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Glial Fibrillary Acidic Protein (GFAP) Immunoreactivity in Rabbit Retina: Effect of Fixation

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The binding of monoclonal and polyclonal antibodies to glial fibrillary acidic protein (GFAP) antigenic sites in the rabbit retina was shown to be sensitive to aldehyde fixation. In chemically unfixed retina, the polyclonal anti-GFAP labeled Müller cells, astrocytes, and unidentified profiles in the outer plexiform layer; the monoclonal anti-GFAP labeled Müller cell endfeet and astrocytes only. The outer plexiform layer label with the polyclonal antibody was lost after fixation for 1 hr in 1% paraformaldehyde; elsewhere, the label was reduced. Fixation also reduced labeling by the monoclonal antibody. Such fixation sensitivity may underlie the different patterns reported for retinal GFAP immunoreactivity in the literature.

Key words: antibody; astrocyte; fixative; glial fibrillary acidic protein (GFAP); immunofluorescence; intermediate filaments; Müller cell; rabbit; retina.

1. Introduction

Glial fibrillary acidic protein (GFAP) is a protein of Mr 47–51 kDa (Rueger et al., 1979; Eng, 1982) which is organized into 10-nm diameter intermediate filaments in astroglial cells of the CNS (Eng et al., 1971; Bignami et al., 1972). Using antibodies, GFAP has also been localized in the normal mammalian retina, primarily in astrocytes (Schnitzer, 1985; Karschin, Wässle and Schnitzer, 1986) and Müller cell (MC) endfeet (Erickson et al., 1987; Ekström et al., 1988). However, considerable disagreement exists in the literature about GFAP immunoreactivity in normal MCs and elsewhere in the normal retina. GFAP expression in MCs increases dramatically upon retinal injury (Bignami and Dahl, 1979), detachment (Erickson et al., 1987), light damage, or inherited degeneration (Eisenfeld, Bunt-Milam and Sarthy, 1984; Ekström et al., 1988).

In the last 4 yr, there have been several reports of GFAP immunoreactivity in the normal vertebrate outer plexiform layer (OPL), perhaps in horizontal cells (HCs). Using antibodies, GFAP has been localized to A-type HCs (Karschin et al., 1986) and to the OPL (Ekström et al., 1988) of the cat retina, to the OPL of teleost retinas (Linser, Smith and Angelides, 1985), to 'horizontal elements' in the OPL of the mouse retina (Ekström et al., 1988), and to the OPL of the rabbit retina (Osborne, 1986). In the set of experiments described here, we used rabbit retina because this species, like the cat, has A-type HCs rich in intermediate filaments (Fisher and Boycott, 1974), and because other studies of rabbit retina with GFAP antibodies have yielded negative results with regard to OPL and MC labeling (Schnitzer, 1985; Kivelä, Tarkkanen and Virtanen, 1986; Schnitzer and Karschin, 1986). In contrast, our study indicates that GFAP may indeed be localized to both the OPL and to MCs of normal rabbit retina, but that positive results with the antibodies used are markedly sensitive to fixation parameters.

2. Materials and Methods

Experimental Animals

Adult, pigmented rabbits maintained on a 12:12 light/dark cycle were deeply anesthetized with rompun and ketamine and killed with an overdose of nembutal administered by intracardiac puncture. The eyes were enucleated and hemisected, and the anterior segments and vitreous bodies were removed.

Biochemistry

Retinas were peeled away from the retinal pigmented epithelium (RPE) and homogenized in phosphate-buffered saline (PBS) containing 0·1 mM phenylmethyl sulfonylfluoride, 2·5% sodium dodecyl sulfate, and 0·5% Triton X-100, and then spun for 30 min in a microfuge. Samples of the supernatant, containing 50 μg of protein, were analyzed by SDS–PAGE on a 7·5%–20% gradient gel under denaturing conditions. Molecular weight standards (Bio Rad) were also analyzed. The proteins were electroblotted onto nitrocellulose, blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS; pH 7·5), and incubated overnight in polyclonal anti-GFAP (Dako Corp.) diluted 1:400 in TBS. To determine the specificity of the anti-GFAP antibody, a monoclonal anti-vimentin (Dako Corp.) was also used, diluted 1:400 in TBS. After washing in TBS containing 0·05% Tween 20, the blots were incubated in HRP-linked secondary antibody (Bio Rad; diluted 1:2000)

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for 2 hr, rinsed, and the labeled bands visualized using HRP Color Development Reagent (Bio Rad).

