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Characterization of cultured rat oligodendrocytes proliferating in a serum-free, chemically defined medium

(growth regulation/nervous tissue culture/myelination-associated event/glia)

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ABSTRACT **A serumless, chemically defined medium has been developed for the culture of oligodendrocytes isolated from primary neonatal rat cerebral cultures. Combined together, insulin, transferrin, and fibroblast growth factor synergistically induced an essentially homogenous population (95–98%) of cells expressing glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activity to undergo cell division. Proliferating cells were characterized by several criteria: (*i*) ultrastructural analysis by transmission electron microscopy identified the cell type as an oligodendrocyte; (*ii*) biochemical assays showed expression of three oligodendrocyte biochemical markers, induction of both glycerol phosphate dehydrogenase and lactate dehydrogenase (EC 1.1.1.27), and presence of 2′,3′-cyclic nucleotide 3′-phosphodiesterase (EC 3.1.4.37); and (*iii*) immunocytochemical staining showed cultures to be 95–98% positive for glycerol phosphate dehydrogenase, 90% for myelin basic protein, 60–70% for galactocerebroside, and 70% for A2B5. Few cells (<5%) stained positive for glial fibrillary acidic protein, and none were detected positive for fibronectin.**

The formation and maintenance of myelin by oligodendrocytes is well established (1), yet the factors regulating the proliferation and differentiation of oligodendrocytes remain obscure. To elucidate these factors and circumvent *in vivo* complexities, investigators have turned to tissue culture techniques. Primary cultures of mixed glia were first described in 1960 (2); however, more than a decade passed before McCarthy and de Vellis (3) observed that a critical density of dissociated brain cells was needed for oligodendrocyte proliferation in mixed cell cultures. Although oligodendrocytes proliferate in mixed glial cultures (4), oligodendrocytes from rat neonatal cerebellum and corpus callosum were found unresponsive to the mitogens brain and pituitary fibroblast growth factor (FGF), epidermal growth factor (EGF), myelin basic protein, and pituitary extracts (5). Little if any proliferation has been demonstrated in isolated cultures of oligodendrocytes (6, 7).

The recent development of serumless, chemically defined media has created manipulatable and defined environments for study of cell proliferation and differentiation. Although first developed for clonal cell lines (8), other defined media have been created for primary neuronal cultures (9) and primary astrocyte (10) cultures.

Here we report the development of a serumless, chemically defined medium that induces a homogenous population of oligodendrocytes, with respect to glycerol phosphate dehydrogenase expression, to reproducibly undergo cell proliferation. These cultures have been characterized biochemically, immunologically, and ultrastructurally and compared to parallel cultures maintained in serum-supplemented medium.

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MATERIALS AND METHODS

Cell Culture. Purified cultures of oligodendrocytes were prepared as described (3) with the additional modification of a preliminary shaking (200 rpm, stroke diameter 3.81 cm; 37°C) for 1 hr to remove and discard dividing astrocytes. After further shaking for 15–18 hr, isolated oligodendrocytes were collected by centrifugation, counted, and seeded in Falcon plastic 24-well (2.1 cm^2/well) culture plates at a density of 10^5 cells per well. Culture medium was Dulbecco–Vogt modified Eagle's medium/Ham's F-12 medium, 1:1 (vol/vol), with 1.2 g of $NaHCO_3$ per liter and 15 mM Hepes buffer (serum-free medium), supplemented with 10% (vol/vol) fetal calf serum. Serum-free medium was routinely made with pyrogen-free, double-distilled water. Stored serum-free medium was supplemented with fresh pyruvate (110 mg/liter) before use.

Oligodendrocyte Defined Medium (ODM). ODM consisted of serum-free medium, insulin (5 μg/ml), transferrin (500 ng/ml), and FGF (75 ng/ml).

Cell Proliferation. Purified oligodendrocytes were seeded in culture medium. After 18–20 hr of incubation to allow cell attachment, cells were washed three times in serum-free medium and the experimental medium was added. A portion of the cells were counted and this cell density was used as the actual seeding density. The day of addition of experimental medium was designated day 0. The medium was changed every 2 days, and unless otherwise noted, cells were counted at day 5. For cell counts, cells were trypsinized with a 1% Enzar-T (40 times concentrated) trypsin concentrate and 0.1 mM EDTA in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution. Values reported represent cell number per ml, which corresponds to cell number per well of a 24-well culture plate (Falcon), and are the mean value of six wells.

