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Astrocytes support incomplete differentiation of an oligodendrocyte precursor cell

G.Keilhauer, D.H.Meier[1], S.Kuhlmann-Krieg, J.Nieke and M.Schachner

Department of Neurobiology, University of Heidelberg, Im Neuenheimer Feld 504, 6900 Heidelberg, FRG

[1]Present address: Neurologische Klinik, University of Tübingen, 7400 Tübingen, FRG

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Glial fibrillary acidic protein-positive astrocytes, but not neurons or fibroblasts, support the differentiation of an oligodendroglial precursor cell expressing O4 antigen and vimentin into an O4 antigen-positive, but vimentin-negative oligodendrocyte. Further maturation into galactocerebroside (O1)-positive oligodendrocytes is, however, not achieved under the culture conditions used, neither in the presence of astrocytes nor neurons.

Key words: astrocytes/oligodendrocyte precursor/differentiation

Introduction

It has been suggested that oligodendrocytes and astrocytes develop from a common precursor cell (Privat *et al.*, 1981; Choi *et al.*, 1983; Raff *et al.*, 1983b). The cellular and molecular signals that underlie the divergent routes of differentiation remain, however, unknown. It is likely that signals from axons influence either directly or indirectly the induction of oligodendrocyte differentiation in the mammalian optic nerve *in vivo* (Privat *et al.*, 1981). *In vitro*, astrocytes have been shown to support proliferation and differentiation of oligodendrocytes according to their time table *in vivo* (Noble and Murray, 1984). This study was undertaken to characterize the influence of astrocytes, neurons and a fibroblast cell line on the differentiation of oligodendrocytes from rat cerebral hemispheres.

Results

Primary monolayer culture of rat cerebral cortex

When monolayer cultures of temporal lobes from zero- to 2-day-old rats are maintained in culture medium containing 10% fetal calf serum, L1 antigen-positive neurons are no longer detectable 4 days after plating. The culture then consists of two major cell populations, a monolayer of glial fibrillary acidic protein (GFA)-positive astrocytes underneath small, phase-dark cells with few slender processes, some, but not all of which express O4, L2 and A2B5 antigens, tetanus toxin receptors, vimentin and the L2 epitope, but not GFA, O1 or L1 antigens (see Table I). The percentage of O4 antigen-positive cells which also express vimentin falls from 55 ± 5% at day 3 *in vitro* to 5 ± 5% at day 13 (Figure 1). Vimentin is a marker for all undifferentiated neuroepithelial cells shortly after closure of the neural tube (Tapscott *et al.*, 1981; Holtzer *et al.*, 1982; Bignami *et al.*, 1982), but remains expressed during later development only in astrocytes and ependyma (Schnitzer *et al.*, 1981). Vimentin has also been found in a glial progenitor cell and ceases to be expressed upon differentiation into a galactocerebroside-positive oligodendrocyte (Raff *et al.*, 1984).

During the culture period of 13 days the percentage of small, phase-dark and O4 antigen-positive cells increases from 3 to 12% of the total cells or from 0.7 to 4.0 × 10^5 cells per flask. The number of small, phase-dark and O4 antigen-negative cells increases from 0.8 to 4.6 × 10^6 cells per flask. These cells are most likely precursors to the O4 antigen-positive oligodendrocytes, since most express A2B5, tetanus receptors, vimentin and some the L2 epitope.

Isolation and immunological characterization of O4 antigen-positive cells from primary monolayer culture

The population of small, phase-dark cells can be enriched to a purity of ~80% by shaking off. After replating these cells in monolayer culture and maintenance for 3 days *in vitro*, the percentage of small, phase dark cells which express O4 antigen is 90–95%. Flat, epithelioid astrocytes which are GFA- and vimentin-positive, and some O4 antigen-negative, small phase dark cells are the contaminant cell populations. The percentage of O4 antigen-positive cells which also express vimentin is ~35% of all O4-positive cells after 3 days *in vitro* after isolation (Figure 2), that is, 13 days after seeding in primary culture. The percentage of O4 antigen-positive cells that also express vimentin is, therefore, higher than in the primary cultures at the equivalent time *in vitro*. Of the O4-positive cells, <0.1% are GFA-positive and ~1% express O1 antigen. Almost all O4 antigen-positive cells are also N-CAM, L2 and A2B5 antigen-positive. By all these criteria, the O4 antigen-positive cells are immature oligodendro-

