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Glial Fibrillary Acidic Protein Increases in Müller Cells after Retinal Detachment

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Retinal detachment, separation of the neural retina from the retinal pigment epithelium (RPE), initiates a series of changes in the eye which result in loss of vision if the retina is not rapidly reattached to the RPE. Many of the complex effects of this separation on the cell biology of the retina have yet to be determined. We report here a change in the amount and location of a specific cytoskeletal protein, glial fibrillary acidic protein (GFAP), within Müller cells after retinal detachment. Cat neural retina and RPE were separated by injecting fluid into the extracellular space between the retina and RPE. Normal retinas and retinas detached for 30 days were fixed and embedded for conventional electron microscopy or immunocytochemistry, or homogenized and processed by SDS–PAGE for immunoblot analysis with anti-GFAP. In normal retinas and in attached retinal regions of eyes with retinal detachment, GFAP was detected only in the end feet of the Müller cells as 10 nm diameter filaments and as a diffuse component over the cytoplasm. By contrast, in regions where the retina was detached from the RPE, GFAP was localized throughout the Müller cells in both of these forms. Immunoblots revealed a significant increase in anti-GFAP labeling of a 51000 MW band from the detached retina.

Key words: Müller cells; GFAP; retina; retinal detachment; immunocytochemistry; immunoelectron microscopy.

1. Introduction

Glial fibrillary acidic protein (GFAP) is a 47000–54000 MW protein (Rueger, Huston, Dahl and Bignami, 1979; Eng, 1982) that was first isolated from multiple sclerosis plaques (Eng, Vanderhaeghen, Bignami and Gerstl, 1971). The amount of this protein increases in certain diseases or injuries to the CNS (Kerns and Hinsman, 1973; Eng and DeArmond, 1981; Miller and Oberdorfer, 1981; Craft et al., 1985). It has also been isolated from and localized to astrocytes in normal brain as well as spinal cord (Eng et al., 1971; Bignami, Eng, Dahl and Uyeda, 1972; Dahl and Bignami, 1973; DeVries, Eng, Lewis and Hadfield, 1976; Eng, DeVries, Lewis and Bigbee, 1976; Eng and DeArmond, 1981; Onteniente, Kimura and Maeda, 1983; Eng, 1985). Glial fibrillary acidic protein usually occurs as 10-nm diameter (intermediate) filaments in fibrous astrocytes but anti-GFAP also labels a diffuse cytoplasmic component (Schachner, Hedley-Whyte, Hsu, Schoonmaker and Bignami, 1977). The diffuse cytoplasmic labeling may be GFAP monomers, since isolated GFAP monomers can polymerize into 10-nm filaments in vitro (Lucas, Bensch and Eng, 1979; Rueger et al., 1979).

Müller cells have some attributes of astrocytes (Uga and Smelser, 1973; Bignami and Dahl, 1979; Ripps and Witkovsky, 1985). For instance, Müller cells have been shown to be GFAP-immunoreactive after a variety of ocular or retinal injuries and degenerations (Bignami and Dahl, 1979; Miller and Oberdorfer, 1981; Shaw and

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Weber, 1983; Eisenfeld, Bunt-Milam and Sarthy, 1984). Although retinal astrocytes have been shown to be GFAP-immunoreactive (Karschin, Wassle and Schnitzer, 1986), there are conflicting reports on the presence of GFAP in Müller cells from normal rat and human retinas (Bignami and Dahl, 1979; O'Dowd and Eng, 1979; Dixon and Eng, 1981; Shaw and Weber, 1983; Eisenfeld et al., 1984; Ohira, Oshima and Kikuchi, 1984; Bjorklund, Bignami and Dahl, 1985; Hiscott et al., 1984).