Immunocytochemistry

Rabbit eyecups were cut into quadrants and then immediately plunged, retina side first, into liquid Freon cooled by liquid nitrogen, where they remained for 5 min. Pieces not immediately sectioned on the cryomicrotome (Lab Tek) were stored at −80°C. Serial 8-μm thick cryosections were cut and air-dried onto clean glass slides. Cryosections termed 'unfixed' were incubated in 0·137 M phosphate buffer (PB; pH 7·4), whereas 'fixed' cryosections were incubated in 1% paraformaldehyde plus 0·1 M lysine in PB for 1, 5, 10, 20, 45 or 60 min. Thorough rinsing in PB preceded immunofluorescence labeling with antibodies to GFAP.

Both fixed and unfixed cryosections were first blocked with normal serum diluted 1:40 in PBS plus 1% BSA (PBS/BSA; pH 7·4) for 20 min. This solution was poured off and replaced with primary antibody (experimentals) or normal serum (controls) diluted in PBS/BSA as follows: polyclonal anti-GFAP (Dako Corp.) at 1:100, monoclonal anti-GFAP (Amersham Corp.) at 1:100, and normal mouse or rabbit serum (Cappel) at 1:100. Sections remained in primary antibody or normal serum solutions for 1 or 12 hr and were then rinsed thoroughly in PBS/BSA. Sections were then incubated for 1 hr in the appropriate affinity-purified, FITC-conjugated secondary in PBS/BSA; sections were mounted in PBS/BSA mixed 1:1 with glycerol plus 5% *n*-propyl gallate to retard photobleaching (Giloh and Sedat, 1983) and viewed with a Zeiss Photomicroscope III equipped for epifluorescence. Images were recorded with manual 20-second exposures onto Kodak Tri-X film, rated ASA 400 but push-developed in Diafine. All micrographs were printed using standardized darkroom settings to demonstrate differences in fluorescent label observable through the microscope.

3. Results

A Coomassie blue-stained gel of rabbit retina and its corresponding Western blots are shown in Fig. 1. The polyclonal anti-GFAP labeled a single band of Mr 51 kDa [Fig. 1(D)], which did not comigrate with the single band of Mr 55 kDa labeled by the monoclonal antibody to vimentin. It also faintly labeled a high molecular weight band [Fig. 1(D)].

Unfixed cryosections probed with the polyclonal anti-GFAP had a distinctive labeling pattern (Fig. 2). Many MCs throughout the retina were labeled, sometimes faintly, in the endfeet and also in the trunks of the cells extending up toward the OPL [Figs 2(A) and 3(A)]. The MC label was particularly evident in areas of one retina exhibiting mild pathology [Fig. 2(B)], namely an accumulation of lipofuscin in the RPE. In the medullary ray region, astrocytes of the nerve fiber layer (NFL) were heavily labeled [Fig. 2(C)]. Significant label in the OPL was also noted throughout the retina [Fig. 2(A), (B), (C)]. The retinas of control sections incubated in normal rabbit serum were unlabeled [Fig. 3(H)].