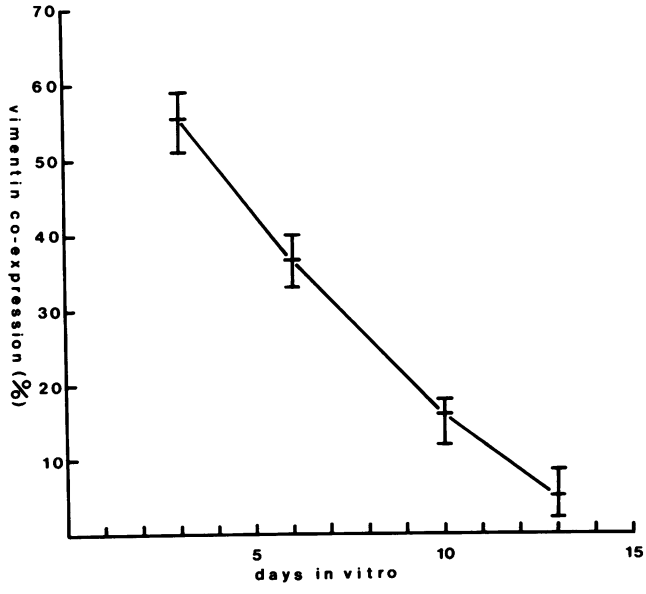

Fig. 1. Expression of vimentin in O4 antigen-positive cells as a function of time in primary cultures of 1-day-old rat cerebral hemispheres maintained in Bottenstein/Sato's medium containing 10% fetal calf serum. Values are means ± SEM from four independent experiments. In each experiment >2000 cells were assessed at each time point.

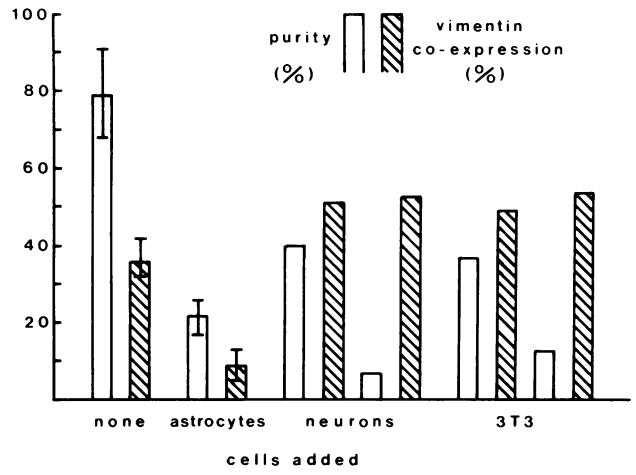

Fig. 2. Expression of vimentin in enriched populations of O4 antigen-positive cells as a function of co-culture with other cell types after 3 days *in vitro*. The purity of O4-positive cells ($O4^+$ cells/total cells) is shown in the white columns. The percentage of O4-positive cells which also express vimentin ($O4^+$, vim^+ cells/total $O4^+$ cells) is shown in the corresponding hatched columns. For O4 antigen-enriched cells (no other cell types added) and for O4-positive cells co-cultured with GFA-positive astrocytes chemically defined medium was used. Mean values ± SEM of five independent experiments are given. Co-cultivation in chemically defined medium resulted in moderate survival of neurons or 3T3 cells and therefore a higher proportion of O4-positive cells (pairs of columns on the left). Co-cultivation in fetal calf serum resulted in better survival of neurons and 3T3 cells and therefore a lower proportion of O4 positive cells (pairs of columns on the right).

cytes. Electronmicroscopic examination of O4 immuno-peroxidase-labeled cells supports this classification (Figure 3a). The presence of intermediate filaments (Figure 3b) is not typical of mature oligodendrocytes. However, not all O4 antigen-positive cells can be identified as oligodendrocytes (Figure 3c) by the criteria of Berg and Schachner (1981). They also do not fall into the morphological categories of glioblasts or immature astrocytes as they have been characterized *in vivo* (Privat *et al.*, 1981; Choi *et al.*, 1983).

