An Immunocytochemical Study of Cat Retinal Müller Cells in Culture

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Müller cells, the specialized radial glia found in vertebrate retinas, were enzymatically dissociated from adult cat retinas and grown in culture. The cells were processed for immunofluorescence microscopy at times ranging from 4 hr to 3 months in culture. Labeling with antibodies specific to glial fibrillary acidic protein, cellular retinaldehyde binding protein, glutamine synthetase, carbonic anhydrase C and alpha crystallin, all proteins known to be found in Müller cells, was detected in the cultured cells. Immunoblot analysis of the cultured cells showed single protein bands corresponding to the appropriate molecular weights of the antigens.

Key words: Müller cells; retina; culture; glial fibrillary acidic protein; cellular retinaldehyde binding protein; glutamine synthetase; carbonic anhydrase C; alpha crystallin; immunocytochemistry.

1. Introduction

The culturing of glial cells derived from vertebrate retina has been the focus of many recent studies. Cultures have been initiated from retinal explants (Hansson, 1971; Messing and Kim, 1979; Burke, 1982; Burke and Foster, 1984), and from dissociated cells of embryonic (Adler, Magistretti, Hyndman and Shoemaker, 1982; Linser and Moscona, 1983; Li and Sheffield, 1986; Trachtenberg and Hokanson, 1986) and adult retinas (Sarthy and Lam, 1978; Trachtenberg and Packey, 1983; Reichenbach and Birkenmeyer, 1984; Oka, Frederick, Landers and Bridges, 1985; Roberge, Caspi, Chan, Kuwabara and Nussenblatt, 1985; Sarthy, 1985). Because the mammalian retina contains several cell types that may assume the morphology of glial cells in culture (e.g. Müller cells, astrocytes, pericytes, and microglia), the exact cellular composition of the culture is often unclear. Also, morphological identification of cells in culture is often not possible since their appearance may be very different from that in vivo. This is certainly true for Müller cells, which have a very distinct morphology (Ramón y Cajal, 1972 translation). Antigenic markers can be useful for identifying cells in culture provided they are cell-type specific and continue to be expressed in vitro. Glial fibrillary acidic protein (GFAP) is an example of a commonly used marker for astrocytes. It is, however, present in both retinal Müller cells and astrocytes and hence is not a specific indicator of Müller cells (Bignami and Dahl, 1979; O'Dowd and Eng, 1979; Ohira, Oshima and Kikuchi, 1984; Björklund, Bignami and Dahl, 1985; Karschin, Wässle and Schnitzer, 1986a, b; Erickson, Fisher, Guerin, Anderson and Kaska, 1987; Lewis, Erickson, Kaska and Fisher, 1988). In the preceding paper we presented immunocytochemical evidence that antibodies to cellular retinaldehyde binding protein (CRALBP) and glutamine synthetase (GS) bind only to Müller cells in adult cat retina (Lewis et al. 1988). In this paper we describe evidence that cells cultured from adult cat retinas express GFAP, CRALBP and GS, as well as carbonic

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anhydrase C (CAC) and alpha crystallin (AC) thus demonstrating that the cultured cells express the same identifiable antigens as Müller cells in vivo.

2. Materials and Methods

Dissociation procedure

Adult cats ($n = 12$) were killed with an overdose of sodium pentobarbitol. Retinas were dissociated according to methods of Vaughan and Fisher (1987). Briefly, eyes were enucleated and placed in 37°C balanced salt solution (BSS) for approx. 20 min to decrease the adhesion between the retinal pigment epithelium and the neural retina. The anterior portion of the eye was then removed and the retinas were peeled away and placed in calcium–magnesium-free BSS supplemented with 0·5 mg ml^{-1} Nagarse (Protease type XXVII, Sigma) for 30 min at 37°C. Retinas were subsequently rinsed in BSS containing calcium, magnesium and 0·5 mg ml^{-1} BSA and transferred to 2 ml of BSS containing calcium, magnesium, 0·5 mg ml^{-1} BSA and 0·1 mg ml^{-1} DNAse 1 (Sigma). After gently pipetting the tissue up and down several times with a wide-bore pipet, the non-dissociated tissue was allowed to settle and the dissociated cells, still in suspension, were withdrawn and placed into a single test-tube. This procedure was repeated until very little non-dissociated tissue was present. The mixed population of dissociated cells was transferred to several dishes containing culture media. The culture dishes were prepared the night before by adding Dulbecco's Modified Eagle's Medium (DMEM) plus 10% fetal calf serum (FCS) to each dish containing three glass coverslips that had been sterilized and coated with 0·1 mg ml^{-1} Poly-L-lysine (Sigma). The dishes were then placed in a 5% CO_2 incubator at 37°C. The exact number of cells placed in each dish was not determined since the population consisted of a mixture of cell types. Cells were fed approximately every 5 days with DMEM plus 10% FCS.

Control cells for the immunofluorescence were processed at the same time as the experimental cells. Normal rabbit serum or PBS–BSA was used in place of the primary antibody.