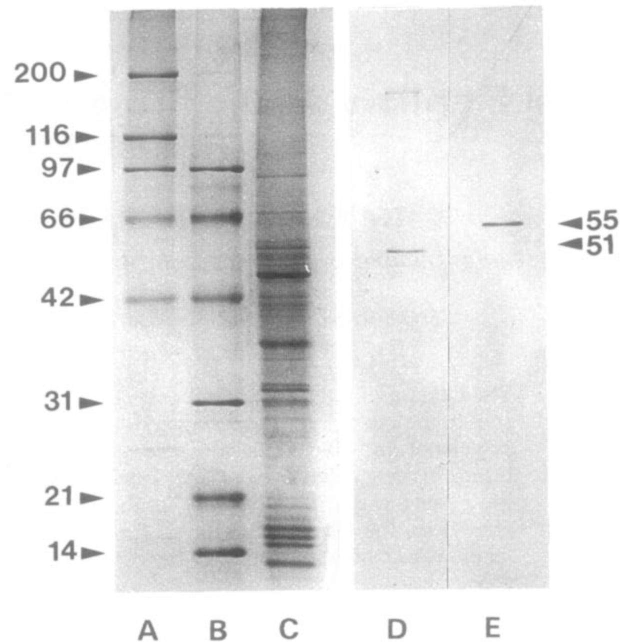

FIG. 1. Coomassie blue-stained polyacrylamide gel showing high (lane A) and low (lane B) molecular weight standards (Mr for each is listed at left, in kDa) and proteins of homogenized rabbit retina (lane C), and the corresponding immunoperoxidase-stained blots of polyclonal anti-GFAP (lane D) and monoclonal anti-vimentin (lane E). The polyclonal anti-GFAP labels a faint, high molecular weight band near the top of the blot and a single, major band at 51 kDa. The monoclonal anti-vimentin labels a single band at 55 kDa.

The monoclonal anti-GFAP also labeled the NFL astrocytes in unfixed cryosections [Fig. 4(A), (C)] and, faintly, the endfeet of MCs in retina apposed to lipofuscin-accumulating RPE (not shown). The OPL did not label with the monoclonal antibody, even when incubation in primary antibody was extended to 12 hr [Fig. 4(C)].

When serial cryosections were fixed for increasing lengths of time and then probed with the polyclonal anti-GFAP, a progressive loss of labeling was seen [Fig. 3(A)–(G)]. Cryosections prefixed for 60 min and then probed with either the polyclonal [Fig. 3(G)] or the monoclonal [Fig. 4(B), (D)] anti-GFAP showed a noticeable diminution of labeling in the MCs and in the NFL astrocytes (compared with unfixed cryosections). In the case of the polyclonal antibody, a complete loss of labeling in the OPL was observed [Fig. 3(G)]. Extending the incubation time in primary antibody from 1 hr to 12 hr did not increase labeling in these fixed sections [e.g. Fig. 4(B), (D)].