Antigen expression by isolated O4 antigen-positive cells after maintenance in vitro

During culture times up to 10 days O4 antigen-positive oligodendrocytes remain O1 antigen-negative in defined and serum-containing culture medium. During this time period the absolute numbers of small, phase-dark cells remain constant ($2.0-2.5 \times 10^4$ cells per coverslip) while GFA-positive astrocytes increase in number in serum containing medium. (In defined medium proliferation of astrocytes is less.) Incorporation of [^{3}H]-thymidine into small, phase-dark cells is $<1\%$ under both culture conditions. The percentage of O4 antigen-positive cells which also express vimentin, tetanus toxin receptors, A2B5 antigen and the L2 epitope also remains constant during this culture time. Likewise, the percentage of these cells which express O1 antigen or GFA remains low, $\sim 1\%$ and $<0.1\%$, respectively. These observations exclude that O1 antigen-positive oligodendrocytes die at the same rate that O4 positive and O1 negative precursor cells are generated. We therefore conclude that O1 does not become expressed by O4 antigen-positive cells.

Influence of other cell types on the differentiation of O4 antigen-positive cells

To investigate whether the decreased expression of vimentin by O4-positive cells in primary cultures depends on interaction with astrocytes or is due to an intrinsic signal, the enriched population of O4 antigen-positive cells was co-cultured with a highly enriched population of GFA-positive astrocytes. These were obtained ($>98\%$ pure) by a short trypsinization step (100 μg/ml trypsin, 1 mM EDTA in Hanks' balanced salt solution, 15 min at room temperature) from the primary cultures after removal of small, phase-dark cells by shaking. Cell densities of co-cultures were kept identical to those of isolated O4 antigen-positive cells, since the percentage of O4 antigen-positive cells which also express vimentin was slightly higher at lower plating densities. After 3 days of co-culture the ratio of astrocytes to O4 antigen-positive cells was 4:1 (Figure 2), when cells were plated in defined medium at a ratio of 1:1 (i.e., 10^4 plus 10^4 cells). Under these conditions the percentage of O4 antigen-positive cells expressing vimentin was lower than in the isolated O4 antigen-positive population and only $\sim 10\%$, i.e., the same level seen in primary culture after 13 days *in vitro* (Figure 1). In contrast, isolated O4 antigen-positive cells expressed vimentin in 36 ± 5% of all cells (Figure 2). This effect was seen when cells were maintained in defined medium or with fetal calf serum. During 3 days the cell densities of O4 antigen-positive cells in the two culture situations remained constant and thymidine incorporation was low ($<1\%$ of O4 positive cells incorporated [^{3}H]thymidine), thus excluding a shift in or selection of subpopulations. We, therefore, conclude that astrocytes are able to induce differentiation of O4 antigen- and vimentin-positive precursor cells into O4 antigen-positive and vimentin-negative oligodendrocytes.

To investigate whether other cell types were also able to reduce expression of vimentin in the oligodendrocyte precursors, highly purified neurons ($>99\%$) from 5- to 7-day-old mouse and rat cerebellum (Keilhauer, *et al.*, 1985) and 3T3 fibroblasts were co-cultured with isolated O4-positive cells at two different ratios (Figure 2). Overall cell densities were again maintained equal. The percentage of O4 antigen-positive cells that express vimentin was not reduced under these conditions (Figure 2). The effect was similar, when co-culture was performed in defined or fetal calf serum-containing medium. Co-expression was found to be even higher in co-cultures with neurons or 3T3 cells than in enriched precursor cell cultures alone. This could be due to the lower overall density of contaminating astrocytes originating from the isolated O4 antigen-positive precursor population. It is noteworthy that cell-cell contact between oligodendrocytes and astrocytes seems to be required for the shift in co-expression, since conditioned media from highly enriched astrocyte cultures did not reduce vimentin expression in O4 antigen-positive precursor cells. Also, solitary O4 antigen-positive cells without cell contact showed more co-expression with vimentin than those in contact with astrocytes.