Antisera

Rabbit anti-bovine-GFAP (Dako Corp.), rabbit anti-bovine-CRALBP (gift from Dr John Saari), rabbit anti-chicken GS and CAC (gifts from Dr Paul Linser) and rabbit anti-mouse-AC (gift from Dr Joram Piatigorsky) were all used at a dilution of 1:100. The mouse monoclonal anti-GFAP (Amersham) was used at 1:500. All antibodies were diluted with phosphate-buffered saline (PBS) containing 0·5% bovine serum albumin (BSA). Normal goat serum (NGS) (Vector Labs – diluted 1:75 with PBS) was used as a blocking agent. Affinity purified goat anti-rabbit IgG–fluorescein, goat anti-rabbit IgG–rhodamine and goat anti-mouse IgG–fluorescein (Cappel) were used as secondary antibodies at a dilution of 1:20.

Immunofluorescence

Cells were processed for immunofluorescence at times ranging from 4 hr to 3 months in culture. Coverslips were first removed from each culture dish and rinsed with PBS–BSA. Cells were fixed in 1% paraformaldehyde in PBS for 30 min, rinsed in PBS–BSA and permeabilized with 0·03% Triton X-100 in PBS for 5 min. After washing with PBS–BSA, NGS was added for 30 min, at which point the excess was removed without drying or rinsing. Cells were exposed to the primary antibody for 1 hr, rinsed in PBS–BSA and exposed to the secondary antibody for an additional hour. For the double labeling experiments, the cells were incubated simultaneously with the monoclonal mouse antibody to GFAP and the polyclonal rabbit antibody to CRALBP. The secondary antibodies, goat anti-rabbit–rhodamine and goat anti-mouse–fluorescein were also added together after the primary antibodies were rinsed off. When the secondary antibody incubation was complete, coverslips were rinsed in distilled water, inverted onto a glass microscope slide containing a drop of glycerol plus 5% n-propyl gallate and sealed with nail polish. The stained cells were studied and photographed with a Zeiss Photomicroscope III equipped for epifluorescence.