When the mammalian retina is separated from the retinal pigment epithelium (RPE) by an experimental retinal detachment, Müller cells show characteristic responses, including the appearance of numerous 10-nm filaments in sclerad regions of the cell not normally containing them (Erickson, Fisher, Anderson, Stern and Borgula, 1983). In addition, subsequent growth of Müller-cell processes into the subretinal space and within the neural retina is similar to astroglial scar formation elsewhere in the CNS (Kerns and Hinsman, 1973; Kao and Chang, 1977; Kao, 1980; Erickson et al., 1983). Growth of Müller cells into the subretinal space interferes with photoreceptor outer-segment regeneration upon retinal reattachment (Anderson, Guerin, Erickson, Stern and Fisher, 1986) and appears to be one of the reasons for poor visual recovery from retinal detachment.

This study was designed to determine: (1) if Müller cells in normal cat retina contain GFAP; (2) if the 10-nm filaments that appear throughout the Müller cells' cytoplasm after retinal detachment are GFAP-immunoreactive, and (3) if GFAP is found in Müller cells in attached retina from eyes with retinal detachments.

2. Materials and Methods

Cat retinas ($n = 4$) were experimentally detached from the RPE for 1 month before they were processed for conventional electron microscopy, immunocytochemistry, or SDS–PAGE and immunoblot analysis. Detailed methods have been published previously (Anderson, Stern, Fisher, Erickson and Borgula, 1983; Erickson, Anderson and Fisher, 1985). Briefly, the lens and vitreous were removed and a 0·5% aqueous solution of Healon (sodium hyaluronate; Pharmacia) was slowly injected (using a micropipette) into the extracellular space between the photoreceptors and RPE. The resulting bullous retinal detachment radiated outward from the retinal hole produced by the micropipette.

Tissue for conventional electron microscopy was fixed overnight with 1% paraformaldehyde–1% glutaraldehyde in sodium phosphate buffer. Secondary fixation (for 1·0 hr) was with 2·0% osmium tetroxide (OsO_4) in the same buffer. The tissue was dehydrated with increasing concentrations of ethanol and embedded in Araldite resin. Thin sections were placed on copper grids and stained sequentially with uranyl acetate and lead citrate.

Tissue for immunocytochemistry was fixed for 1·0 hr with 1% paraformaldehyde–1% glutaraldehyde in sodium phosphate buffer. The tissue was not post-fixed with OsO_4. Dehydration with increasing concentrations of *N*,*N*-dimethyl formamide preceded embedding in Lowicryl K4M resin (Polysciences). Some tissue from normal cat retina was dehydrated in methanol, stained en bloc with uranyl acetate, and embedded in LR White resin (Polysciences).

Light-microscopic immunocytochemistry was performed at room temperature on 1·0-micrometer Lowicryl sections on Formvar-coated glass slides. The sections were covered by a drop of normal goat serum (Vector Laboratories) diluted 1 :75 with phosphate-buffered saline (PBS) for 20 min. The normal goat serum was blotted from the sections which were then incubated overnight with the anti-GFAP (rabbit anti-bovine-GFAP, IgG fraction; DAKO Corp.). The anti-GFAP was diluted 1 :250 with PBS and contained 0·5% bovine serum albumin (BSA). After a 10-min rinse in PBS, the sections were incubated with biotinylated goat anti-rabbit-IgG (Vector Laboratories; diluted 1 :250 with PBS) for 30 min. The slides were subsequently rinsed in PBS for 10 min and incubated with avidin–biotin conjugate (Vector laboratories; 1 :100 dilution in PBS) for 1·0 hr. After rinsing for 10 min in PBS,

incubating with 3-amino-9-ethylcarboazole peroxidase substrate solution (Polysciences) for 1·0 hr, and rinsing for 5 min in glass distilled water, a coverslip was applied to the sections with glycerol.

