Culture of rat cerebral oligodendrocytes in a serum-free, chemically defined medium

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Oligodendrocytes were isolated from the cerebra of young rats (5–10 days old) by trypsinization of the
tissue followed by cell separation on Percoll gradients. The isolation was carried out in physiological,
isotonic media. The cell yield was $2-4 \times 10^6$ cells per brain; the plating efficiency was $\geqslant 70\%$. Isolated
cells were seeded on poly-L-lysine-coated culture dishes and maintained in a serum-free, chemically
defined medium for at least 30 days. After 10 days in culture $67 \pm 10\%$ of the surviving cells were
oligodendrocytes, as judged by immunocytochemical and morphological criteria, whereas most of the
other cells reacted positively with antiserum against glial fibrillary acidic protein. The expression of
typical oligodendrocyte markers (2':3'-cyclic-nucleotide 3'-phosphodiesterase, galactocerebrosides and
myelin basic protein) was greatly enhanced under these serum-free conditions as compared with cultures
in serum-containing medium. The antigenic markers (galactocerebrosides, myelin basic protein) were
absent in the freshly isolated cells but could be detected after 3 days in culture by immunocytochemistry.
The activity of 2':3'-cyclic-nucleotide 3'-phosphodiesterase increased from 75 nmol min^{-1} mg^{-1} protein
on day 4 to 400 nmol min^{-1} mg^{-1} protein on day 14 in culture.

Introduction

Oligodendrocytes produce myelin in the central nervous system. Isolation and
maintenance in culture of this cell type is therefore of great importance for the
biochemical investigation of the synthesis of myelin-specific compounds and their
assembly in myelin membranes. Several procedures have been described for the

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isolation of oligodendrocytes; some are only intended for the bulk isolation of these cells (Poduslo and Norton, 1972), whereas other methods have been developed with the aim of obtaining a pure culture of oligodendrocytes (McCarthy and de Vellis, 1980; Hirayama et al., 1983).

Our aim was the isolation of metabolically active oligodendrocytes which could be maintained in a serum-free, chemically defined culture medium so as to study their lipid metabolism. Such media are advantageous for the investigation of the metabolic effects of hormones. Serum-free media for primary cultures of neural cells have been used previously (e.g. Honneger et al., 1979; Snyder and Kim, 1979; Morrison and de Vellis, 1981; Romijn et al., 1982; Brunner et al., 1982).

This paper describes a method for the isolation of an oligodendrocyte-enriched cell population from the brains of rat pups. The cells can be maintained in culture for at least one month in a chemically defined medium and express specific oligodendrocyte markers (see Mirsky et al., 1980), such as galactocerebrosides, myelin basic protein and 2':3'-cyclic-nucleotide 3'-phosphodiesterase (EC 3.1.4.37) to a greater degree than cells cultured in serum-containing medium.

Materials and methods

Materials

Putrescine and biotin were obtained from Serva (Heidelberg, F.R.G.); Percoll was supplied by Pharmacia (Uppsala, Sweden). Goat anti-rabbit-IgG conjugated to fluoroisothiocyanate (FITC) was bought from Miles-Yeda (Rehovot, Israel). Rabbit anti-galactocerebroside serum was prepared by injecting rabbits with an emulsion of galactocerebrosides (Type I, Sigma, St. Louis, MO), bovine serum albumin (Sigma A 8022) and complete Freund's adjuvant, following the procedure described by Bologa et al. (1981). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco (Grand Island, NY); Ham's F12 medium and Newborn Calf Serum (Lot no. 29101128) were obtained from Flow (Irvine, U.K.). Deoxyribonuclease (D0751), trypsin (T 8253), soybean trypsin-inhibitor (Type II-S), poly-L-lysine (P 2636) and all other biochemicals were supplied by Sigma (St. Louis, MO). Albumin was further purified as described by Chen (1967).

Animals

Wistar rats, 13 days pregnant, were obtained from the Central Institute for the Breeding of Laboratory Animals (Zeist, the Netherlands). The litters were trimmed to 10 pups one or two days after birth. Five-to-10-day-old pups were used for the isolation of oligodendrocytes.