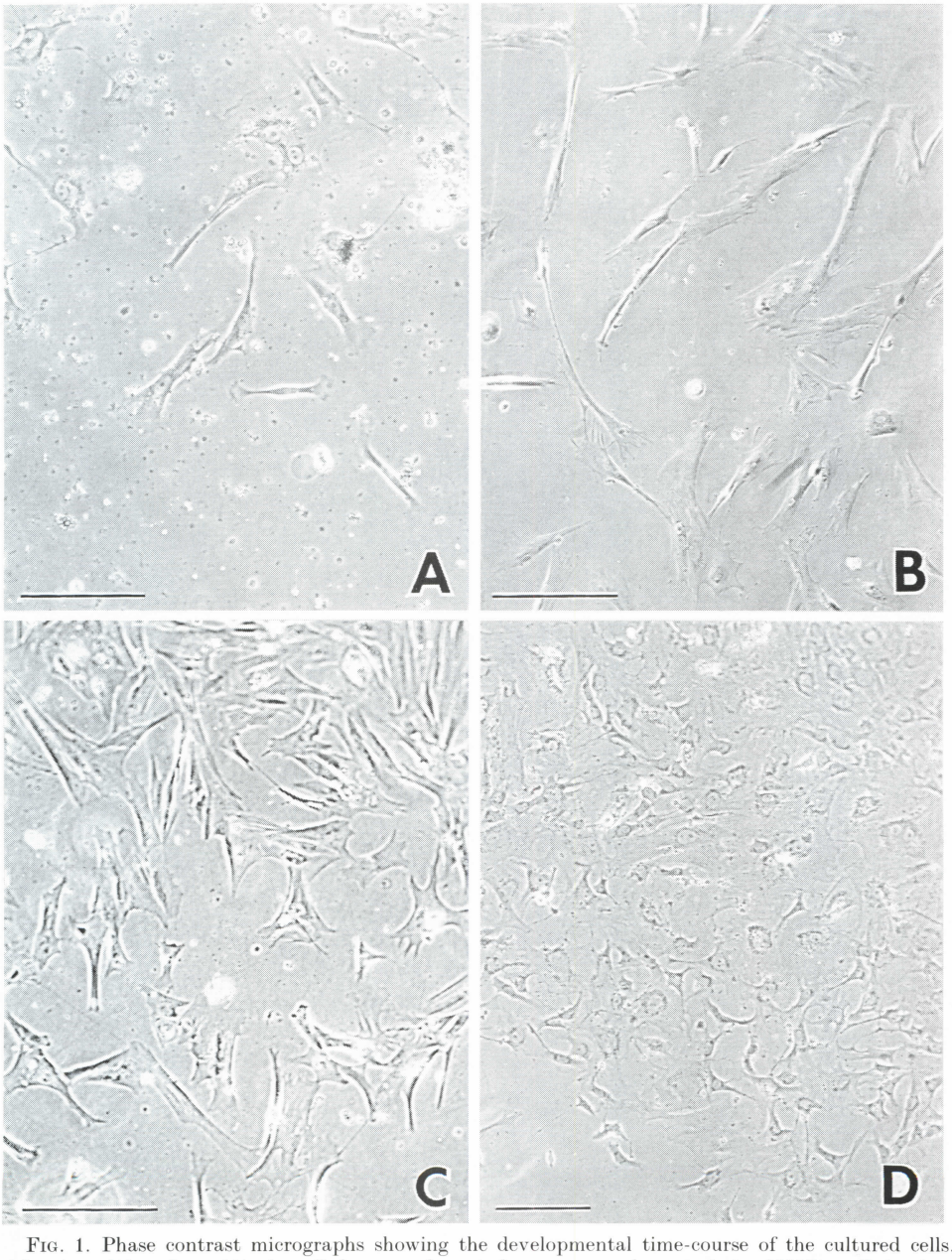

FIG. 1. Phase contrast micrographs showing the developmental time-course of the cultured cells. After 1 week in culture (A), the cells have an elongated appearance characteristic of Müller cells in vivo. Note the presence of cellular debris, most probably neurons, still present from the dissociation. Most cells retain this elongated morphology for several weeks in culture (B, 2 weeks), and are attached to the dish along their entire length. By 4 weeks in culture (C), when the cells are approaching confluency, they begin to appear more spread out, eventually becoming polygonal in shape by 2 months (D) (Scale bars represent 200 μm.)

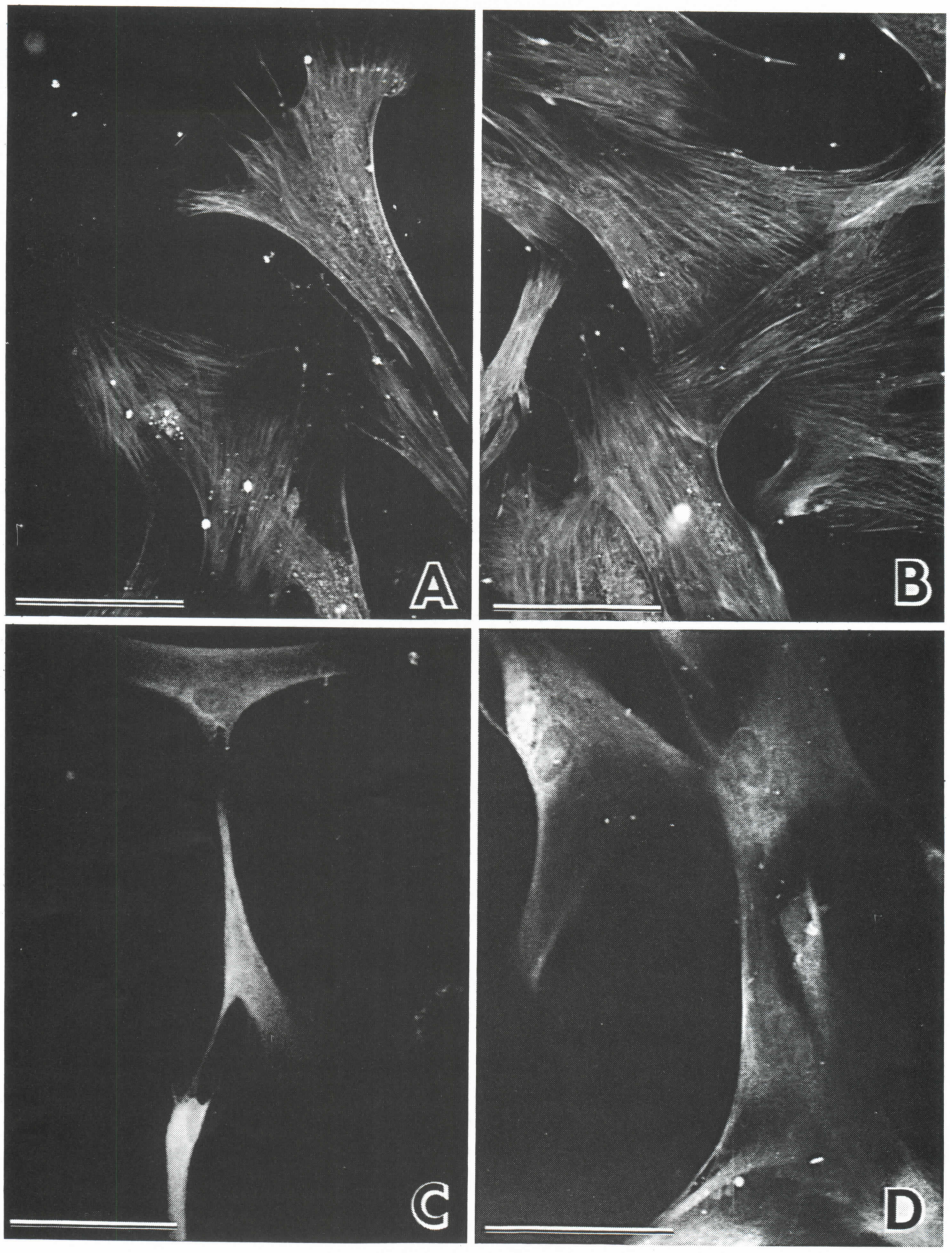

FIG. 2. Immunofluorescence micrographs of the cultured cells using anti-GFAP (A, B) and anti-CRALBP (C, D). Anti-GFAP labels both a filamentous and diffuse cytoplasmic component of the cells at 1 week (A) and 4 week (B). Anti-CRALBP diffusely labels the cytoplasm, being concentrated many times near or around the nucleus (C, 1 week; D, 4 weeks). (Scale bars represent 50 μm.)