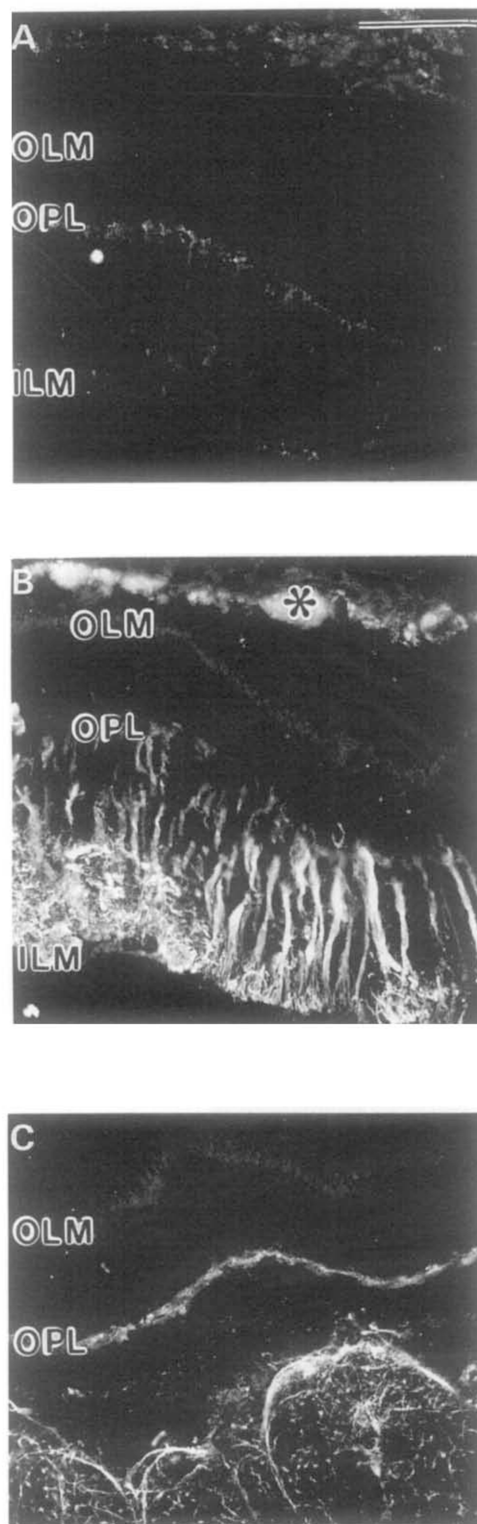

FIG. 2. Chemically unfixed cryosections from different parts of the rabbit retina, labeled with polyclonal anti-GFAP for 1 hr. OLM: outer limiting membrane; OPL: outer plexiform layer; ILM: inner limiting membrane. Scale bar = 50 μm. (a) The OPL and many Müller cells (MCs) label with anti-GFAP. MC label is faint, but extends from the endfeet (adjacent to the ILM) up to the OPL. See also Fig. 3(A). (B) From an area of retina in which the RPE has accumulated lipofuscin (*); MC label is especially pronounced. (C) From the medullary ray region of the retina; the OPL is brightly labeled, as are the nerve fiber layer astrocytes at the bottom of the micrograph.

4. Discussion

The Western blots illustrate the specificity of the polyclonal antibody to GFAP. The 51-kDa band labeled by the GFAP antibody does not coincide with the band labeled by the vimentin antibody (which has previously been shown not to cross-react with any other intermediate filament protein: Osborn, Debus and Weber, 1984), nor is it in the molecular weight range of the neurofilament triplet proteins. These data agree with that from Western analysis of the same polyclonal anti-GFAP against cat retina homogenates (Erickson et al., 1987). It is reasonable to conclude that the polyclonal antibody recognizes GFAP epitopes in rabbit retina.

The polyclonal antibody also faintly labeled a high molecular weight band. We have three possible explanations for this band. It could be due to epitopes on an unrelated polypeptide that are exposed only upon the denaturing conditions required for immunoblotting. It could be of GFAP origin (e.g. as an aggregation artifact of the denaturing procedures of SDS gel electrophoresis). In neither of these instances does the existence of the high molecular weight band detract from our conclusion that our polyclonal antibody recognizes GFAP. Alternatively, the high molecular weight band could be due to epitopes on an unrelated polypeptide that are present both in blots and in tissue sections. Importantly, this alternative would detract from our conclusion only if *all* the observed tissue immunoreactivity was due to the putative, unrelated high molecular weight molecule. This is doubtful since Western analysis showed the high molecular weight polypeptide to be considerably less abundant than the 51-kDa GFAP polypeptide. Moreover, we have evidence, in a manuscript now in preparation, that this is not the case. Using an electron microscope immunocytochemistry protocol (Erickson et al., 1987), the presence of 10-nm intermediate filaments was demonstrated in normal rabbit Müller cells and the same polyclonal antibody to GFAP was used to immunogold-label these filaments. Therefore, we are persuaded that intermediate filaments composed of GFAP exist in normal rabbit Müller cells and that a portion, at least, of the immunoreactivity reported in the present study is indeed due to 51-kDa GFAP. Epitopes in the high molecular weight band are probably only a minor source of tissue immunoreactivity.

Despite repeated attempts, we were unable to label any band(s) in Western blots with the monoclonal anti-GFAP. One interpretation of this result is that the monoclonal antibody is unable to bind to its GFAP epitope when the protein is denatured. Such differences between blot and tissue immunoreactivity have been observed in other instances with monoclonal antibodies to GFAP (Dahl, Grossi and Bignami, 1984).