Isolation of oligodendrocytes

The procedure described here combines two methods (Snyder et al., 1980; Gebicke-Harter et al., 1981; see also Hirayama et al., 1983). The procedure is carried out at room temperature under sterile conditions. Ten pups were decapitated and the heads were immersed in 70% ethanol, washed with Hank's balanced salts solution

(HBSS, without $CaCl_2$, $MgCl_2$ and $NaHCO_3$, but with 5 mM HEPES and 100 U/ml penicillin and 100 μg/ml streptomycin). The cerebra were removed and collected in HBSS, transferred to a dry Petri dish and subsequently minced to pieces of about 1 mm^3 in 0.5 ml of HBSS containing 0.5 mg of deoxyribonuclease. The mince was dispersed in 40 ml of HBSS containing 60 mg trypsin and transferred to a 100 ml flask which was shaken during 45 min at 37°C. Subsequently the suspension was centrifuged for 2 min at 275 g. After removal of the supernatant and the superficial layer of red blood cells, the remaining tissue was washed twice with HBSS and finally suspended in 10 ml of HBSS containing 30 mg soybean trypsin inhibitor and 0.5 mg deoxyribonuclease. The tissue was then dissociated carefully by pipetting with a glass 10 ml pipette (orifice approx. 0.5 mm) until clumps of tissue were no longer visible (10–20 passes). The obtained cell suspension was sieved through a double layer of nylon screen (100 μm pores), adding HBSS to facilitate the sieving, to a final volume of 90 ml. This suspension was then mixed with 30 ml of undiluted Percoll.

Six "step-gradients" were prepared by layering 15 ml of a 20% Percoll dilution in HBSS over 20 ml of the cell suspension (25% Percoll) (see Fig. 1). The gradients were centrifuged during 30 min at 9150 g_{max} in a Sorvall GSA rotor using special adaptors for 50 ml tubes. After centrifugation the top 15 ml, including the floating layer of debris and dead cells, were removed by suction. The distinct band of red blood cells at the bottom (Fig. 1) was removed using a Pasteur pipette. The remaining suspension of cells (about 20 ml per tube) was sieved through nylon screen (20 μm pores) and diluted with 3 volumes of HBSS. The cells were then pelleted by centrifugation (10 min at 1465 g_{max}) in a Sorvall GSA rotor. The supernatant was removed, and the cell pellet was suspended in DMEM containing 10% (v/v) newborn calf serum. An appropriate dilution of the cell suspension was counted using a hemocytometer under a phase contrast microscope. The yield was usually between 2 and 4×10^6 cells per brain. The whole procedure took 3.5–4 h.
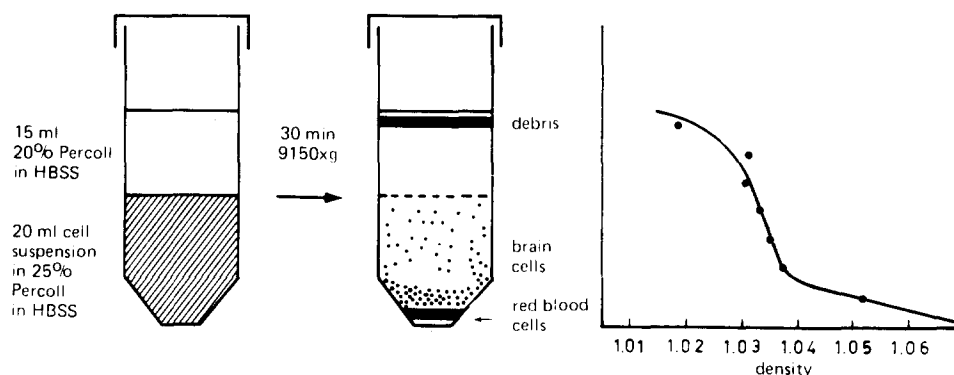

Fig. 1. Step-gradient centrifugation, showing the position of debris, red blood cells and brain cells and the change in density profile resulting from centrifugation.

