside had first appeared.

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