In chemically unfixed cryosections of normal rabbit

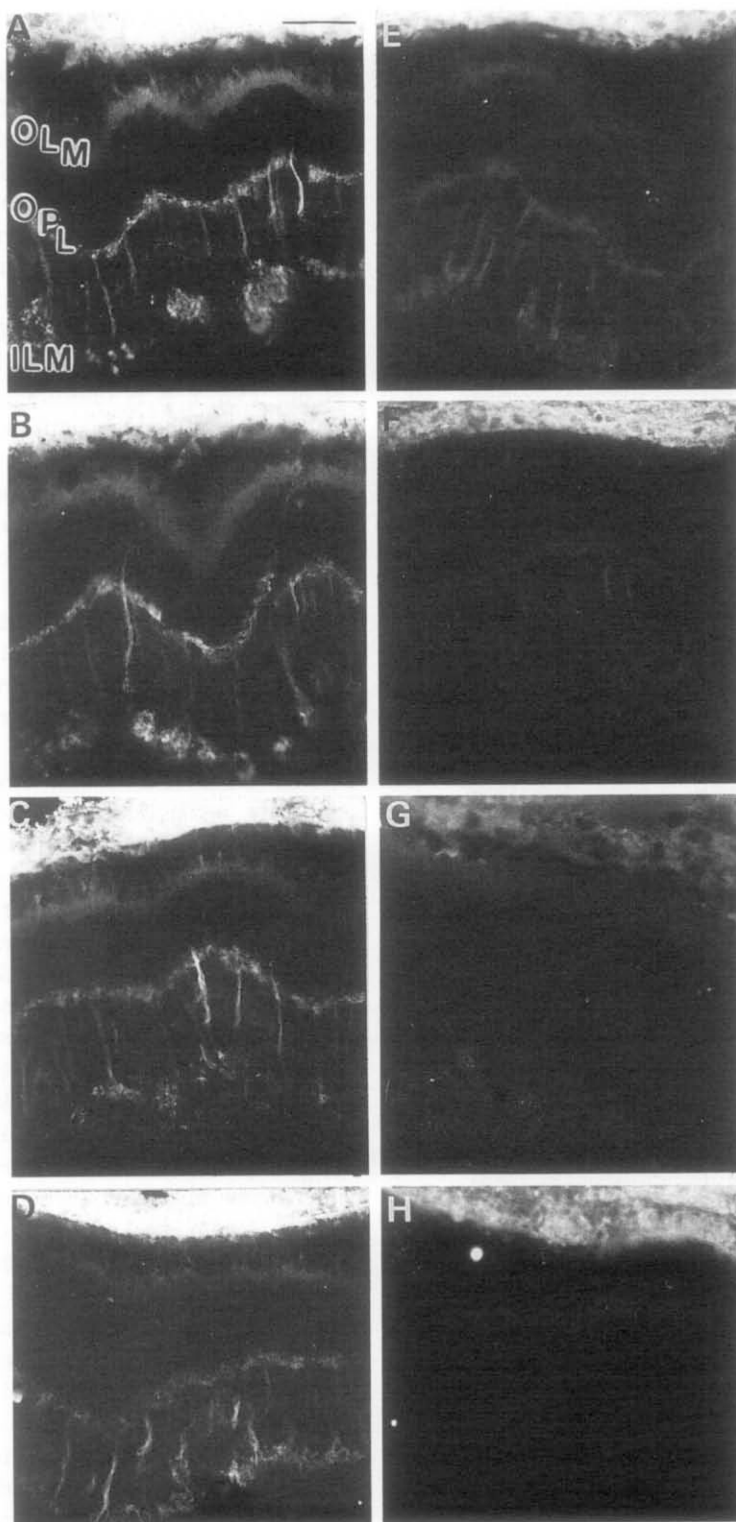

FIG. 3. Serial cryosections of rabbit retina fixed for various times in 1% paraformaldehyde (or not at all) and then immunolabeled with polyclonal anti-GFAP. All micrographs were made with manual 20 sec exposures and printed with standardized darkroom settings. Progressive loss of labeling occurs with increasing time in fixative. Scale bar = 50 μm. (A) Chemically unfixed; as in Fig. 2(A), the OPL and many MCs label. (B) Fixed for 1 min; labeling is the same as in (A). (C) Fixed for 5 min; labeling is the same as in (B). (D) Fixed for 10 min; labeling is less than in (A), (B) or (C). (E) Fixed for 25 min; labeling is less than in (D). (F) Fixed for 45 min; labeling is barely above the background fluorescence of photoreceptor inner segments at the top of the micrograph. (G) Fixed for 60 min; labeling has disappeared. (H) Chemically unfixed (normal rabbit serum) control; retina is unlabeled.