Culture conditions

Cells were plated in plastic tissue culture dishes (Nunc, Denmark) that had been coated with poly-L-lysine (5 μg/ml; 0.1 ml/cm^2) for 1 h and air dried. The cell density was 3×10^4 cells per cm^2 in 1.5 ml DMEM (0.15 ml/cm^2) containing final concentrations of 10% newborn calf serum, 25 mM glucose, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES and 8 mM sodium bicarbonate. The medium was replaced after 24 h by 1.5 ml of a new chemically defined medium (CDM; Table I) developed by Romijn et al. (unpublished results) on the basis of the hormone-supplemented medium formulated by Bottenstein and Sato (1979). Cytosine-1-β-D-arabinoside (Ara-C; 10^{-5} M) was added after another day in culture. Ara-C (10^{-5} M) was also added when the medium was changed after 5 days in vitro. Subsequently the medium was changed every 4 days. The cultures were maintained at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2.

Immunofluorescence

Cultures were washed 3 times with HBSS (which was also used for all further washes), followed by a 30 min incubation at room temperature with 4% formaldehyde in HBSS. After fixation the cultures were washed again 3 times. Before incubation with the antisera against myelin basic protein and glial fibrillary acidic

TABLE I

COMPOSITION OF THE CHEMICALLY DEFINED MEDIUM

DMEM : Ham's F12 : H$_2$O *	67.5 : 22.5 : 10
Ascorbic acid	50.0 mg/liter
Biotin	0.1
Bovine serum albumin (approx.)	23 $\times 10^3$
DL-Carnitine	2.0
D-(+)-Galactose	7.5
Glutamine	33.0
Hydrocortisone	20.0×10^{-3}
Insulin	2.0
linolenic acid **	1.0
linolenic acid **	1.0
Na$_2$SeO$_3$	7.9×10^{-3}
Progesterone	6.3×10^{-3}
Putrescine	16.0×10^{-3}
Retinol **	0.1
Retinyl acetate **	0.1
DL-α-Tocopherol **	1.0
DL-α-Tocopheryl acetate **	1.0
Transferrin	50.0
Triiodothyronine	2.0×10^{-3}

* The final glucose concentration was 20 mM; HEPES (20 mM), NaHCO$_3$ (8 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml) were added. Final pH at 37°C was 7.4. Water was added to bring the osmolarity of the medium to 300 mOsm.

** As their albumin complexes at molar ratios of 0.2 (retinol, retinyl acetate), 1.6 (tocopheryl, tocopheryl acetate) and 5 (fatty acids).

protein, the cultures were incubated for 10 min with a 0.1% solution of Nonidet P_{40} in HBSS, followed by three washes. For incubation with anti-galactocerebroside serum, fixed or unfixed cultures were used without further treatment. They were incubated with the antisera for 30 min at room temperature followed by 3 washes. The cultures were then incubated for 30 min with goat anti-rabbit-IgG FITC conjugate and washed again. Stained cultures were inspected with a Zeiss microscope with FITC-fluorescence and phase-contrast optics.

Other methods

2':3'-Cyclic-nucleotide 3'-phosphodiesterase (EC 3.1.4.37) was measured in homogenates of rat cerebra and in sonicates of cultured cells using the method described by Sims and Carnegie (1978). Protein was estimated using the method of Lowry et al. (1951).

Results

Development of cells in culture

The procedure for cell isolation, described in this paper, gives a yield between 2 and 4×10^6 cells per cerebrum. The cells attach very rapidly to the bottom of the poly-L-lysine-coated culture dishes. On uncoated dishes this attachment is delayed, so that cells clump together and are lost when the medium is changed. Aggregation also occurred when the cells were seeded too densely.

The percentage of cells that remains attached after 24 h is variable (70–90%) and depends at least partially on the age of the animals.

