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Induction of oligodendrocyte-like properties in a primitive hypothalamic cell line by cholesterol, an eye derived growth factor and brain extract

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A serum-free medium has been devised which permits proliferation of the mouse primitive nervous cell line F7. When cholesterol, eye-derived growth factor and brain extract are added in this medium for 48 h, 80–90% of oligodendrocyte-like cells are generated. These cells have diminished substrate adhesion. They acquire the capacity to synthesize carbonic anhydrase II and myelin basic protein, two specific proteins of oligodendrocytes. These observations suggest that F7 clonal cell line, which has been previously shown to be a neurophysin cell precursor, is also a precursor for oligodendrocytes, and represents a bipotent stem cell line for both neuronal and glial cell lineages.

Key words: oligodendrocyte/differentiation/cholesterol/serum-free medium/stem cells

Introduction

Generation of cell diversity is one of the major problems underlying brain development. Although much is known about the features of nerve cell types during their terminal differentiation, little information has been obtained concerning the molecular mechanisms which underlie their emergence during development. Among glial cell lineages oligodendrocytes play a key role in the central nervous system, since they elaborate the myelin sheaths around the axons that are essential for normal neuronal functions. Thus we have searched for factors which induce a primitive hypothalamic nerve cell line in culture to acquire some of the morphological and biochemical properties of oligodendrocytes.

In vivo, oligodendrocyte differentiation is accompanied by characteristic morphological changes (see review in Bunge, 1981) and by biochemical events such as the appearance of myelin basic protein antigen (MBP) (Cohen and Guarnieri, 1976; Delasalle *et al.*, 1981) or of an isozymic form of carbonic anhydrase (CA II, EC 4.2.1.1.), which was found localized in this glial cell type by immunohistochemical studies (Delaunoy *et al.*, 1977; Ghandour *et al.*, 1980). MBP is accumulated before the onset of myelination (Bunge, 1981). CAII is mainly unattached to cytoplasmic organelles in young rats, whereas 40% of the total enzyme activity resides in the membrane fraction in adult brain (Delaunoy *et al.*, 1977; Ghandour *et al.*, 1980).

During maturation of primary cultures of mouse or rat brain, probably under exogenous signals, loosely attached cells appear, which have been characterized by ultrastructural and immunocytochemical criteria (Delaunoy *et al.*, 1980) and by enzymatic marker studies (Mc Carthy and de Vellis, 1980), as oligodendrocytes.

Primitive nerve cell lines, such as clone F7 used here, provide homogeneous cultures which, in a defined medium, would represent a unique model system for the analysis of how the expression of a particular brain phenotype is controlled under the influence of exogenous inducers. We obtained the mouse hypothalamic primitive nerve cell clone F7 by viral transformation of foetal mouse hypothalamic cells (de Vitry *et al.*, 1974). It is a stable line which has been characterized as a primitive nervous cell by virtue of its ultrastructural features (Tixier-Vidal and de Vitry, 1976). We have already established that this line is a progenitor of the neuronal cell lineage (de Vitry, 1977). It shares some of the features of hypothalamic foetal cells *in vivo*, such as its ability to synthesize somatostatin and metenkephalin (Cesselin *et al.*, 1982).

The aim of this study was first to devise a serum-free medium able to support the survival or the proliferation of this primitive nerve cell. We then characterized agents, which, if added to this basal defined medium, induce oligodendrocyte differentiation such that the cells become loosely attached, round up, and acquire the capacity to express CA II and MBP antigens. Whether these inducers mimic signals acting *in vivo*, and whether F7 cells represent stem cells for oligodendrocyte lineage are discussed.

Results

Maintenance of F7 cells phenotype in basal serum-free medium (MS⁻)

F7 cells grown in serum medium (MS⁺), exhibited a flat 'undifferentiated' morphology, and their doubling time was ~24 h; such cultures remained stable as already described (de Vitry, 1977) and no rounded cell appeared in these cultures. When the plates were seeded at a density of 5 x 10^4 cells per 30-mm dish, the cultures became confluent after 2–3 days.