Immunoelectron microscopy was performed at room temperature on thin sections placed on nickel grids. The grids were incubated on drops of normal goat serum (Vector Laboratories; diluted 1 : 75 with PBS) for 20 min, blotted, and incubated overnight with rabbit anti-bovine-GFAP (IgG fraction; DAKO) diluted 1 : 400 with PBS and containing 0·125 mg ml^{-1} sodium azide and 0·5 % BSA. The grids were rinsed with PBS–BSA, transferred to drops of goat anti-rabbit-IgG-20 nm gold (1 : 20 dilution; Janssen Pharmaceutica) for 1·0 hr. After rinsing with PBS–BSA and then double-distilled water, the grids were stained sequentially with uranyl acetate, lead citrate, and OsO_4 vapors (2·0 % OsO_4 in sodium phosphate buffer; 1·0 hr).

Normal retina and regions of retina detached for 30 days were homogenized in PBS containing 0·1 mM phenylmethyl sulfonylfluoride. Samples containing 50 μg protein were analysed by SDS–PAGE on 7·5–15 % gradient gels under reducing conditions. In addition, proteins of known molecular weights (Bio-Rad) were run as standards. The retinal proteins were transferred by electroblotting to nitrocellulose paper, blocked with 3 % BSA in Tris-buffered saline (TBS; 20 mM Tris–HCl, pH = 7·5, 500 mM NaCl) and incubated overnight with rabbit anti-bovine GFAP diluted 1 : 100 in TBS. After washing in TBS containing 0·05 % Tween 20 (TTBS) the blot was treated with horseradish peroxidase (HRP) labeled goat-anti-rabbit IgG (Bio-Rad Labs) for 1·0 hr. The blot was then washed in TTBS followed by TBS and incubated in HRP Color Development Reagent (containing 4 chloro-1-naphthol; Bio-Rad) for 15 min. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr and Randall (1951).

Normal cat retinas ($n = 6$) were used as controls for the retinal detachment experiments. Immunocytochemistry controls included replacement of the anti-GFAP with non-immune rabbit IgG (1 : 100 dilution for light microscopic immunocytochemistry and 1 : 400 dilution for immunoelectron microscopy) or PBS–BSA.

3. Results

By light-microscopic immunocytochemistry, anti-GFAP labeling was detected only in detached retina (Fig. 1). The reaction product appeared as strands extending from the border of the vitreous cavity to the outer plexiform layer (OPL) of the retina (Fig. 1 D). No anti-GFAP labeling was found in Müller cells of normal retina or in regions of attached retina from an eye with a retinal detachment. Immunocytochemical controls were also devoid of labeling.

By immunoelectron microscopy, anti-GFAP labeling was detected in Müller cells of normal retinas near the vitreous border (Fig. 2 A). The gold particles were sparse over the cytoplasm and more frequent over 10-nm diameter filaments. This area of the cell (Müller cell end foot) was not full of filaments and consequently, filaments were not found in every cell in each plane of section. However, filaments were sometimes detected from the vitreous border to the region of the ganglion-cell axons. Other cellular regions showed no evidence of intermediate filaments or anti-GFAP labeling. Astrocytes in normal and detached retinas labeled with anti-GFAP but neurons did not, although some neurons had intermediate filaments in their cytoplasm. In normal cat retina, filament location and immunolabeling was the same in tissue embedded in Lowicryl or LR White resins. The immunocytochemistry control sections were essentially devoid of gold particles (Fig. 2 B).

Müller cells in retinas detached for 1 month exhibited a dramatic increase in the number of intermediate filaments and anti-GFAP labeling (Fig. 3). The most striking difference between the experimental and control eyes was the appearance of intermediate filaments throughout most of the Müller cells' cytoplasm after the 30-day