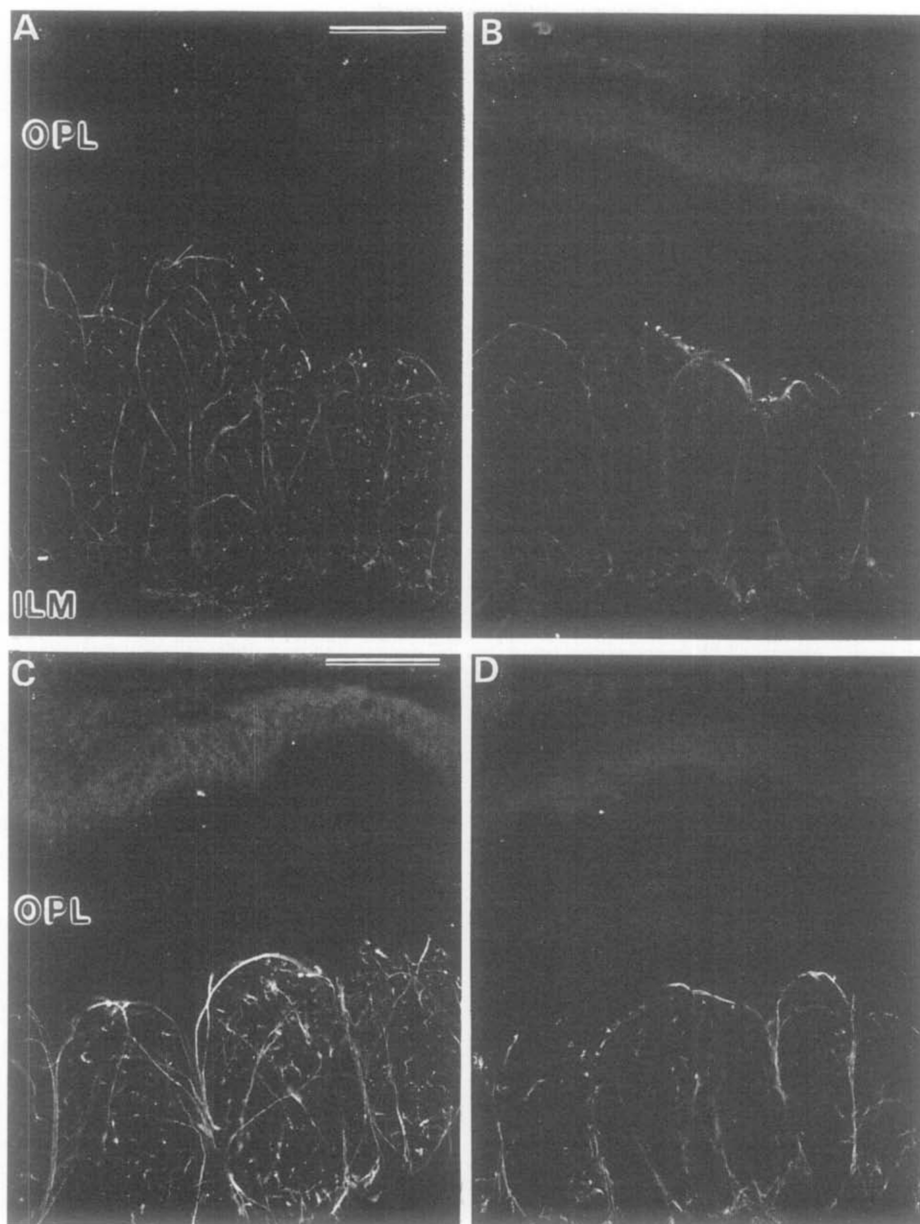

FIG. 4. Serial cryosections of rabbit retina from the medullary ray region labeled with the monoclonal anti-GFAP, illustrating the effects of fixation (60 min in 1% paraformaldehyde) and of incubation time on labeling. All micrographs were obtained as before. At short or long incubation time in primary antibody, fixation resulted in loss of labeling, particularly in the finer astrocytic fibers wrapping the nerve fibers. Scale bar = 50 μm. (A) Chemically unfixed section incubated in primary antibody for 1 hr. Labeling is restricted to the astrocytes; the OPL was never labeled by this antibody. (B) Fixed section also incubated in primary antibody for 1 hr. Labeling is less than in (A). (C) Chemically unfixed section incubated in primary antibody for 12 hr. Labeling is more vigorous than in (A), probably reflecting the increased incubation time, but it is still restricted to astrocytes. (D) Fixed section incubated in primary antibody for 12 hr. Labeling is less than in (C).

retina, we observed anti-GFAP labeling of the OPL, agreeing with one previous report (Osborne, 1986), but at odds with several others (Shaw and Weber, 1984; Schnitzer, 1985; Kivelä et al., 1986; Schnitzer and Karschin, 1986). As shown in Table I, both positive and negative results with regard to OPL-labeling by GFAP antibodies have been reported.

With one exception (Karschin et al., 1986; A-type HCs in cat retina), unequivocal identification of GFAP-immunoreactive profiles in the OPL has not been possible (cf. Ekström et al., 1988), though HCs are often proposed as the site of labeling. Intermediate filaments have not been demonstrated in photoreceptor synaptic terminals, bipolar cell dendrites, or interplexiform cell processes, making them unlikely candidates for the OPL labeling. It is also unlikely that this label is due to the presence of astrocytes in the OPL, since that cell type is unknown in the mammalian OPL (Dräger, 1983). Another possibility, recently proposed by Osborne (1986), is that microglia