Immunoblots

At various times during growth, the cells were washed extensively in PBS, scraped off the culture dish and homogenized in 0·1 mM phenylmethylsulfonylfluoride. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Approximately 50 μg of protein was analyzed by SDS–PAGE using a 10% gel. Proteins of known molecular weights (Bio-Rad) were used as standards.

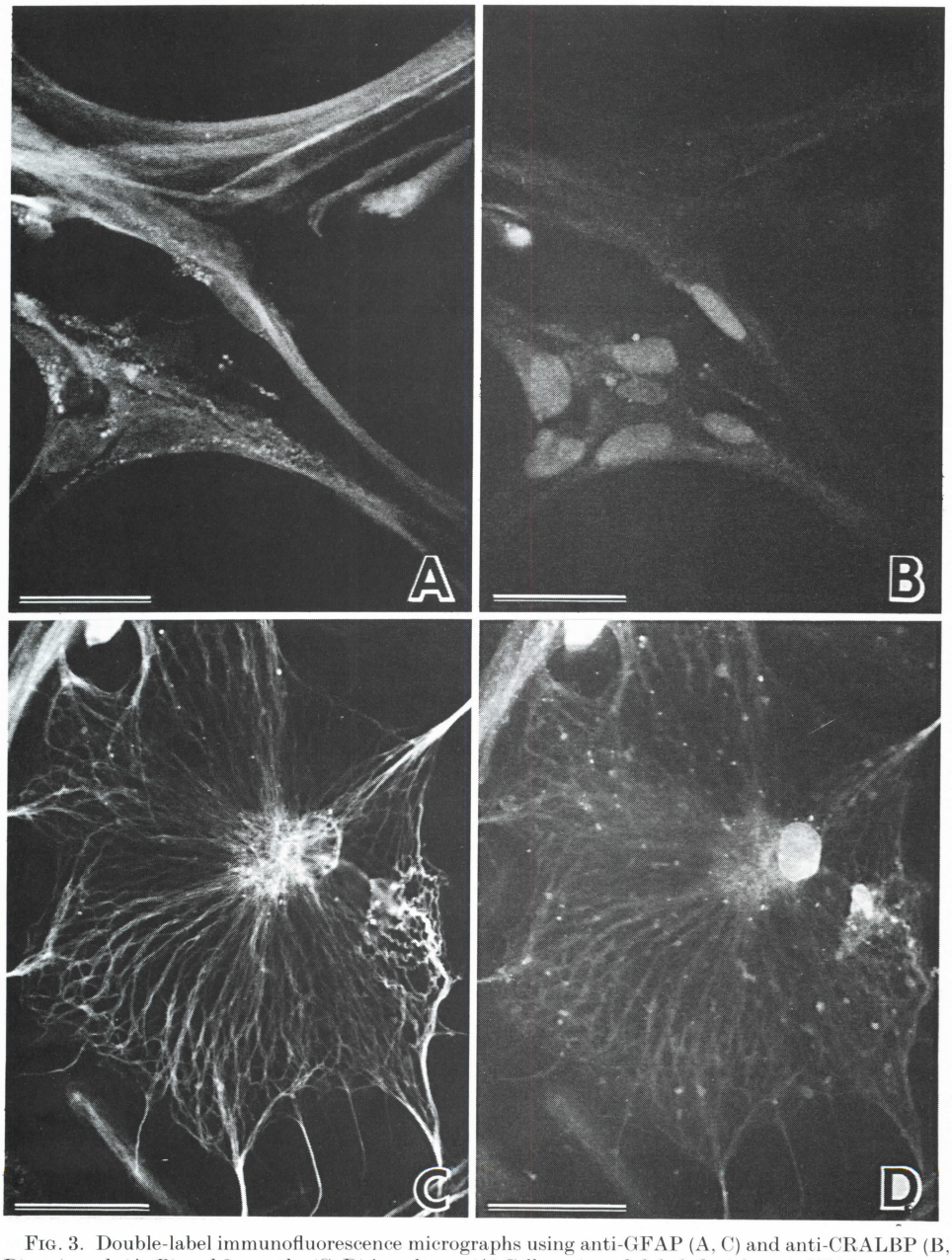

FIG. 3. Double-label immunofluorescence micrographs using anti-GFAP (A, C) and anti-CRALBP (B, D) at 1 week (A, B) and 3 months (C, D) in culture. (A) Cells at 1 week labeled with anti-GFAP revealing the filamentous and diffuse component of this protein; the same cells are shown in (B) labeled with anti-CRALBP where the label is mainly cytoplasmic and nuclear. At this time the cells still have their characteristic in vivo elongated appearance; at 3 months in culture, however, even though the cells now have a more polygonal shape, they still express both of the proteins. These double-label experiments demonstrate that the same cell labels with both anti-GFAP and anti-CRALBP, implying that they are most probably Müller cells. (Scale bars represent 25 μm.)