In MS⁻ medium, growth of F7 cells was approximately as good as in MS⁺ medium, with a doubling time ~24 h. As is shown in Figure 1, cells exhibited a flat morphology comparable to the one observed in serum-containing medium, except that they had lost their cytoplasmic granulations. When the plates were seeded at a density of 5 x 10^4 cells per 30-mm dish, the cells divided 2–3 times and reached confluency within 2–3 days, as did the cultures grown in MS⁺. MS⁻ medium, however, did not permit F7 cells to attach to the plastic dish. Seeding in MS⁺ medium was necessary to achieve cell attachment before any experiment could be run in serum-free medium. In MS⁻ medium, as in MS⁺ medium, F7 cells were neither recognized by MBP nor by CA II antibodies (Table I). For the various supplements of MS⁻, dose-response curves have been established (unpublished data), to define the optimal conditions for growth. Simultaneously, immunocytochemical tests were performed to verify the maintenance of the basal functional properties of F7 cells, such as the presence of somatostatin and of metenkephalin observed in the cell cultures in MS⁺ medium (Cesselin *et al.*, 1982).

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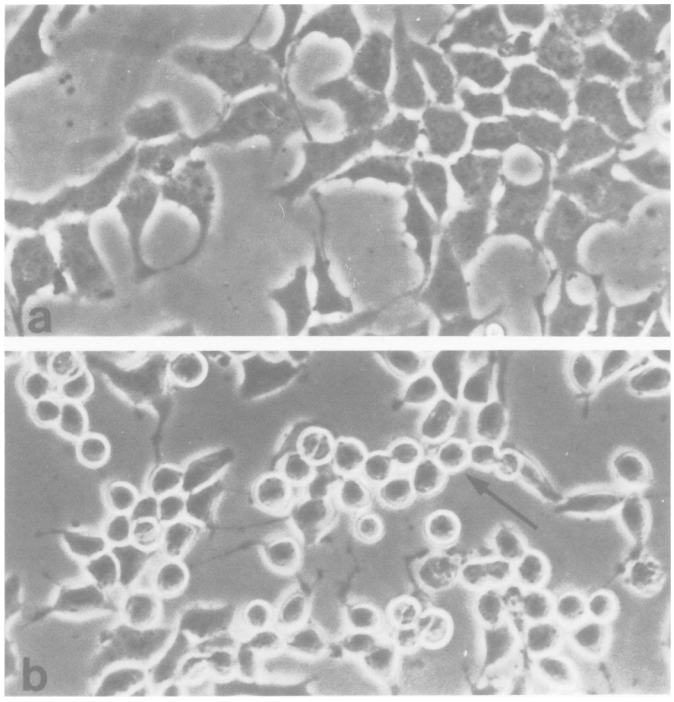

Fig. 1. F7 cells: phase contrast: x 240. **(a)** Cells grown in serum-free medium (MS^-). **(b)** In serum-free medium + cholesterol + retinal factor + brain extract (MOL): see row of cells (→).

Table I. Analysis of the effect of various combinations of inducers on MBP and CA II levels in F7 cells after 2 days as measured by RIA

Media	Rounded cells[a] (% of total)	MBP pg/μg protein Experiment number 1	2	3	4	CA II μg/mg protein 1	2	3
MS^+	0	S	S	S	S	S	S	S
MS^-	0	S	S	S	S	20	S	S
MS^- + cholesterol	8–10	S	S	S	S	S	S	S
MS^+ + cholesterol + brain extract[b]	10	S	S	S	S	403	391	137
MS^- + cholesterol + EDGF	30–40	S	S	S	S	ND	ND	199
MS^- + brain extract	5–10	ND	ND	ND	ND	ND	154	126
MOL	80–90	450	500	1000	800	285	229	275
Newborn rat primary cultures in 10% serum + brain extract	–	ND				93		

[a]Rounded and elongated retracting cells: 10 fields counted at magnification x 150.
S, below sensitivity of the assay. ND, not done.
[b]Less than 10 ng/mg protein of CA II was found in brain extract.