The development of the cultures varies with the composition of the culture medium: in DMEM/10% newborn calf serum the cells develop a morphology, as seen by phase optics, which is similar to the astrocyte-enriched cultures described by Hansson et al. (1980). Surprisingly, more than 90% of the cells in our preparation were glial fibrillary acidic protein negative and only few galactocerebroside-positive cells were present (cf. Raff et al., 1983). After 10–14 days in culture, small, phase-dark cells appear on top of the confluent cell layer; after this time the appearance of the culture remains constant (Fig. 2). Similar cells have been observed by McCarthy and de Vellis (1980). In our culture system the small dark cells remain galactocerebroside-negative. However, the activity of cyclic-nucleotide phosphodiesterase increased with the age of the culture (from 20 nmol min^{-1} mg^{-1} at 3 days in vitro to 150 nmol min^{-1} mg^{-1} protein at 14 days in vitro). Because cyclic-nucleotide phosphodiesterase activity is very high in brain and preferentially associated with myelin and myelin-related cellular structures (see Sprinkle et al., 1980; Nishizawa et al., 1981; Pfeiffer et al., 1981; Hansson et al., 1982; and refs. therein), this result might indicate that precursor cells of oligodendrocytes do develop in the presence of serum, expressing cyclic-nucleotide phosphodiesterase activity but lacking galactocerebrosides. Hansson et al. (1982) showed that the cyclic-nucleotide phosphodiesterase activity is low in astrocyte cultures and does not increase with the age of the culture.
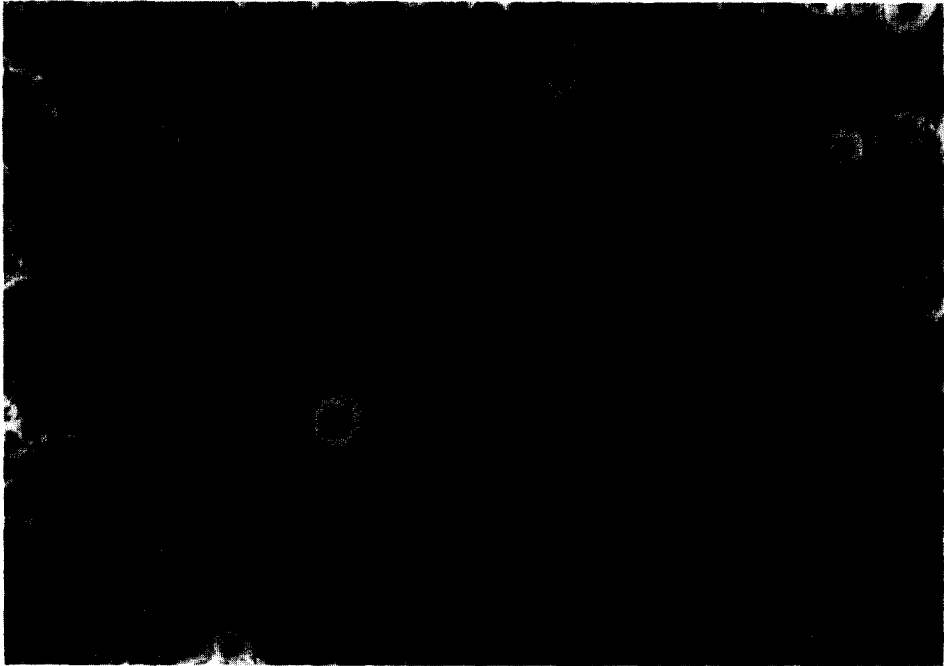
Fig. 2. Culture of brain cells in DMEM/10% newborn calf serum after 28 days in culture. Note the confluent layer of cells with small dark cells on top.

We plated our cells in serum-containing medium because the cultures deteriorated gradually if plated directly in CDM. The cells developed quite differently when the DMEM/10% newborn calf serum was replaced by CDM after 24 h. After 48 h in vitro two main cell types predominated (Fig. 3). The most abundant is a cell type that develops a very elaborate and highly branched system of processes, which became increasingly complex with the age of the culture (Figs. 4 and 5). These cells are galactocerebroside-positive (Fig. 6) and myelin basic protein positive (Fig. 7). Using anti-galactocerebroside serum, the processes were more intensely fluorescent, whereas anti basic-protein fluorescence was observed predominantly in the peri-karya. The oligodendrocytes make up $67 \pm 10\%$ of the cells after 10 days in culture. Most of the other cells were astrocytes (glial fibrillary acidic protein positive).