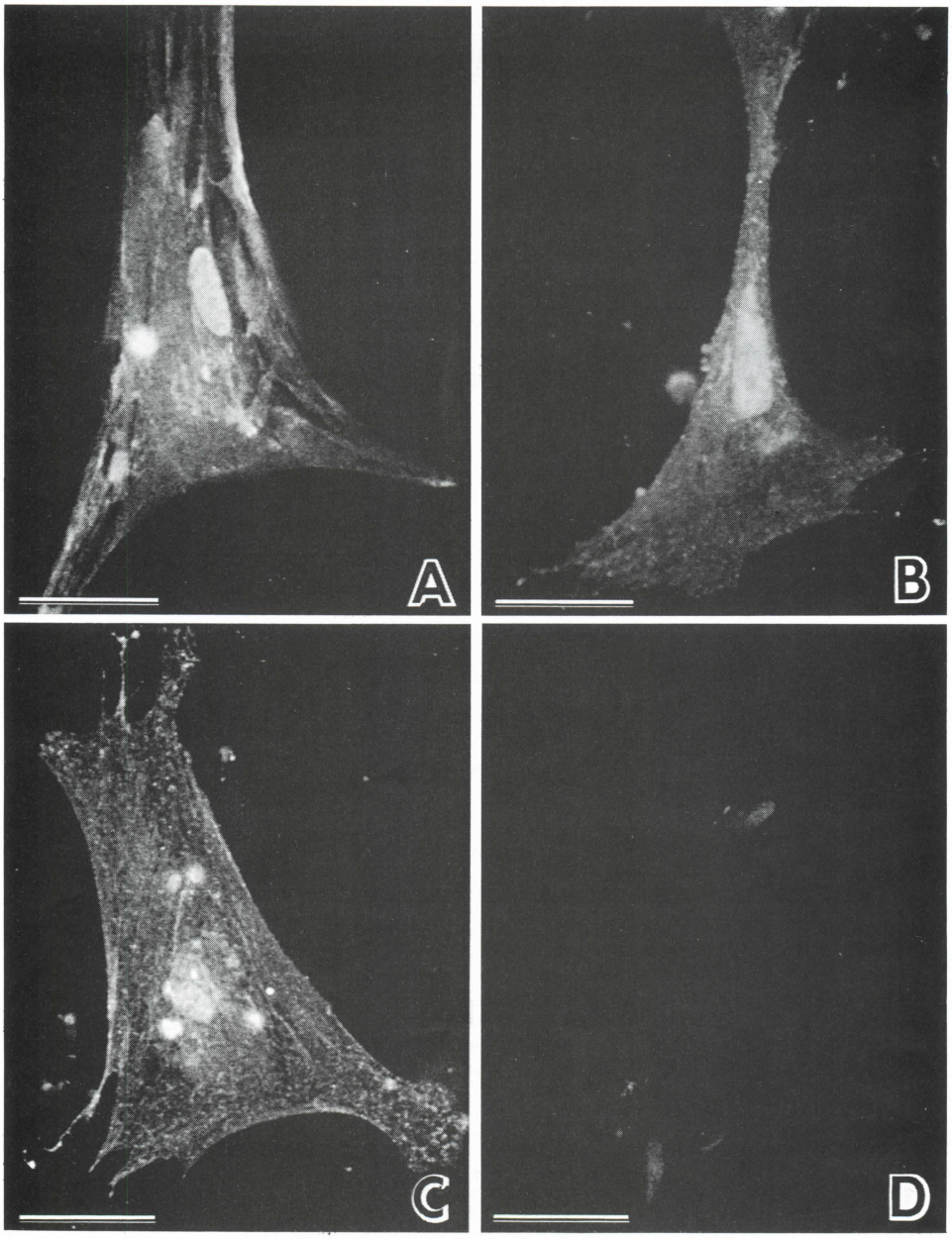

FIG. 4. Immunofluorescence micrographs of the cells cultured for 2 weeks using anti-GS (A), anti-CAC (B) and anti-AC (C). These antibodies label a diffuse cytoplasmic and nuclear portion of the cells. Immunocytochemistry controls (D) show essentially no label present in the cells. (Scale bars represent 25 μm.)

Immunoblots were performed by transferring the glial cell proteins to nitrocellulose paper, blocking with 3 % BSA in Tris-buffered saline (TBS) and incubating overnight in the primary antibodies (all diluted 1 : 100 in 1 % BSA in TBS). The blots were then washed in Tween–Tris-buffered saline (TTBS) (0·05 % Tween 20) and incubated with horseradish peroxidase (HRP) labeled goat anti-rabbit IgG (Bio-Rad) for 1 hr. Blots were rinsed in TTBS and then in TBS and placed in HRP Color Development Reagent (Bio-Rad) for 15 min.