The morphology of the O4 antigen-positive oligodendrocytes expressing vimentin was less elaborate than those not expressing vimentin (Figure 4a). Often, strongly vimentin-positive cells were weakly O4 antigen-positive (Figure 4a). In O4 antigen-positive cells with 'hairy-eye-ball' morphology observed previously in mature, galactocerebroside-positive oligodendrocytes (Schachner and Willinger, 1979a, 1979b), vimentin expression was either absent or reduced to a small whirl in the perinuclear region (Figure 4a, b). Oligodendrocytes cultured in the presence of astrocytes showed the more elaborate, mature morphology, whereas those co-cultured with neurons or 3T3 cells were in general less mature in appearance (Figure 4c, d).

To investigate whether the O4 antigen-positive, but vimentin-negative oligodendrocytes are able to differentiate further into O1 antigen-positive, mature oligodendrocytes, co-cultures were

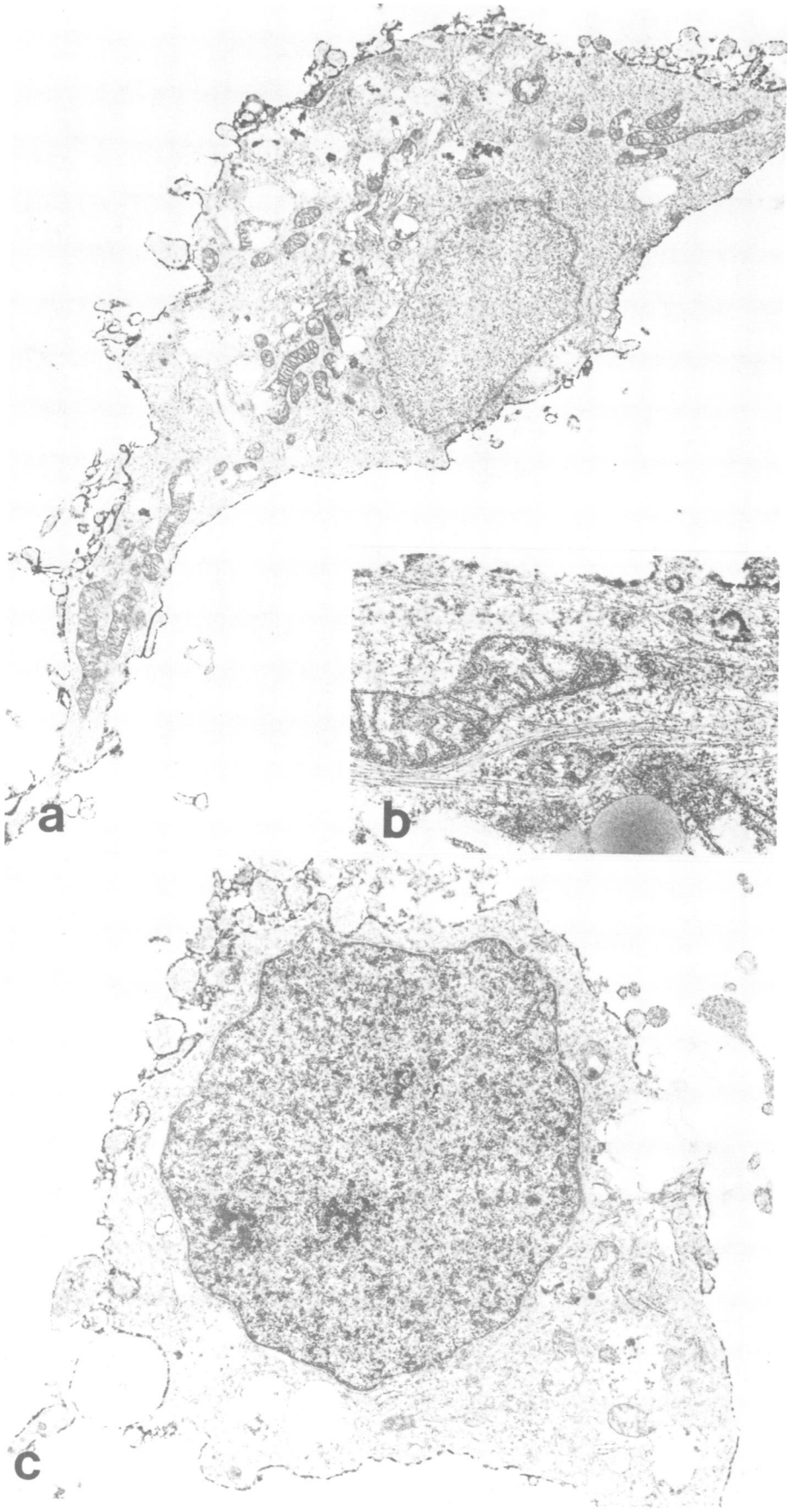