These astrocytes proliferated in CDM, but more slowly than in serum-containing medium. In addition to oligodendrocytes and astrocytes, a small number of phago-cytic cells was usually present, as judged by the uptake of Indian ink. Hansson et al. (1980) also reported the presence of macrophages in their primary cultures. Initially some small bipolar cells also developed but they disappeared after 7–10 days in vitro. We do not know if this is a population of neurons or of oligodendrocyte precursor cells as reported by Raff et al. (1983).

The activity of cyclic-nucleotide phosphodiesterase increased from 75 nmol min^{-1} mg^{-1} protein after 3 days in CDM to 400 nmol min^{-1} mg^{-1} after 14 days in vitro.
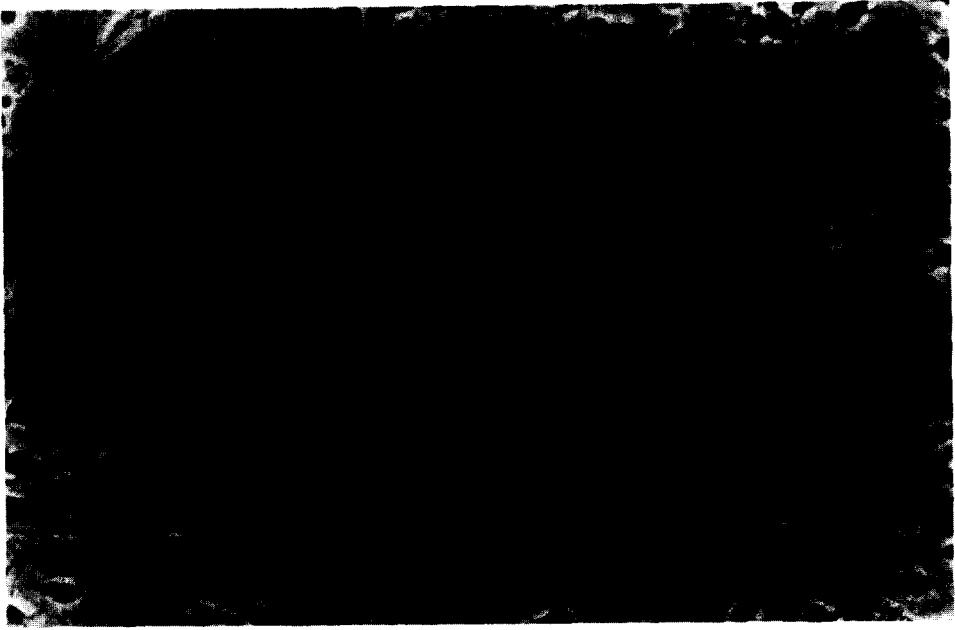
Fig. 3. Culture of brain cells after 2 days in vitro. Two cell types have become apparent: flat, elongated cells and small round cells that bear many processes. The cells were seeded in DMEM/10% newborn calf serum. After 24 h this medium was replaced by CDM. (For further culture conditions see Methods section.)
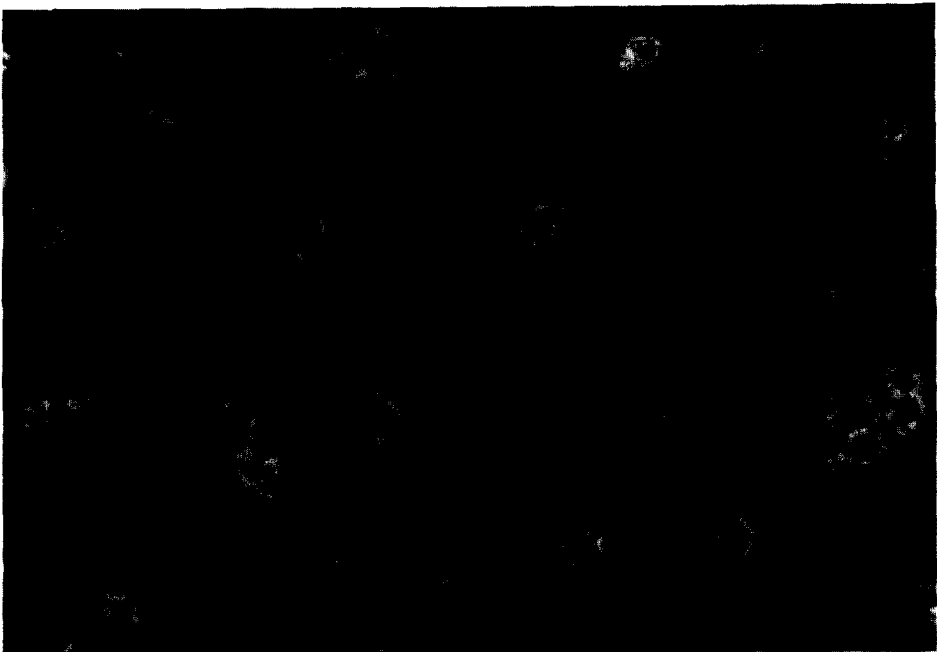