Enzyme Assays. Cell cultures were grown in either ODM or serum-supplemented medium for 5 days and subsequently assayed for glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8), lactate dehydrogenase (LDH; EC 1.1.1.27), and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (cNPase; EC 3.1.4.37). Methods used to prepare culture homogenates and assay GPDH and LDH have been described (11, 12). Homogenates were assayed for cNPase activity by a fluorometric method (13). The inducers hydrocortisone, for GPDH, and N^6,$O^{2'}$-dibutyryl AMP, for LDH and cNPase, were prepared and used as described (3).

Protein Assay. Protein concentration was determined by the method of Bradford (14). Crystalline bovine serum albumin was used as the standard.

Abbreviations: ODM, oligodendrocyte defined medium; GPDH, glycerol-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; cNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; FGF, fibroblast growth factor; EGF, epidermal growth factor; GFAP, glial fibrillary acidic protein.

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Electron Microscopy. Cell cultures were prepared as described (3) and subsequently embedded *in situ* and processed for transmission electron microscopy (15).

Immunocytochemical Staining. Cells were grown on 15-mm glass coverslips contained in multiwell culture plates for 5 days in either serum-supplemented medium or ODM. All staining was by indirect immunofluorescence with fluorescein-conjugated goat anti-rabbit/mouse IgG/IgM as the second antibody. For controls, preimmune sera or, when preimmune sera were not available, normal sera were used. For surface staining, live cells were incubated with the primary antibody. For cytoplasmic staining, cells were first fixed in 4% (wt/vol) paraformaldehyde and then processed, stained, and examined through a Zeiss fluorescence microscope equipped with FITC (fluorescein isothiocyanate) filters and mercury-vapor epi-illumination (16, 17).

The following rabbit polyclonal and mouse monoclonal antibody preparations were used: rabbit anti-GPDH. (1:200 dilution), rabbit anti-glial fibrillary acidic protein (GFAP) (1:500 dilution), rabbit anti-galactocereboside (1:30 dilution), rabbit anti-fibronectin (1:200 dilution), rabbit anti-myelin basic protein (1:200 dilution), mouse anti-A2B5 (1:200 dilution), mouse anti-RBA1 (1:10 dilution), mouse anti-RBA2 (1:10 dilution), and mouse anti-JP-1A9 (1:40 dilution).

Combined Immunocytochemical Staining and Autoradiography. Cells were grown in plastic multiwell culture plates and assayed for [^{3}H]thymidine uptake by GPDH positive cells. After serum-supplemented medium was removed, cells were incubated in ODM containing 1 μM hydrocortisone for 24 hr and subsequently in fresh ODM containing [^{3}H]thymidine (1 μCi/ml; 1 Ci = 37 GBq) and 1 μM hydrocortisone for 24 hr. The cells then were washed three times with phosphate-buffered saline, fixed with 4% paraformaldehyde for 1 hr, and processed for GPDH immunocytochemical detection as described above. Stained cultures were processed for autoradiography (18) and evaluated for silver grains and GPDH staining.

Materials. Unless otherwise stated, all chemicals were purchased from Sigma. Other materials were obtained from the following sources: FGF and endothelial cell growth supplement from Collaborative Research (Waltham, MA); progesterone, testosterone, growth hormone, and dihydroxyacetone phosphate from Calbiochem–Behring; glucose-6-phosphate dehydrogenase from Boehringer Mannheim; Ham's F-12 medium (H-17), Dulbecco's modified Eagle's medium (H-12), Hanks' balanced salt solution (Mg^{2+}- and Ca^{2+}-free), and fetal calf serum from Irvine Scientific; trypsin (1% Enzar-T) from Reheis (Kankakee, IL); lymphocyte hybrid cell line A2B5, clone 105 from American Type Culture Collection; fluorescein isothiocyanate-conjugated goat anti-rabbit/mouse IgG/IgM from Bio-Rad; and the myelin basic protein radioimmunoassay test kit from Diagnostic Systems Laboratories (Webster, TX). The following were gifts: nerve growth factor from J. R. Perez-Polo (University of Texas Medical Branch); EGF and anti-fibronectin rabbit antisera from H. Herschman (University of California, Los Angeles); anti-galactocerebroside rabbit antisera from L. Bologa (City of Hope, Duarte, CA); anti-myelin basic protein and anti-JP-1A9 mouse antisera from G. Dutton (University of Iowa); and anti-GFAP rabbit antiserum from L. Eng (Stanford University).