Induction of oligodendrocyte-like differentiation

Since the isolation procedure used to separate oligodendrocytes in dissociated primary cultures is generally based on their diminished cell adhesion, rounding of cells was taken as a criterion for selecting for oligodendrocyte presumptive cells. As indicated in Table I, when medium MS^- was supplemented with cholesterol and 17α oestradiol rounded cells developed, after one to two days of culture they became progressively detached. Such cells represented only ~10% of the F7 population. Addition of eye-derived growth factor (EDGF) to cholesterol medium produced spectacular effects. Division appeared to be unaffected, but the number of rounded floating cells was increased to ~40% of the population. Rounded cells were already visible after 3–4 h. An effect of EDGF was seen only in the low-density cell zone; it had no effect in the area of confluent cells. If brain extract was added to the 'cholesterol-EDGF MS^-' (MOL medium) cell division was stimulated and the yield of rounded cells was of the order of 80–90% of the cell population (Figure 1). Brain extract by itself did not induce rounded cells. Addition of 17α oestradiol in ethanolic solution had no visible effect. As in the case of 'EDGF-cholesterol-MS^-' medium, in MOL medium, rounded cell formation was triggered if cells were sparsely seeded. No inductive effect of MOL was observed on confluent cells. The 2–3 rounds of cell division in MOL medium appeared to be necessary to obtain the highest yield of morphologically differentiated cells. Beyond this time, the survival of cells declines. If oligodendrocyte-like cells were plated back in MS^+, some cells degenerated in the presence of serum, other cells began to proliferate after a lag period, and progressively regained their 'undifferentiated' morphology.

Immunocytochemical characterization of oligodendrocyte-like cells

As seen in Figure 2, in MOL medium, nascent rounded cells were recognized by CA II and MBP antisera. The staining was mostly confined to overlying cells, or retracting elongated cells, while flat attached cells were faintly coloured. If cells were treated with sera preadsorbed with their corresponding antigens, staining was nearly absent (Figure 2). In the case of CA II, nearly 100% of the cell population displayed a faint but specific immunoreactivity in MS^- medium supplemented with cholesterol and brain extract. The intensities of the immunocytochemical reactions obtained using different combinations of inducers were compared, and showed that immunoreactivity was maximum in MOL medium. MOL medium was therefore selected for the biochemical characterization of rounded cells.

Immunochemical and biochemical characterizations of oligodendrocyte-like cells

Quantitative determination of antigens by radioimmunoassay (RIA). CA II: as shown in Table I, CA II was found in three different experiments in oligodendrocyte-like cells, while no CA II was found in MS^- cells. Levels of CA II obtained in cells grown in MOL medium for 48 h were of the same order or even higher than that reached in primary glial cell cultures of newborn rats grown for 20 days in the presence of added brain extract and 10% foetal calf serum medium. Various combinations of inducers such as brain extract alone, or EDGF plus cholesterol added to MS^- medium induced the presence of CA II in F7 cells.

MBP: Figure 3 illustrates a dilution experiment where it appears that immunoreactive mouse MBP reacts with human antibody, but its reactivity is four orders less than that of human MBP. A complete parallel is observed between the dilution curves obtained with mouse and human MBP, respectively. MBP was found in all of the mouse cell extracts of F7 cells grown in MOL medium (Table I). The presence of all inducers (cholesterol, EDGF and brain extract) was necessary for detection of MBP.