Fig. 4. Culture of brain cells, 4 days after isolation. Note the highly branched oligodendrocytes, interspersed with astrocytes. The cells were seeded in DMEM/10% newborn calf serum. After 24 h this medium was replaced by CDM. After 48 h Ara-C was added. (For further culture conditions see Methods section.)
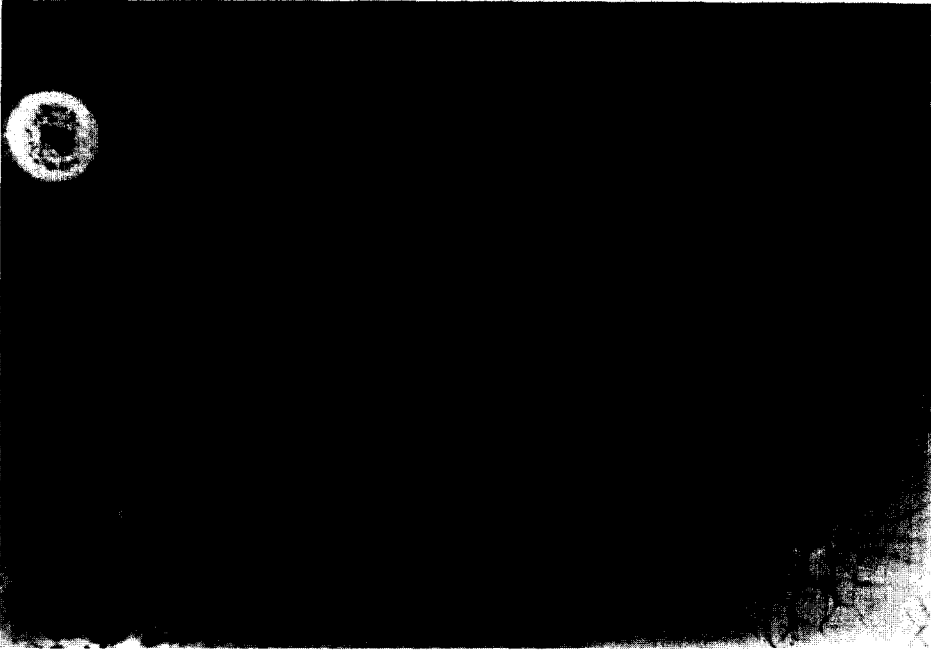
Fig. 5. Oligodendrocytes after 10 days in culture. The cells were seeded in DMEM/10% newborn calf serum. After 24 h this medium was replaced by CDM. After 48 h Ara-C was added. (For further culture conditions see Methods section.)