RESULTS

Cell Proliferation. When seeded at an initial density of 10^5 cells per 2.1 cm^2, oligodendrocytes cultured in serum-supplemented medium underwent little or no cell proliferation over a 5-day growth period. However, in the presence of ODM, the number of cells tripled (Fig. 1). The absence of insulin, transferrin, or FGF or the addition of transferrin

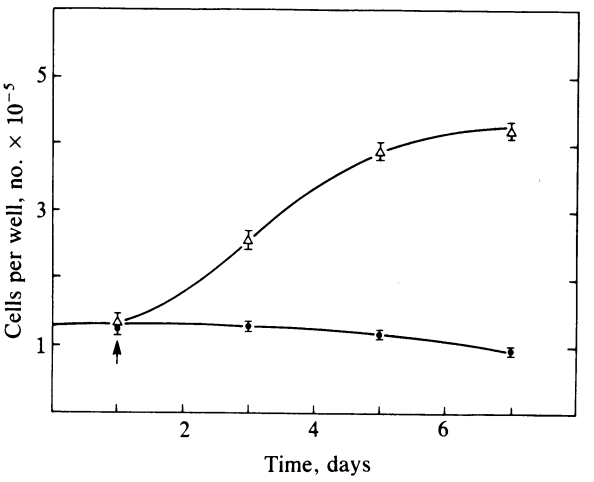

FIG. 1. Growth curve of isolated oligodendrocytes. Cells were grown in either serum-supplemented medium (●) or ODM (△). Data represent cell numbers per well, values are the mean of six wells ± SEM. Arrow indicates when ODM was added to cultures.

alone resulted in limited survival after 5 days in culture. When added individually to serum-free medium, insulin maintained cell number near original seeding density whereas FGF induced limited cell proliferation over a 5-day growth period (Fig. 2). However, when combined together, the supplements acted synergistically, producing an increase in cell number (Fig. 2). The addition of ODM supplements to serum-supplemented medium induced a similar increase in cell number (data not shown). This suggests either that serum does not contain the critical supplements for proliferation or that their concentration may be limiting.

The effect on cell proliferation of varying the concentration of each added supplement is shown in Fig. 3. Optimal concentrations of FGF, insulin, and transferrin were 75 ng/ml, 5 μg/ml, and 500 ng/ml, respectively. In addition, there was a requirement for fresh pyruvate (0.11 mg/ml) in ODM. The need for pyruvate is probably the result of low activities of pyruvate dehydrogenase (19) and hexokinase (20).

In addition to the components described above, we tested a number of other compounds for their ability to promote

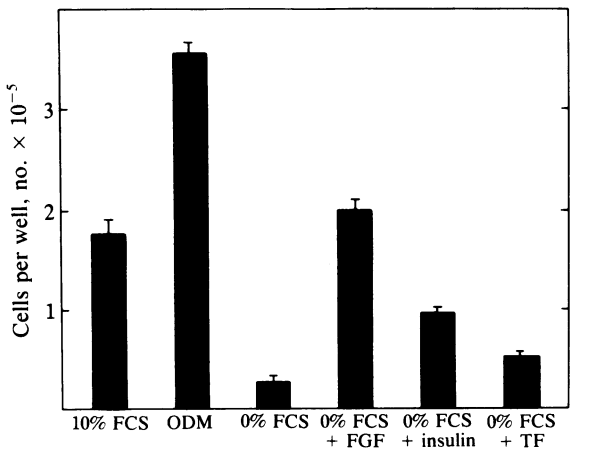

FIG. 2. Response to individual components of ODM. Cells were seeded and washed as described in *Materials and Methods*. Medium added at this time was either serum-free medium (0% FCS), serum-free medium plus one of the three supplements [FGF, insulin, or transferrin (TF)], ODM (all of the supplements), or serum-supplemented medium (10% fetal calf serum, FCS). Cell number was determined 5 days later. Values represent the mean of six wells ± SEM.