Fig. 3. Immunoelectron microscopy of enriched populations of O4 antigen-positive cells. (**a**) O4-positive cell classified as oligodendrocyte according to cytological appearance (27); magnification ×8000. (**b**) O4-positive cell process filled with intermediate filaments; magnification ×24 000. (**c**) O4-positive cell not classified as oligodendrocyte; magnification ×12 000.

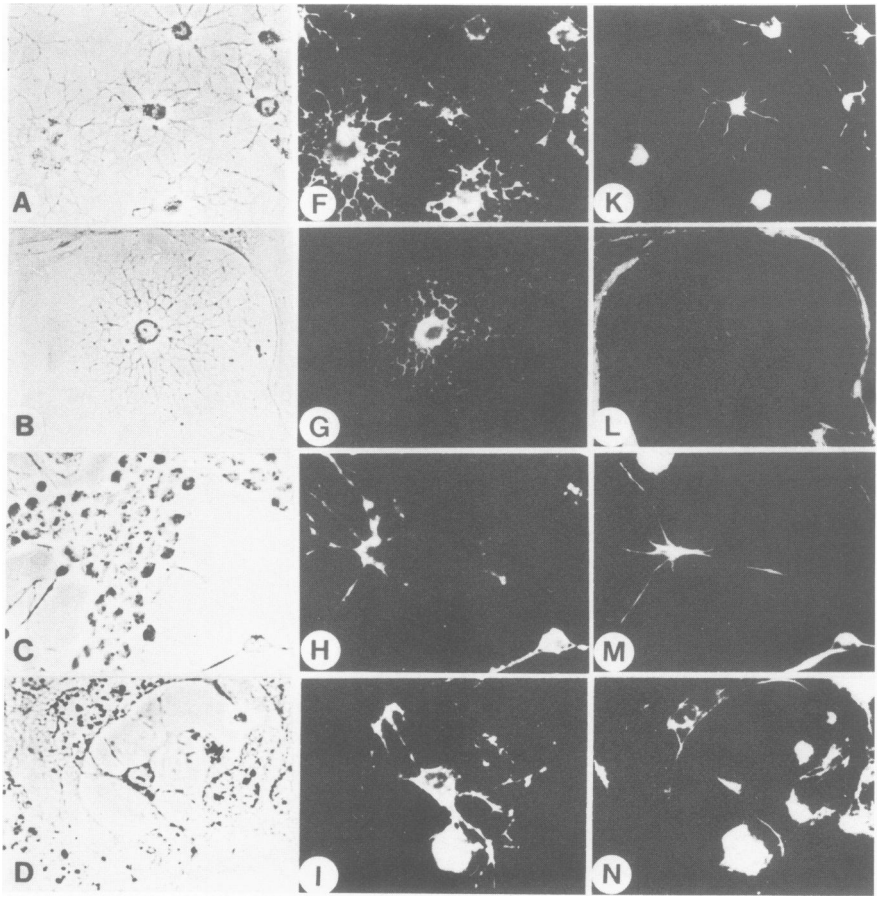

Fig. 4. Double immunolabeling for O4 antigen (middle column) and vimentin (right column) in enriched populations of O4-positive cells (**A**), co-cultured with astrocytes (**B**), neurons (**C**) and 3T3 cells (**D**). Corresponding phase contrast micrographs to middle and right columns are in the left column. Cultures were maintained for 3 days in defined medium (**A,B**) and in the presence of 10% fetal calf serum (**C,D**). Magnification ×160.

maintained for up to 16 days. Neither in the presence of highly purified neurons or astrocytes, nor 3T3 cells was the percentage of O4 antigen-positive cells also expressing O1 antigen significantly increased (not shown).