Fig. 6. Oligodendrocytes fixed and stained for galactocerebroside by the indirect fluorescent antibody technique.
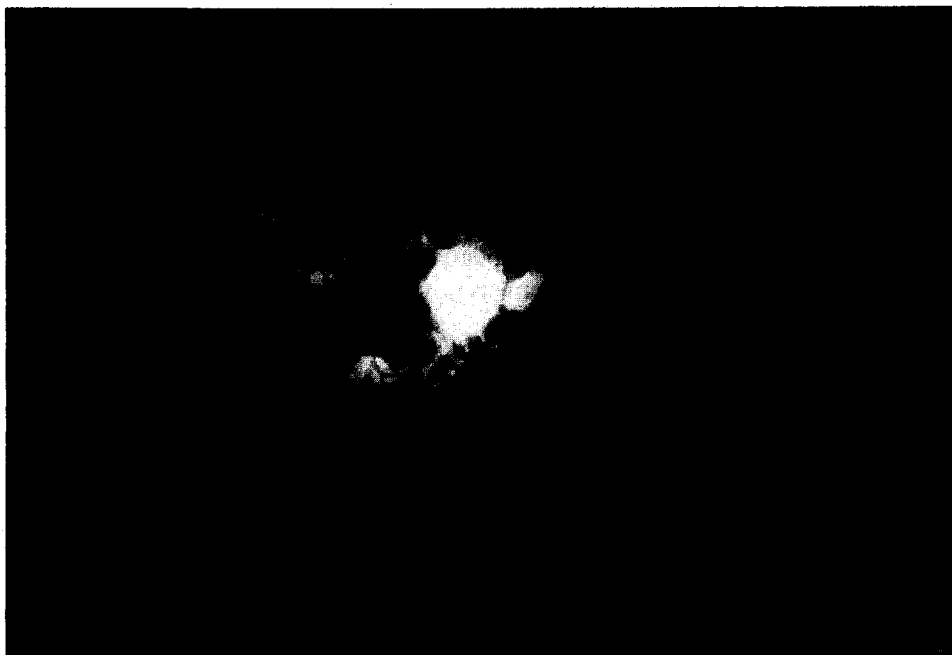
165

Fig. 7. Oligodendrocytes fixed and stained for myelin basic-protein by the indirect fluorescent antibody technique.

Parallel experiments showed that cyclic-nucleotide phosphodiesterase activity in whole cerebrum homogenates increased from 100 nmol $\text{min}^{-1}\text{ mg}^{-1}$ protein at day 6 to 1000 at day 21.

An interesting observation was that oligodendrocytes in CDM tended to form domains in culture (see Fig. 4); preferably in the open spaces between proliferating astrocytes. Apparently oligodendrocytes produce a more elaborate network of processes when they are attached directly to the coated surface of the dish than when they grow on top of a layer of astrocytes.

Cultures were maintained during at least 30 days. However, the percentage of oligodendrocytes slowly decreased due to astroglial overgrowth.

Discussion

Oligodendrocytes are the cells that produce and maintain myelin in the central nervous system (reviewed by Norton, 1983). In rat pups, myelination peaks at the end of the suckling period, in the third postnatal week. Myelin has a high lipid content with cholesterol, phospholipids and galactocerebrosides in a molar ratio of 4:3:2. Hence it can be anticipated that oligodendrocytes have a high capacity for lipid synthesis. Dissociation of brain tissue into isolated cells results in the rupture of

processes, tight junctions etc. As a consequence, it is essential that the isolated cells are allowed to recover in culture from this sustained damage, before their metabolic properties are investigated. Moreover, long-term cultures will be required for the study of myelination, which is not a short-term process. A successful cell isolation method should, therefore, employ physiological conditions in order to maintain viability both during the isolation procedure and the subsequent culture. To achieve this goal, oligodendrocytes were isolated just before the stage of active myelination, but after the development of sufficient oligodendrocyte precursor cells (Raff et al., 1983) that will continue their development in vitro.