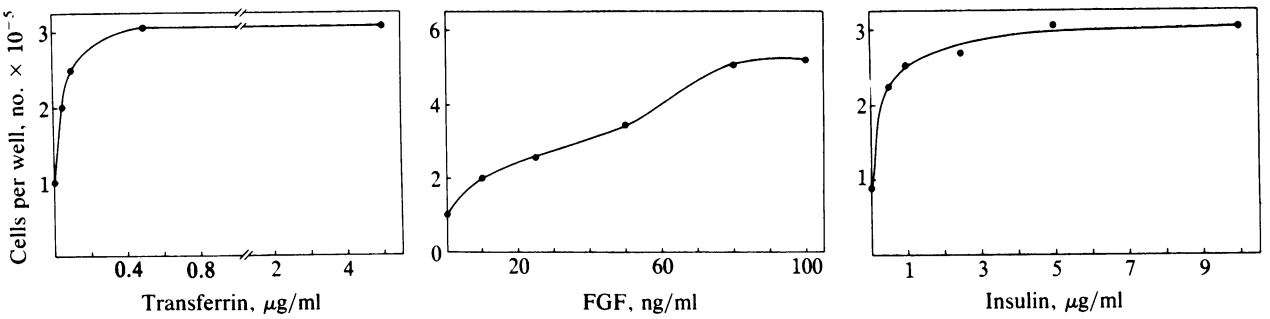

FIG. 3. Cell proliferation as a response to various supplement concentrations. In each experiment, the concentration of two of the three supplements was held constant while the concentration of the third was varied over the range indicated. Values represent the mean of six wells. SEM was less than 10% of the mean for all points.

oligodendrocyte proliferation: thyroxine (10 nM), triiodothyronine (10 nM), progesterone (20 nM), testosterone (20 nM), 17β-estradiol (20 nM), selenium (30 nM), 2-hydroxybutyrate (100 mg/ml), bovine serum albumin (1 mg/ml), biotin (8 μg/ml), ascorbic acid (10 μg/ml), prostaglandin E_1 (500 ng/ml), prostaglandin $F_{2\alpha}$ (500 ng/ml) linoleic acid (10 μg/ml), oleic acid (10 μg/ml), arachidonic acid (1 μg/ml), 4,7,10,13,16,19-docosahexaenoic acid (1 μg/ml), growth hormone (100 ng/ml), EGF (100 ng/ml), and nerve growth factor (100 ng/ml). When each of these compounds was added to ODM, no effect on cell number was seen after 5 days of culture. Endothelial cell growth supplement was toxic to oligodendrocytes cultured in ODM.

Combined Immunocytochemical Staining and Autoradiography. In duplicate experiments, >90% of GPDH positive cells had silver grains over their nucleus (Fig. 4), indicating that the supplements in ODM induced proliferation of almost the entire population, not a subpopulation, of GPDH-positive cells.

Morphological Characterization. After 5 days in culture, the oligodendrocytes cultured in ODM had less elaboration of processes compared to cells grown in serum-supplemented medium (Fig. 5). Transmission electron microscopy of the cells grown in ODM revealed ultrastructural features typical of immature oligodendrocytes (21). Cells possess an eccentric round nucleus with finely clumped heterochromatin. The abundant cytoplasm contains distinctive organelle configurations: stacked cisternae of rough endoplasmic reticulum, prominent perinuclear Golgi apparatus, and microtubules (Fig. 6).

Biochemical Characterization. The modulation of the specific enzyme activities of GPDH, LDH, and cNPase by induction treatments were characterized in ODM and serum-supplemented medium (Table 1). Although enzyme activities of GPDH and LDH were inducible in both culture conditions, the extent of GPDH induction was much greater in the presence of serum. Conversely, cNPase basal activity doubled in ODM and was significantly induced only in ODM.

Immunocytochemical Characterizations. Several different antisera were used to characterize both oligodendrocytes proliferating in ODM and those maintained in serum-supplemented medium (Fig. 7, Table 2). Cultures induced with hydrocortisone in the presence of serum were more intensely stained for GDPH than those induced in ODM. This difference in staining intensity accurately reflected the level of GPDH induction determined biochemically (Table 1). The larger number of myelin basic protein-immunoreactive cells in ODM correlated with myelin basic protein content determined by radioimmunoassay (212 ng/mg of protein for cultures grown in ODM compared to 69 ng/mg of protein in serum supplemented cultures). In cultures grown in ODM, the intensity of reaction to galactocerebroside antiserum varied; the majority of the cells that were stained were decorated only in distinct areas on cell body surfaces and processes.