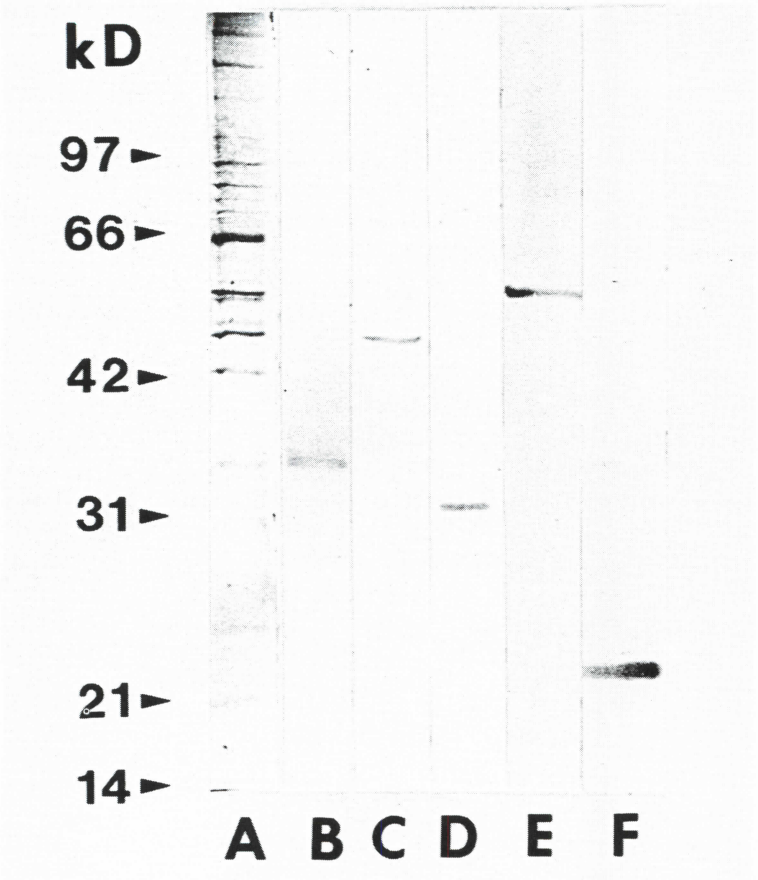

FIG. 5. Coomassie Blue-stained SDS–PAGE gel of proteins from the cultured cells (lane A) and Western blots (lanes B–F). Bands at the appropriate molecular weights are shown for all antibodies used. Anti-CRALBP labels a band at 33 kDa (lane B), anti-GS at 45 kDa (lane C), anti-CAC at 31 kDa (lane D), anti-GFAP at 45 kDa (lane E) and anti-AC at 22 kDa (lane F).

3. Results

Initially, most of the retinal cells in suspension in the culture medium retained their in vivo morphology. Within a few hours, however, many of the cells rounded up and did not attach to the culture dish. These floating cells, most probably neurons, were left in the culture dishes until either the medium was changed or the dishes were processed for immunofluorescence. By 4 hr after dissociation and plating, the Müller cells, still with their elongated appearance, were observed to adhere to the culture dish. Prior to this they were not securely attached and could not be processed for immunofluorescence. Figure 1A shows cells that have been cultured for 1 week with debris from other cell types still present. At this time and for the next several weeks the cells were attached to the dish along their entire length and had a bipolar or elongated shape (Fig. 1B; 2 weeks). The majority of these cells retained their elongated shape for several months in culture (Fig. 1C; 4 weeks). After approx. 2 months in culture, however, the now confluent cells became more spread-out and polygonal in shape (Fig. 1D).

Immunofluorescence labeling was usually performed at 1 week in culture, which allowed time for the cells to proliferate but not to become confluent. All of the antibodies, however, labeled the cells at any time between 4 hr and 3 months in primary culture. Anti-GFAP was found to label both a filamentous and a diffuse cytoplasmic component in the cells at all observed time points (Fig. 2A, 1 week; 2B, 4 weeks). Anti-CRALBP diffusely labeled the cytoplasm of all cells, being especially concentrated near or around the nucleus and, in some cases, within the nucleus (Fig. 2C, 1 week; 2D, 4 weeks). To show that the same cells expressed both of these antigens a double-labeling experiment was performed using these two antibodies. The results are shown at both 1 week in culture, when the cells have an elongated appearance (Fig. 3A, anti-GFAP; 3B, anti-CRALBP) and at 3 months in culture, when they have a more polygonal appearance (Fig. 3C, anti-GFAP; 3D, anti-CRALBP). Antibodies to GS, CAC and AC were also found to label the cultured cells (Fig. 4A–C, respectively). All three of these antigens appeared diffusely throughout the cytoplasm of all cells and the nuclei of some cells. The controls showed essentially no label present in the cells (Fig. 4D). Other cell types, initially present in the cultures, did not adhere to the dishes and were subsequently washed away during processing for immunofluorescence.

The Western blots of the cell homogenates showed a single band corresponding to the appropriate molecular weight of each antigen (Fig. 5): anti-CRALBP labeled a band at 33000 kDa, anti-GS at 45000 kDa, anti-CAC at 31000 kDa, anti-GFAP at 51000 kDa and anti-AC at 22000 kDa.