Discussion

The present study has shown that an oligodendrocyte precursor cell expressing O4 and A2B5 antigens, the L2 epitope, N-CAM, tetanus toxin receptors and vimentin is able to differentiate into an O4 antigen-positive but vimentin-negative oligodendrocyte in the presence of mature astrocytes, but not in the presence of neurons or fibroblasts or upon cultivation for up to 2 weeks. The expression of A2B5 antigen, L2 epitope, N-CAM and tetanus toxin receptors does not change significantly under co-culture conditions. Further differentiation into mature O4 and O1 antigen-positive oligodendrocytes is, however, not achieved in the presence of either astrocytes or neurons alone. This block in differentiation of O4 antigen-positive oligodendrocyte precursor cells (Sommer and Schachner, 1982) into O1 antigen-positive oligodendrocytes is not due to a general deficiency in our culture conditions. Cultures from rat optic nerve prepared according to Raff *et al.* (1983a) yielded the expected number of O1 antigen-positive oligodendrocytes (>95% of all O4 antigen-positive cells) under serum-free conditions. In fetal calf serum-containing medium the absolute numbers of oligodendrocytes was reduced in comparison with serum-free conditions, but again almost all O4 antigen-positive cells also expressed O1 antigen.

On the basis of the present experiments we postulate that mature astrocytes regulate by cell-to-cell contact the development of a precursor cell that shares several cell surface markers with immature astrocytes into an oligodendrocyte. Further differentiation, however, depends on other, as yet unknown, signals. Alternatively, oligodendrocyte precursor cells may not need a signal which induces differentiation, but are inhibited under the conditions of the present study. Cooperative effects between neural cells enhancing the differentiation of oligodendrocytes *in vitro* have been suggested in previous studies (Bologa *et al.*, 1982; Valat *et al.*, 1983; Walker *et al.*, 1984), but have not been traced to individual cell types. *In vivo*, oligodendrocytes from rat optic nerve develop after most astrocytes have withdrawn from the mitotic cycle and have started to differentiate (Valat *et al.*, 1983). Furthermore, the presence of intact axons is required to induce the differentiation of the full complement of oligodendrocytes, as could be shown by denervation. In contrast to our observations, galactocerebroside-positive oligodendrocytes developed in cultures from embryonic and early post-natal rat optic nerve in the absence of neurons and in the presence of astrocytes (Raff *et al.*, 1983a, 1983b). Furthermore, a glial progenitor cell with the same antigenic marker profile as the one described here differentiated into an astrocyte or a galactocerebroside-positive oligodendrocyte depending on the culture conditions (Raff *et al.*, 1983b). In our experiments, however, a specific effect of fetal calf serum on the preferential differentiation of astrocytes which lead to the observed differences remain to be elucidated. It is likely that not only the fine details of culture conditions play an

Table I. Antigenic markers used for characterization of cell type-specificity and developmental stage

Marker	L1	Tetanus toxin/ anti-tetanus toxin antibody	GFAP	Vimentin	N-CAM	O1	O4	L2 epitope	A2B5
Cell type specificity and developmental stage	Mature neuron	Neuron, some astrocytes, immature oligodendrocyte	Mature astrocyte	Mature and immature astrocyte, fibroblast-like cells	Neurons, immature glia	Mature oligodendrocyte	Immature and mature oligodendrocyte	Subpopulations of neurons, astrocytes and oligodendrocytes	Neuron, immature astrocyte and oligodendrocyte
References describing the antibodies	Rathjen and Schachner (1984)	Dimpfel *et al.* (1977); Schachner and Willinger (1979a); Raff *et al.* (1983a); Schachner *et al.* (1983); Abney *et al.* (1983)	Bignami *et al.* (1972)	Schnitzer *et al.* (1981)	Noble *et al.* (1985); Silver and Rutishauser (1984); Keilhauer *et al.* (1985) Edelman (1983); Goridis *et al.* (1983); Rutishauser (1984)	Sommer and Schachner (1981, 1982); Sommer *et al.*, in preparation		Kruse *et al.* (1984); Wernecke *et al.*, 1985	Eisenbarth *et al.* (1979); Berg and Schachner (1982); Abney *et al.* (1983); Schnitzer and Schachner (1982)

important role, but that the developmental committment of the two glial precursor cells with the same marker phenotype is different. The signals which lead to a divergent deployment of glia might be due to different cellular and molecular environments as have been suggested to exist for differentiated astrocytes which differ from one brain region to another in their response to neurotransmitters (Hamberger *et al.*, 1977), in their influence on neuronal morphology (Denis-Donini *et al.*, 1983) and in biochemical composition (Raff *et al.*, 1983a; Schachner, 1982). By use of cell type- and stage-specific markers and highly enriched neural cell populations the decisive signals that lead to complete differentiation of an oligodendrocyte from a precursor cell is open to analysis *in vitro*.