Cells cultured in either ODM or serum-supplemented medium did not express reactivity to fibronectin antiserum, a marker for fibroblasts. Monoclonal antisera to vimentin (RBA1), GFAP, and desmin (RBA2) did not react with oligodendrocytes cultured in ODM. Those cells cultured in ODM that do react with GFAP antiserum have a morphology that distinguishes them from the cells positive for GPDH and myelin basic protein. The GFAP-positive cells have a larger cell body and many long branching and tapering processes. These observations suggest that the GFAP-positive cells are astrocytes (23). In serum-supplemented medium, in addition to the flat and polygonal cell type reacting with GFAP antiserum, 20–30% of the smaller phase-dark cells expressed GFAP immunoreactivity. Addition of serum to ODM at the start of isolated culture resulted in positive GFAP staining of

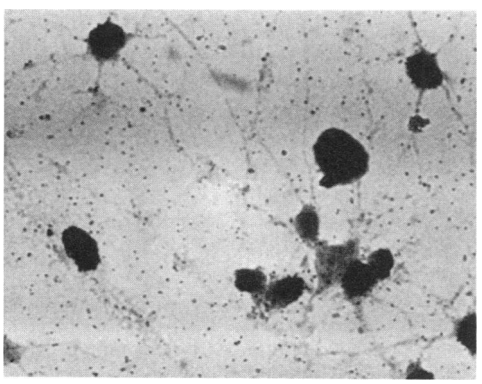

FIG. 4. Autoradiograph of oligodendrocytes 48 hr after replating. The cells were incubated with [^{3}H]thymidine and processed as described in *Materials and Methods*. (×110.)

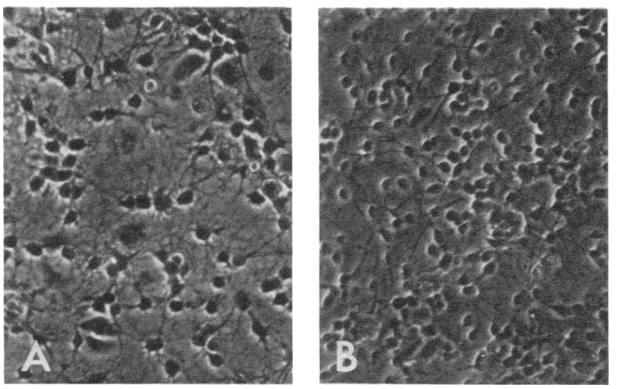

FIG. 5. Phase-contrast micrographs of isolated oligodendrocytes grown for 5 days in serum-supplemented medium (*A*) or in ODM (*B*). (×190.)

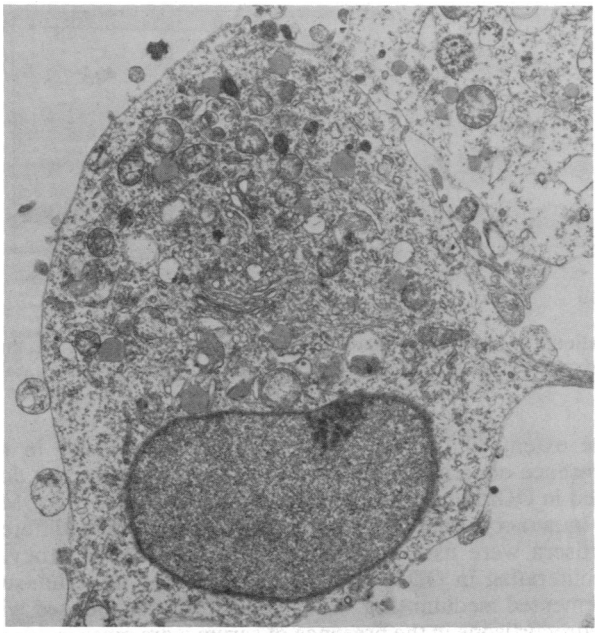

FIG. 6. Transmission electron micrograph of isolated oligodendrocytes after 5 days in ODM. (×11,250.)