A number of reports on the isolation of oligodendrocytes has been published during the last decade (e.g. Poduslo and Norton, 1972; Fewster et al., 1973; Chao and Rumsby, 1977; Szuchet et al., 1978; Snyder et al., 1980; McCarthy and de Vellis, 1980; Bhat et al., 1981; Lisak et al., 1981; Gebicke-Harter et al., 1981; Hirayama et al., 1983). As far as rat-brain oligodendrocytes are concerned, the method described by McCarthy and de Vellis (1980) is the only one using physiological conditions as much as possible and yielding enough cells for biochemical investigations. Bhat et al. characterized oligodendrocytes isolated by a modification of this procedure and reported a purity of 99%. A drawback of this method is, that the primary cultures must be maintained for two weeks, before oligodendrocytes can be harvested and subcultured. This precludes the study of the early steps in the differentiation of oligodendrocytes and myelination. Other authors use unphysiological media, e.g. hypertonic sucrose gradients for cell separation (Szuchet et al., 1978; Snyder et al., 1980). More recently iso-osmotic Percoll gradients were introduced for the isolation of oligodendrocytes from cat brain (Gebicke-Harter et al., 1981), bovine brain (Lisak et al., 1981) and rat brain (Hirayama et al., 1983). The isolation method described in this paper uses physiological media and isotonic gradients and yields an oligodendrocyte-enriched culture with cells that exhibit typical oligodendrocyte markers within 3 days after isolation. The age of the rat pups (5–10 days old), is critical for the quality of the preparation. If older rats were used, the yield and viability dropped rapidly; from younger rats oligodendrocytes could also be isolated, but the purity of the preparation was lower.

The most important feature of our method is the utilization of a new serum-free culture medium (CDM) which was recently formulated for the growth of cerebral cortex neurons by Romijn (unpublished results). Since the pioneering work by the group of Sato (see Barnes and Sato, 1980), serum-free, hormone-supplemented culture media have been used also for the culture of neural cells (Honneger et al., 1979; Snyder and Kim, 1979; Morrison and de Vellis, 1981; Romijn et al., 1982; Brunner et al., 1982). One of the advantages of a chemically defined medium over a serum-containing medium is the possibility to study quantitatively and reproducibly the influence of hormones upon myelination. Studies on cellular secretion products and intercellular messengers might be facilitated in serum-free media because it has been suggested that serum possibly interferes with these processes (Bhat et al., 1981). The expression of oligodendrocyte-specific markers indicates that the early development of oligodendrocytes in this CDM parallels their development in vivo (see also Abney et al., 1981 and Hansson et al., 1982). The further development of oligo-

dendrocytes may well depend on signals from other cell-types (Bhat et al., 1981; Bologa et al., 1982; Mithen et al., 1983). It seems likely that central myelination requires signals derived from neuronal perikarya and/or their axons, similar to what has been shown for Schwann cells in the periferal nervous system (Wood and Bunge, 1975).

Ideally, our chemically defined medium should select only for the survival of oligodendrocytes and prevent any proliferation of other cell types (cf. Barnes and Sato, 1980). From that point of view the chemically defined medium described in Table I is not selective for the growth of oligodendrocytes because astrocytes do still proliferate, although their growth is substantially restricted compared to cultures in serum-containing medium. In order to block astrocyte proliferation, we have added cytosine-1-β-D-arabinofuranoside (Ara-C), an inhibitor of mitosis, to the culture medium after 2 days in vitro. Addition of Ara-C before this time resulted in a much lower yield of oligodendrocytes (results not shown), indicating that oligodendrocytes or their precursor cells are still mitotic.

We found little effect of leaving out any of the individual components of the CDM (Table 1), with the exception of transferrin and insulin. This might indicate a cooperative effect of the components of the CDM (cf. Morrison and de Vellis, 1981; Michler-Stuke and Bottenstein, 1982). The absence of serum, however, appears to be the crucial factor responsible for the successful maintenance of intact oligodendrocytes. It is interesting to note in this context that the absence of serum may be the determining factor in the development of a bipotential glial precursor cell into an oligodendrocyte as has been reported by Raff et al. (1983).

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