4. Discussion

The presence of GFAP, an intermediate filament (10-nm diameter) protein (Rueger, Huston, Dahl and Bignami, 1979; Eng, 1982), was used initially to identify the cells described in this study as being glial in nature. Retinal microglia do not express GFAP (Kivela, Tarkkanen and Virtanen, 1986), whereas both retinal astrocytes (Björklund, Bignami and Dahl, 1985; Karschin, Wässle and Schnitzer, 1986a, b) and Müller cells do (Bignami and Dahl, 1979; O'Dowd and Eng, 1979; Ohira, Oshima and Kikuchi, 1984; Björklund, Bignami and Dahl, 1985; Erickson, Fisher, Guerin, Anderson and Kaska, 1987).

To determine if the cells in our culture were astrocytes or Müller cells, or a mixture of cell types, we used the information obtained from our immunocytochemical study of intact cat retina (Lewis et al., 1988). In that study we showed that CRALBP, a retinoid binding protein (Futterman, Saari and Blair, 1977) and GS, an enzyme used for the conversion of glutamate to glutamine (Meister, 1974), labeled the Müller cells but not the astrocytes and thus could be used to differentiate between the two glial cell types. Since both of these antibodies label the cultured cells, it is likely that the cells are Müller cells and not astrocytes. Other immunolabeling studies have shown CRALBP to be present in Müller cells in the neural retina (Bunt-Milam and Saari, 1983; Bok, 1985; Anderson et al., 1986) but this is the first published account showing that CRALBP can be expressed in cultured cells. Glutamine synthetase has also been localized in the retina and has been shown to be an antigen specific to Müller cells (Riepe and Norenberg, 1978; Sarthy and Lam, 1978; Linser and Moscona, 1979; Moscona and Degenstein, 1981; Trachtenberg and Packey, 1983; Moscona, 1983; Linser, Smith and Angelides, 1985). Moscona (1983) showed that the level of GS in the retina is low initially during prenatal development and, as development proceeds,

gradually increases to that found in the adult. The increase of GS in the retina was shown to follow systemic elevation of adrenal corticosteroids (Piddington, 1967). Subsequently, it was found that when chick embryos are given cortisol injections, GS levels prematurely increase in the retina, demonstrating that GS is influenced by corticosteroid levels (Piddington and Moscona, 1967). A similar response to cortisol was seen with isolated retinal tissue in organ culture; that is, cortisol was necessary for the induction of GS (Moscona and Piddington, 1966; Moscona and Degenstein, 1981). In dissociated cell cultures, however, it was found that separated glial cells not only required cortisol but also required direct contact with neurons for the expression of GS (Linser and Moscona, 1983). The cells cultured in this study were initially in contact with neurons and had access to corticosteroid hormones present in the serum which was apparently sufficient to maintain GS at a level detectable by immunofluorescence and immunoblot analysis.

Two other known glial proteins, CAC and AC were tested to characterize further the cultured cells. CAC, an enzyme that catalyzes the hydration of carbon dioxide to carbonic acid (Carter, 1972), has been shown to be present in retina in vivo (Bhattacherjee, 1971; Musser and Rosen, 1973; Bhattacharjee, 1976; Kumpulainen, 1980; Parthe, 1981; Linser and Moscona, 1981; Lutjen-Drecoll and Lonnerholm, 1981; Parthe, 1981; Kumpulainen, Dahl, Korhonen and Nystrom, 1983; Lutjen-Drecoll, Lonnerholm and Eichhorn, 1983; Linser, Smith and Angelides, 1985) and in vitro in cultures of dissociated chick retina (Linser and Moscona, 1981, 1983). Carbonic anhydrase C, however, is regulated independently of GS and does not appear to require corticosteroid hormones or neuronal contact to be expressed (Moscona, 1983). Antibodies to AC, a major lens protein (Piatigorsky, 1981), has also been shown to bind molecules expressed in Müller cells. Although the identity of the molecules is not yet known (Moscona, Fox, Smith and Degenstein, 1985), AC does have an amino acid sequence showing some homology with heat shock proteins (Ingolia and Craig, 1982). While further characterization of this antibody is under way, it does serve the purpose of showing similar immunocytochemical results in the cultured cells as in the intact retina. Although Western blot analysis of the cells using AC showed the presence of a single band, blots of retinal tissue showed a doublet. These two bands have previously been identified as the alpha and beta subunits of AC (Bloemendal, 1981). The presence of a singlet in the cultured cells may signify a loss of one of these subunits. In vitro labeling of crystallins has previously only been done after embryonic cells, dissociated from the retina, formed aggregates of spherical cells (Moscona, Brown, Degenstein, Fox and Soh, 1983). The aggregates had an inner core of glial cell origin with lentoidal morphology and stained positively with antibodies to crystallins. Here we show that the Müller cells in culture express a molecule that binds this antibody to AC without forming lentoids or lens-like structures.