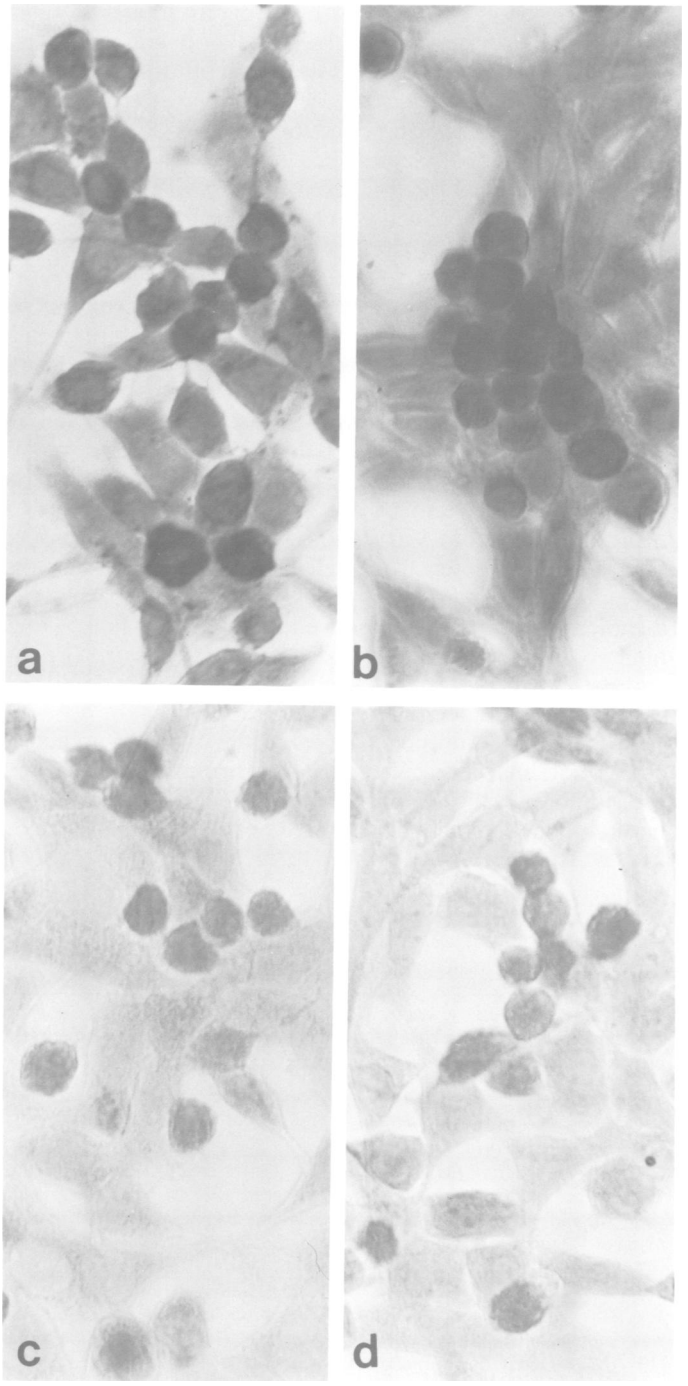

Fig. 2. Immunocytochemical staining of oligodendrocyte-like F7 cells grown in serum-free medium + cholesterol + retinal factor + brain extract (MOL). **(a)** CA II antiserum; **(b)** MBP antiserum; focus on overlying cells (x 340) see positive cells (→); **(c,d)** controls respectively to **a** and **b** with antisera previously adsorbed by homologous antigen.

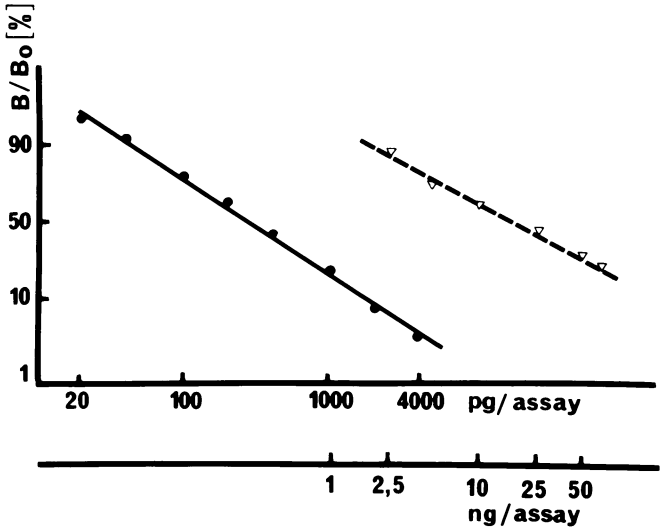

Fig. 3. Comparison of cross activity of human MBP (△_ _ _△) expressed in pg/assay and mouse MBP (●———●) expressed in ng/assay, as studied by RIA using a human MBP antibody.