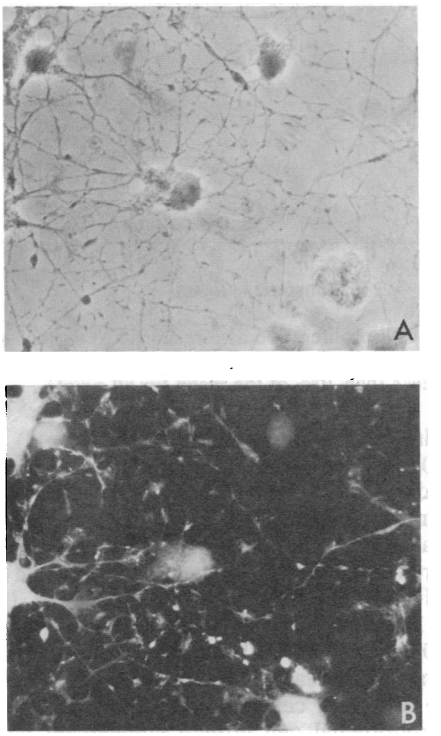

FIG. 7. GPDH immunofluorescence of oligodendrocyte cultures after 5 days in ODM. Cultures were induced with 1 μM hydrocortisone for the last 2 days, and 10% fetal calf serum was present during the last day to enhance GPDH levels (see Table 1). More than 95% of the cells seen in phase contrast (*A*) stained positive for GPDH (*B*). In the absence of serum, the immunostaining was less intense but retained the same distribution (see Table 2 for details). Control sera under both conditions did not stain. (×365.)

some of the phase-dark cells. Therefore, the expression of GFAP is likely regulated by a serum factor(s).

DISCUSSION

We have demonstrated that isolated primary oligodendrocytes from postnatal rat cerebrum can proliferate in a serumless, chemically defined medium. The entire population (>90%) of isolated oligodendrocytes proliferate and homogeneously stain for GPDH, an enzyme exclusively expressed in oligodendrocytes in rat brain. Proliferating cells were judged to be oligodendrocytes by ultrastructural, biochemical, and immunological criteria.

The purified oligodendrocyte cultures (3) are established from primary mixed glial cultures at a time which corresponds to the stage of active oligodendrocyte proliferation *in vivo* (24). However, at the seeding densities employed in this study, isolated oligodendrocytes do not proliferate significantly in serum-supplemented medium. Other studies have also indicated that isolated oligodendrocytes do not proliferate (6, 7). Although oligodendrocytes in mixed glial cultures have been shown to proliferate (4), known potent mitogens, such as fibroblast growth factor, epidermal growth factor, and bovine pituitary extracts, were reported to have no effect on oligodendrocytes (5). The lack of proliferation may have been caused by an inadequate milieu. For instance, ODM supplements induce equivocal cell proliferation when added individually to serum-free medium or to medium supplemented with serum. Another explanation may be the selection of a population of cells no longer capable of

Table 1. Regulation of oligodendrocyte marker enzymes

Enzyme/inducer	Specific activity*	
	ODM	Serum-supplemented medium
GPDH		
− Hydrocortisone	49.3 ± 15.1	62.6 ± 13.3
+ Hydrocortisone	149.1 ± 35.4	612.4 ± 45.5
LDH		
− Bt_2cAMP	818.8 ± 58.7	1138.0 ± 125.1
+ Bt_2cAMP	1258.0 ± 86.1	1930.2 ± 363.0
cNPase		
− Bt_2cAMP	576.2 ± 42.1	270.4 ± 41.0
+ Bt_2cAMP	663.4 ± 32.6†	278.6 ± 10.5

*Specific activity is expressed in units which are defined as nmol of substrate converted per min per mg of protein at 30°C. The final concentration of hydrocortisone was 1.0 μM and N^6,$O^{2'}$-dibutyryl cyclic AMP (Bt_2cAMP) was 1.0 mM. Each inducer was present for a total of 48 hr, with fresh inducer medium added after 24 hr. Enzyme assays were determined after 5 days in culture. Each value represents the mean of six cultures ± SD.
†Significant ($P < 0.05$, $n = 6$).