TABLE I

GFAP immunoreactivity in normal mammalian retina

Species	Müller cells	OPL	Astrocytes
Cat	+ : 4, 5, 6, 13	+ : 6	+ : 4, 5, 6, 12, 13
	− : 12	− : 4, 5, 12, 13	− : none
Rabbit	+ : 13	+ : 8	+ : 9, 10, 13
	− : 7, 9, 10, 12	− : 7, 9, 10, 12	− : 7, 12
Rat	+ : 1*, 2, 13	+ : none	+ : 2, 13
	− : 3, 11	− : 2, 13	− : none
Mouse	+ : 4	+ : 4	+ : 4, 12
	− : 11	− : 11	− : none

1. Bignami and Dahl (1979).
2. Björklund et al. (1985).
3. Eisenfeld et al. (1984).
4. Ekström et al. (1988).
5. Erickson et al. (1987).
6. Karschin et al. (1986).
7. Kivelä et al. (1986).
8. Osborne (1986).
9. Schnitzer (1985).
10. Schnitzer and Karschin (1986).
11. Shaw and Weber (1983).
12. Shaw and Weber (1984).
13. Stone and Dreher (1987).

* Although this paper (Bignami and Dahl, 1979) is often cited in support of a lack of GFAP immunoreactivity in MCs of normal mammalian retina, its authors did, in fact, observe GFAP immunoreactivity in the MC endfeet (the 'glia limitans') of normal rat retina.

in the OPL express GFAP. Microglia in the mammalian retina are, in fact, often adjacent to HCs in the OPL (see Gallego, 1986, for a discussion) and have actually been mistakenly identified as HCs (see Boycott and Hopkins, 1981; Gallego, 1976). It seems unlikely that these cells of mesodermal origin express GFAP. Furthermore, recent evidence indicates that adult rabbits, such as those used in the present study, lack microglia in the OPL (Schnitzer, 1989). It has also been suggested that GFAP immunoreactivity in the OPL is due simply to horizontally oriented processes of MCs (Ekström et al., 1988). Our data do not resolve this issue.