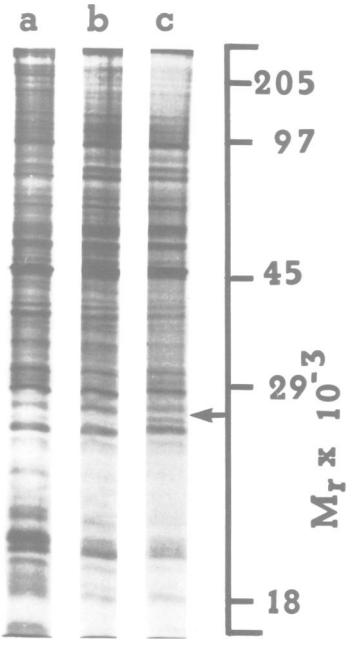

Fig. 4. Autoradiogram of soluble proteins of F7 cells grown in different [^{35}S]methionine supplemented media, in unidimensional gel electrophoresis. Labelled cells from one dish were lysed in 100 μl of phosphate buffered saline containing 100 μg of bovine serum albumin, 10 μl of Trasylol, 150 μg of L-methionine and 0.2% of NP-40, centrifuged at 500 *g* to discard the nuclei and at 10 000 *g* for 10 min. Samples of supernatant (200 000 [^{35}S] c.p.m.) were applied on the gel. **Lane a**, cells grown in MS$^+$. **Lane b**, cells in MS$^-$. **Lane c**, cells in MOL. The arrow indicates the 24 000 mol. wt. polypeptide.

Comparative analysis of total labelled proteins of oligodendrocyte-like cells and control cells. As shown in Figure 4, in a unidimensional gel electrophoresis, a significant difference was seen in electrophoretic profile: a polypeptide with a mol. wt. of 24 000 appeared repetitively amplified in oligodendrocyte-like cells (Figure 4) and was present, but in a lower amount, in control cells. Proteins whose mol. wt. was >45 000 were more intensely labelled in cells grown in MOL medium (lane c), than in those grown in MS$^+$ medium (lane a) or in MS$^-$ medium (lane b), while proteins of 20 000 mol. wt. were less labelled in oligodendrocyte-like cells than in control cells.

To find more subtle differences between a differentiated cell and its immature precursor, we have performed a two-dimensional gel electrophoresis of differentiated and control cell polypeptides in the acidic pH range 6.8–4.7. It revealed >150 polypeptide spots (unpublished data). However, no significant change could be observed between immature and

oligodendrocyte-like cells. The 24 000 mol. wt. polypeptide found in unidimensional electrophoresis must have had such a basic pI, that it was not revealed in this pH range.

Discussion

A preliminary step in this study consisted of defining a serum-free medium which was able to support proliferation of F7 primitive cells for short duration experiments. Parathyroid hormone required for some endocrine cell lines (Barnes and Sato, 1980), was also found to be essential here. The main experiments we describe show that when primitive nervous cell line F7 is transferred to this serum-free medium supplemented with adequate inducers, differentiated cells exhibiting oligodendrocyte-like characteristics become the majority of the cell population, while flat primitive cells progressively disappear. No such rounded oligodendrocyte-like cells appear in the absence of inducers.