Table 2. Immunocytochemical characterization of isolated oligodendrocytes after 5 days in culture

Specific antiserum to	% positive cells	
	ODM	Serum-supplemented medium
GPDH	95–98	95–98
Myelin basic protein	90	10
Galactocerebroside	60–70	20–30
A2B5	70	30
JP-1A9*	5	5
RBA1 (vimentin)	0	ND†
RBA2 (desmin)	0	ND†
GFAP	2–5	20–30
Fibronectin	0	0

Percentage of cells within the culture that were stained in indirect immunofluorescence was determined by counting cells within four separate fields, with a minimum of 100 cells counted. Values represented are means of four separate experiments, each with duplicate experimental cultures.
*JP-1A9 is specific for oligodendrocytes (22).
†Not done.

undergoing cell division. The oligodendrocytes obtained from optic nerve and corpus callosum, characterized as galactocerebroside-positive cells, were nonresponsive to FGF (5). Raff *et al.* (25) have suggested that galactocerebroside expression, a marker for oligodendrocytes, represents a more differentiated cell type. The oligodendrocytes, cultured in serum, used in our study did not express significant levels of galactocerebroside (Table 2). These data, considered together with the narrow time span of oligodendrocyte proliferation *in vivo* (24) and the varying times of differentiation between different brain regions, suggest that the lack of FGF responsiveness may have been the result of culturing a differentiated oligodendrocyte no longer capable of cell division. However, galactocerebroside-positive and, to a lesser extent, myelin basic protein-positive cells in mixed glial cultures from mice are able to incorporate [^{3}H]thymidine (4). Thus, both the culture milieu and the developmental stage of the brain area used to establish cultures may be important in developing a model system for the study of oligodendrocyte proliferation and differentiation.

The sets of components needed for oligodendrocyte and astrocyte cell proliferation are distinct. ODM does not induce significant proliferation of astrocytes (unpublished data). The unique components present in astrocyte defined medium [prostaglandin $F_{2\alpha}$ (500 ng/ml), putrescine (100 nM), and hydrocortisone (50 nM)] or in ODM [transferrin (500 ng/ml) and pyruvate (0.11 mg/ml)] had no effect on the other cell type. This suggests that, in addition to biochemical, ultrastructural, and immunological differences between the two cell types (3, 10), the critical milieu needed for cell proliferation is distinct between the two neonatal cerebral glial cells.

Although both serum-supplemented medium and ODM maintain oligodendrocyte cytological features, they differentially affect the expression of several biochemical and immunological parameters (Tables 1 and 2). The oligodendrocyte markers inducible GPDH (26), LDH (27) and cNPase (28) were expressed, but the magnitude of the induction and specific enzymic activities varied between culture conditions. GPDH decreased in ODM whereas cNPase increased. Serum appears to contain a factor(s) which enhances GPDH inducibility without affecting the distribution of GPDH-positive cells (Table 1, Fig. 7). On the other hand, the number of cells positive for myelin basic protein, galactocerebroside, and A2B5 was dramatically increased by ODM. These observations suggest that multiple regulatory agents are involved in oligodendrocyte differentiation.

Unlike serum-supplemented medium (4, 23, 25, 29–31), ODM favors a wide range of oligodendrocyte differentiation stages. Oligodendrocytes cultured in ODM are proliferative, a state that corresponds to a less differentiated oligodendrocyte *in vivo* (24). Furthermore, the induction of cNPase by dibutyryl cAMP (28), the reduction in number and elaboration of processes (32), and the immunocytochemical expression of A2B5 (33) indicate that the components in ODM induce the expression of less differentiated traits. However, the simultaneous expression of myelin basic protein and galactocerebroside suggests a more differentiated state (29, 33). Although the expressions of both are enhanced in ODM, the expression of myelin basic protein is more prevalent than that of galactocerebroside (Table 2), indicating that these two markers of oligodendrocyte differentiation may be under separate regulatory controls. Thus, there may be a variety of signals or factors controlling each of the multiple events leading to myelination.

We have found that 30% of isolated oligodendrocytes cultured in serum-supplemented medium express GFAP immunoreactivity. However, 90-Å glial filaments have not been seen in transmission electron micrographs of these cells (3). The low galactocerebroside expression (20–30%) suggests that isolated oligodendrocytes from rat cerebrum cultured in serum are developmentally immature. Others have demonstrated GFAP immunoreactivity in immature oligodendrocytes from human fetal spinal cord (34) and in a glial precursor cell when cultured in serum-supplemented medium but not in serumless medium (25). Similarly in our study, the addition of serum to ODM at the time of oligodendrocyte isolation resulted in phase-dark cells that expressed GFAP reactivity. Taken together, these data suggest that isolated oligodendrocyte cultures derived from cerebrum of 1- to 2-day-old rats are developmentally immature and that a subpopulation can be induced to differentiate into GFAP-positive cells by manipulation of the culture environment.

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