In our study, a retina from one particular rabbit exhibited especially heavy anti-GFAP labeling of MCs in portions of the retina directly apposed to lipofuscin-accumulating RPE, concurring with earlier studies of pathological retinas of rat (Bignami and Dahl, 1979; Eisenfeld et al., 1984), cat (Erickson et al., 1987; Ekström et al., 1988), and mouse (Ekström et al., 1988). In this animal, we also observed that anti-GFAP labeling in the OPL differed from that of the MCs, inasmuch as no increase in OPL labeling was associated with the retinal pathology. If OPL labeling is due to a cell type other than the MC, we may conclude that an increase in GFAP expression is not a universal reaction of cells that contain GFAP intermediate filaments. If, on the other hand, OPL labeling is due to horizontally oriented MC processes, then the increase in GFAP expression may be restricted to other parts of the cells, namely the endfoot and main trunk. The function of GFAP intermediate filaments in MCs from normal retina is not understood. Although the function of increased GFAP expression in MCs from pathological retina is not completely understood, it seems likely to play a role in the extensive transformations in cell size and shape that occur in MCs in many of these conditions (e.g. Erickson et al., 1987; Lewis et al., 1989).

The heaviest anti-GFAP labeling that we observed was in the astrocytes that wrap the ganglion cell axons in the medullary ray region, as reported by Schnitzer (1985).

We also observed that many MCs in normal rabbit retina labeled with the polyclonal anti-GFAP, whereas rabbit MCs were previously reported to lack GFAP immunoreactivity (Shaw and Weber, 1984; Schnitzer, 1985; Kivelä et al., 1986; Schnitzer and Karschin, 1986). Table I also summarizes conflicting data on GFAP immunoreactivity in MCs. Such conflicts have recently been recognized by others besides ourselves (Björklund, Bignami and Dahl, 1985; Kivelä et al., 1986; Stone and Dreher, 1987; Ekström et al., 1988; Lewis et al., 1989). Several explanations have been offered—the greater range of GFAP antibodies in use since the first study in retina (Stone and Dreher, 1987), the possible masking of epitopes by hetero-polymerization of GFAP with some other intermediate filament protein (Bignami, 1984; Ekström et al., 1988), or an antigenic dissimilarity between Müller cell GFAP and CNS GFAP (Björklund et al., 1985). Additionally, one report notes the 'unreliability' of GFAP antibodies, attributing problems to delay after fixation and freezing (Stone and Dreher, 1987). Without addressing, or even necessarily contradicting, these speculations, we wondered if fixation sensitivity could also be a factor, especially since one report included a warning about fixation sensitivity to acetone (Björklund et al., 1985).

Our results have shown that anti-GFAP labeling in the rabbit OPL, MCs and astrocytes is significantly affected by chemical fixation. Treatment of unfixed cryosections for even 60 min in a relatively mild fixative (1% paraformaldehyde) drastically reduced

detection of GFAP epitopes in the OPL and in MCs, and significantly reduced labeling in astroctyes. Increasing the length of time in primary antibody (either polyclonal or monoclonal) did not reverse the loss of immunoreactivity with fixation. Our data, in conjunction with the published conflicting data on GFAP immunoreactivity in retinal MCs and in the OPL, lead us to conclude that GFAP epitopes recognized by these antibodies are sensitive to fixation (cf. Walker et al., 1984). This sensitivity to fixation could be due to cross-linking between GFAP and other proteins (e.g. intermediate filament associated proteins) during the fixation process, as shown by Bell et al. (1987) for their monoclonal antibody to GFAP. If true, the hypothesis that MC intermediate filaments are heteropolymers of GFAP with another protein, such as vimentin (Bignami, 1984; Ekström et al., 1988), could help to explain the phenomenon of fixation sensitivity.

Species differences in GFAP immunoreactivity patterns in the retina have been observed when the same antibodies and standardized preparations were used (e.g. Linser et al., 1985), so species differences certainly can be a factor in the various patterns reported. Our results suggest that, while other proteins may or may not suffer from fixation sensitivity, such a phenomenon may contribute to the disparate GFAP distribution patterns reported in vertebrate retina.

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