This differentiation of F7 to oligodendrocyte-like cells is accompanied by morphological events similar to those associated with differentiation of oligodendrocytes *in vivo* (Cohen and Guarnieri, 1976; Delaunoy *et al.*, 1977; Sternberger *et al.*, 1978, Hartman *et al.*, 1979; Ghandour *et al.*, 1980) or in primary cultures of neonatal rat or mouse brain: for example, spherical cell bodies often align in rows, or overlie a basal monolayer and show diminished cell adhesion (Delaunoy *et al.*, 1980; Mc Carthy and de Vellis, 1980; Bhat *et al.*, 1981). As *in vivo*, appearance of CA II and MBP, two protein markers of oligodendrocytes, accompany these morphological changes. In F7 cell cultures, in the presence of the complete set of inducers, the level of CA II is ~250 ng/mg protein, corresponding to the level reached *in vivo* around day 13 post-natally (Delaunoy *et al.*, 1980). Concerning MBP, this was not detected by RIA in rat cerebrum before birth (Cohen and Guarnieri, 1976), and was first seen 5–6 days post-natally in rat brain sections. F7 differentiated cells appear to achieve, with respect to both oligodendrocyte markers, a degree of maturation similar to that of 5–10 days old mouse brain.

Immunocytochemical observations were in agreement with the results of RIA concerning the presence of CA II and MBP, and the absence of these markers in cells cultured in MS^-. The appearance of CA II immunoreactivity in F7 cells treated with brain extract is consistent with an earlier report that neural extract increases CA II in primary glial cell cultures grown in serum (Delaunoy *et al.*, 1980).

The differentiation of F7 cells does not appear to involve any significant major difference in the pattern of total proteins, as judged by both uni- and two-dimensional electrophoresis analysis, with one exception. A unidentified polypeptide of mol. wt. 24 000 is repeatedly observed by unidimensional electrophoresis. Its amount is higher in differentiated cell extracts than in control cell extracts. Surprisingly this polypeptide has a mol. wt. similar to the basic proteolipid protein, the major constituent of myelin, which accounts for 50% of the total myelin membrane proteins (Jolles *et al.*, 1977, Agrawal *et al.*, 1977). CA II and MBP were not detected on the unidimensional autoradiogram, since their level in the cells is under the threshold of sensitivity of this method.

These experiments give some insight into the mode of action of cholesterol, EDGF and factors from the brain. Cholesterol could act here by altering membrane fluidity and modifying the accessibility of membrane receptors to external signals (Heron *et al.*, 1980) or by acting as an axonal signal for oligodendrocyte differentiation (Wood and Boegman, 1980). One should note that myelin, a constituent of oligodendrocytes, is the membrane richest in cholesterol (28% of dry weight). The inducing effect of EDGF on F7 cells seems unique to this retinal factor, since up to now neither brain extract nor a purified fraction from brain extract (unpublished data) could mimic its action. Concerning brain extract, further purification and characterization of the active compounds in the extract should facilitate studies of its mechanism of action. Finally it should be interesting to see whether other clonal cell lines which are developmentally multipotent (such as teratocarcinomas) respond to these external stimuli.

The capacity to switch from an immature nervous cell to an oligodendrocyte-like cell under defined experimental conditions has been shown here. Previously we had shown the capacity of this precursor cell line F7 to switch towards a neuronal lineage (de Vitry, 1977). Thus the F7 cell line has features of a bipotent stem cell line of the central nervous system and, depending on a variety of signals, is able to make a choice between two alternative developmental fates. Other binary choices have already been observed *in vitro* (Landis and Patterson, 1981) or *in vivo* (Le Douarin, 1980; de Vitry *et al.*, 1980) during nervous system development.

In conclusion, our model appears to be suitable for analysis at the molecular level, of the mechanisms underlying the expression of a particular phenotype in the brain, and the exact nature of the interacting signals.

Materials and methods

Materials

Bovine insulin, human transferrin, cholesterol succinate and 17α oestradiol were obtained from Sigma. Synthetic human parathyroid hormone (sequence 1–34) was generously given by Dr.Mouptard (Hopital St. Antoine, Paris, France). [^{35}S]Methionine was purchased from Amersham. For experiments cells were seeded in Falcon 3 cm dishes.