We were fairly certain at this point that the cultures consist solely of Müller cells since all of the cells label with all five antibodies and Western blot analysis gave the same results as with intact retina. However, the possibility that other cell types were also present in the cultures needed to be examined. Although the neural retina was separated from the retinal pigment epithelium (RPE), there was the possibility that RPE cells adhered to the retina and subsequently proliferated in culture. RPE cells express CRALBP in vivo (Blunt-Milam and Saari, 1983; Anderson et al., 1986) and in vitro (Anderson pers. comm.). Retinal astrocytes, which could have proliferated in the cultures, also express GFAP. The double-labeling experiment using GFAP and CRALBP together showed that all cells labeled, to some degree, with both of these

antibodies. Since GFAP has not been shown to be present in RPE cells and CRALBP is not in glial cells other than Müller cells, the case is greatly strengthened for the cells in culture being Müller cells. It is unlikely that the cultures are contaminated with retinal neurons since neurons do not contain all of the antigens studied here and after about 1 week in culture, all of the cells present label with all of the antibodies.

The labeling pattern seen with anti-GFAP was mostly filamentous, and thus represents the 10-nm-diameter filaments present in Müller cells. A diffuse cytoplasmic component was also detected, however. This diffuse labeling has been seen in vivo, where it has been suggested that it may represent GFAP monomers (Schachner, Hedley-Whyte, Hsu, Shoonmaker and Bignami, 1977). Because GFAP monomers will assemble into 10-nm-diameter filaments in vitro (Lucas, Bensch and Eng, 1977; Rueger, Huston, Dahl and Bignami, 1979), the suggestion that the diffuse cytoplasmic labeling may be monomers of GFAP is consistent with the observation that this diffuse labeling only occurs in cells that contain GFAP intermediate filaments.

While GFAP labels mainly a filamentous component of the cells, the labeling pattern observed with the other four antibodies is distinctly different. Each of these produces a diffuse labeling of the cytoplasm, and in many instances the nucleus as well. Nuclear labeling with CRALBP has previously been reported using embedded tissue sections (Bok, 1985) and it has been suggested that CRALBP, like other binding proteins normally found in the nucleus of Müller cells, may be involved in the control of gene expression (Bok, Ong and Chytil, 1984; Chytil and Ong, 1984). Carbonic anhydrase C has also been found in the nuclear fraction of some cells (Karler and Woodbury, 1959). Histochemical (not immunocytochemical) techniques for CAC localization, however, have produced mixed results with nuclear staining only occurring in sections of fixed tissue. When unfixed tissue is used the staining is limited to the cytoplasm (Hansson, 1967, 1968; Lutjen-Drecoll and Lonnerholm, 1981; Lutjen-Drecoll, Lonnerholm and Eichhorn, 1983). On the basis of these results it has been suggested the nuclear localization of CAC is due to non-specific diffusion into the nucleus. Although the in vitro and in vivo (Lewis et al., 1988) immunocytochemistry and the labeling of a specific band on the immunoblots make it likely that the nuclear staining does not represent binding to some other molecular species, it does not rule out the possibility that these soluble proteins diffuse into the nucleus of some cells after a relatively mild fixation.

These results show that dissociated mammalian Müller cells are able to survive in long-term cultures. Although they do not have the highly differentiated appearance of Müller cells in the retina they do retain many of their in vivo antigenic characteristics. There are several variants of standard procedures used in this study that may have contributed to the success of these cultures. First, the dissociation procedure used has been shown to be a very gentle one, producing retinal cells that are morphologically intact and similar to their in vivo appearance (Vaughan and Fisher, 1987). Second, we combined techniques that were specifically designed to produce optimal culturing conditions for glial cells. For example, it has been shown that plating cells at an intermediate density (Bottenstein, 1983) in DMEM with 10% serum (Trachtenberg and Hokanson, 1986) is most suitable for culturing glial cells. Indeed, our experience, using a mixed population of cells, confirmed that very high or very low density produced fewer viable glial cells in the cultures. As in other studies with glia (Adler, Magistretti, Hyndman and Shoemaker, 1982), pretreatment of the culture dishes with serum containing media enhanced the ability of the cells to attach to the dish. Other studies have also suggested that neuronal cell elements present may

contribute to the initial activation and subsequent proliferation of glial cells in culture (Burke and Foster, 1984). As previously discussed, neuron–glia contact, along with serum hormones, was shown to be necessary for the induction of GS in neural retina (Linser and Moscona, 1979) and cultured cells (Linser and Moscona, 1983). Since retinal neurons were initially present in our cultures they may well have contributed to the growth of the cells. Apparently, however, once the glial cells are activated and begin proliferating, neuronal cells are no longer required; we see continued cellular proliferation and expression of antigens after the neuronal cells have died and were washed away.

In vitro, retinal Müller cells assume a completely different morphology from that in the retina. Thus, specific markers are needed to identify conclusively these cells in culture. In this study we identified cells in culture as most probably Müller cells by using a panel of antibodies which, in combination, are unique to Müller cells among all the cell types in the retina. This study also demonstrates that the cells continued to express these, and other glial-specific proteins, for several months in culture. Many specific questions can now be asked about the function of these proteins and of retinal Müller cells in experimental conditions free from surrounding cells.

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