Materials and methods

Cultures (Table I)

Primary cultures from temporal lobes of 0–2-day-old Sprague-Dawley rats were maintained according to McCarthy and de Vellis (1980) in medium containing 10% fetal calf serum (Boehringer, Mannheim, FRG, lot no. 673234 OL). The fetal calf serum batch was selected for optimal maintenance of small, phase dark cells. Dissociated cells were plated into poly-L-lysine-coated 75 cm^2 tissue culture flasks (Greiner, Nürtingen, FRG) at a density of 30×10^6 cells in 10 ml culture medium. Medium was renewed every 3 days. After 7 days macrophages were removed by shaking for 2 h at 37°C and 150 r.p.m. After 9–11 days *in vitro* small cells were shaken off from the layer of flat astrocytes as described (McCarthy and de Vellis, 1980) and subcultured in the same medium or in chemically defined Bottenstein-Sato medium containing 200 μM progesterone, 2.5 μg/ml insulin and the other ingredients as indicated (Bottenstein and Sato, 1979). The yields per culture flask were usually $\sim 2 \times 10^5$ small phase dark cells. Plating efficiency was ~50%, when cells were seeded at 5×10^4 cells/50 μl per poly-L-lysine-coated glass coverslip, 1.6 cm in diameter.

Immunocytochemical procedures

Indirect immunofluorescence procedures for O1, O4, L1, L2 and A2B5 antigens, tetanus toxin receptors, glial fibrillary acidic protein (GFA) and vimentin were carried out as described previously (Schnitzer and Schachner, 1981). Monoclonal O1 antibodies recognize galactocerebroside and bind to the cell surface of more mature oligodendrocytes (Sommer and Schachner, 1981, 1982; Schachner *et al.*, 1981; Sommer *et al.*, in preparation). Monoclonal O4 antibodies recognize sulfate and bind to the less mature oligodendrocytes in addition to the more mature galactocerebroside-positive ones (Sommer and Schachner, 1981, 1982; Schachner *et al.*, 1981; Sommer *et al.*, in preparation). Cell surface L1 antigen is specifically expressed by post-mitotic neurons in the central nervous system (Rathjen and Schachner, 1984). Cell surface antigen A2B5 is expressed by neurons and immature astrocytes and oligodendrocytes (Abney *et al.*, 1983; Schnitzer and Schachner, 1982; Berg and Schachner, 1982; Eisenbarth *et al.*, 1979). Monoclonal antibody L2 recognizes a developmentally early appearing epitope on neurons and glia (Schachner *et al.*, 1983; Kruse *et al.*, 1984). Tetanus toxin receptors are expressed by neurons (Dimpfel *et al.*, 1977; Mirsky *et al.*, 1978), certain astrocytes (Schachner and Willinger, 1979a; Raff *et al.*, 1983a) and immature oligodendrocytes (Schachner *et al.*, 1983; Abney *et al.*, 1983). In the central nervous system, GFA is the protein subunit of intermediate filaments specific for mature astrocytes (Bignami *et al.*, 1972). Vimentin is expressed in all cells of the neuroepithelium shortly after closure of the neural tube (Tapscott *et al.*, 1981; Holtzer *et al.*, 1982; Bignami *et al.*, 1982), but remains expressed during later development only in astrocytes and ependyma (Schnitzer *et al.*, 1981). [^{3}H]-Thymidine incorporation (16 h) and autoradiography in combination with immunofluorescence was performed as described previously (Leutz and Schachner, 1981). Combined indirect immunoperoxidase and electron microscopy were carried out as described (Berg and Schachner, 1981).

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