Cell cultures

Cultures in serum-containing medium (MS^+). They were made as previously described (de Vitry, 1977).

Serum-free medium (MS^- and MOL). Cells were first seeded at 4–6 x 10^4 cells per dish in MS^+, which was replaced after 24 h by experimental medium, and grown for 48 h. Control cells were transferred in MS^- medium made of 1:3 mixture of Ham's F 12 and Dulbecco's modified Eagle's medium (DME) media supplemented with insulin (10 μg/ml), transferrin (25 μg/ml), 2.4 mM L-glutamine, glucose (10^{-2} M) and parathyroid hormone (2 ng/ml). For induction of oligodendrocyte differentiation, preceding medium MS^- was supplemented with cholesterol succinate (10^{-5} M), 17α oestradiol (10^{-9} M), EDGF (10 μl/ml) and brain extract (100 μl/ml). This medium was called MOL medium. Cholesterol and EDGF were added every day. If not mentioned, the addition of 17α oestradiol accompanies the addition of cholesterol.

Preparation of inducers. Cholesterol and 17α oestradiol were added in an ethanolic solution (1 μl/ml medium).

Brain extract was prepared as previously described (Delaunoy *et al.*, 1980).

EDGF was prepared from bovine eye retina. The fraction used was the acetic acid purified EDGF as prepared in Barritault *et al.* (1983). 10 μl containing 10 μg of EDGF, corresponding to 20 stimulating units, were added to the cultures every day for maximal inductive effect.

Labelling of cells. The radioactive precursor was added ([^{35}S]methionine, 13.5 Ci/mmol, 35 μCi/ml) when the cells were transferred to experimental medium and maintained for 48 h. The cells were scraped, centrifuged at 500 *g* and frozen until use.

Preparation of cell extracts. This will be given in the legend of Figure 4.

Immunochemical techniques

Antisera. Rabbit antisera against mouse CA II, bovine and human MBP were obtained as previously described (Delaunoy *et al.*, 1978; Dupouey *et al.*, 1979).

Immunocytochemical localization of MBP and CA II antigens. The indirect immunoperoxidase technique already described (de Vitry, 1977) was used with minor modifications. Cells were rinsed, fixed at 4°C for 2 h in 8% neutral formaldehyde, then treated for 5–10 s at 4°C with Nonidet P 40 0.2% and rinsed for 2 h. In the case of CA II, cells were directly fixed without prior rinsing. Cells were incubated in the specific sera (anti-mouse CA II or anti-bovine MBP I/400) for 10–12 h at 4°C. Control sections were treated with normal rabbit serum, as well as with specific antisera previously adsorbed by the homologous protein. This treatment produced no staining.

RIA. Cells of 10 tissue cultures dishes grown for 48 h in MS^- or in MOL media, were collected.

CA II determination was carried out as previously described (Delaunoy *et al.*, 1978). The reliable sensitivity of the assay is 0.5 ng.

MBP determination. The pellet of cells was delipidated with chloroform: methanol (2:1 v/v) and centrifuged 3 min at 10 000 *g*. Preparation of MBP samples and RIA were carried out as described (Delasalle *et al.*, 1981), using antiserum against human MBP. The reliable sensitivity of the assay is 10 fmol.

Electrophoresis analysis

SDS-polyacrylamide gel electrophoreses were performed in 12.5–33% w/v acrylamide-bisacrylamide slab gels (Laemmli, 1970). Two-dimensional gel electrophoresis was carried out following the procedure of O'Farrell (1975). Isoelectric focusing was carried out in 2.5 x 130 mm tubes containing 5% w/v acrylamide, and ampholine (LKB) in the ratio 1.6% pH 5–8, and 0.4 % pH 3.5–10. This gel was run in the second dimension in a 190 mm long SDS slab gel of 12.5% acrylamide. Dried gels were autoradiographed on X ray film (Kodak Industrex).

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