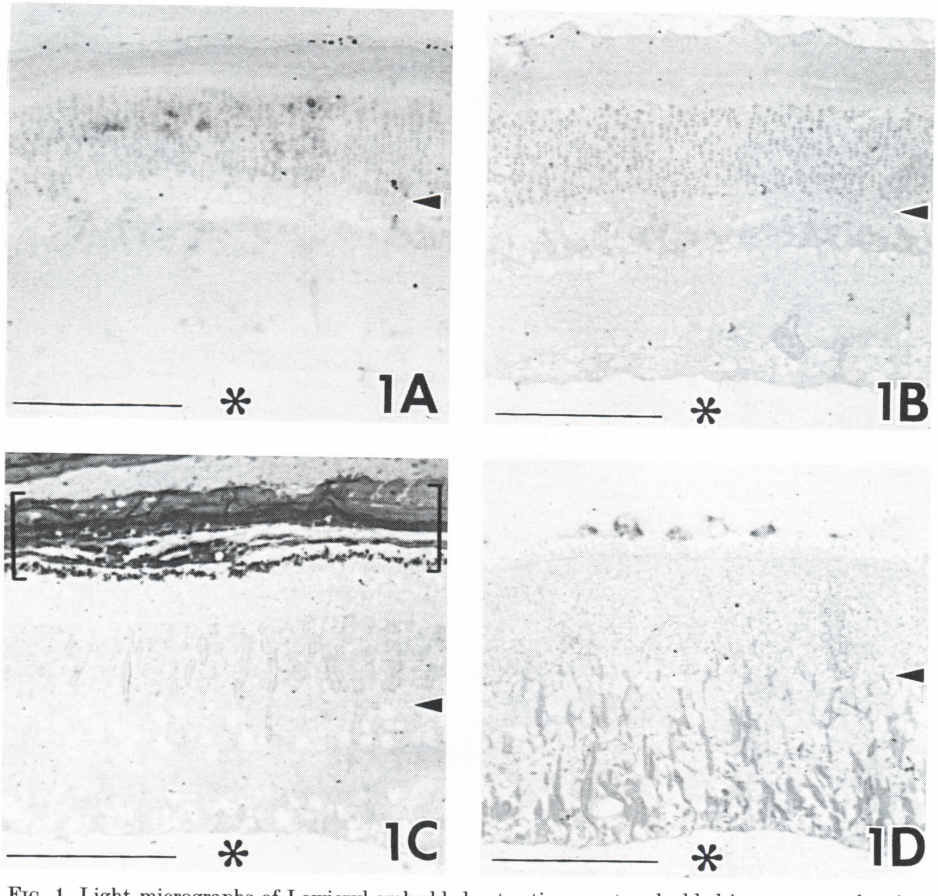

FIG. 1. Light micrographs of Lowicryl-embedded cat retina; post-embedded immunocytochemistry performed on 1·0-micrometer thick sections, avidin–biotin-conjugate immunoperoxidase technique; asterisk indicates vitreous cavity, arrowhead denotes outer plexiform layer, bars represent 10·0 μm. A, Normal retina; non-immune IgG control; B, normal retina; anti-GFAP; C, attached retina from an eye with a 30-day retinal detachment (same eye as in D); anti-GFAP. The dark area, within the brackets, is pigment in the RPE and choroid that is found in non-tapetal areas. D, Region of retina detached for 30 days (same eye as in C); anti-GFAP. Heavy labeling of the Müller cells in the detached retina extends from the vitreous cavity to the outer plexiform layer.

detachment interval. Figure 4A shows an example of a Müller cell whose nucleus has translocated to the OPL and whose cytoplasm is filled with 10-nm filaments. Figures 4B and 5 show anti-GFAP labeling of filaments in this region of a Müller cell.

A major band at 51000 MW reacted with anti-GFAP on immunoblots prepared from both normal and 1-month detached retinas (Fig. 6). When equal amounts of protein from retinal homogenates were analysed, there was an increase in the anti-GFAP labeled band from the detached retina. An additional band (47000 MW) labeled faintly with anti-GFAP. This pattern is characteristic of immunolabeling using either polyclonal or monoclonal anti-GFAP (Eng, 1985). The 47000 MW band is probably a degradation product caused by a Ca^{2+}-activated protease (Schlaepfer and Zimmerman, 1981; Eng, 1985).

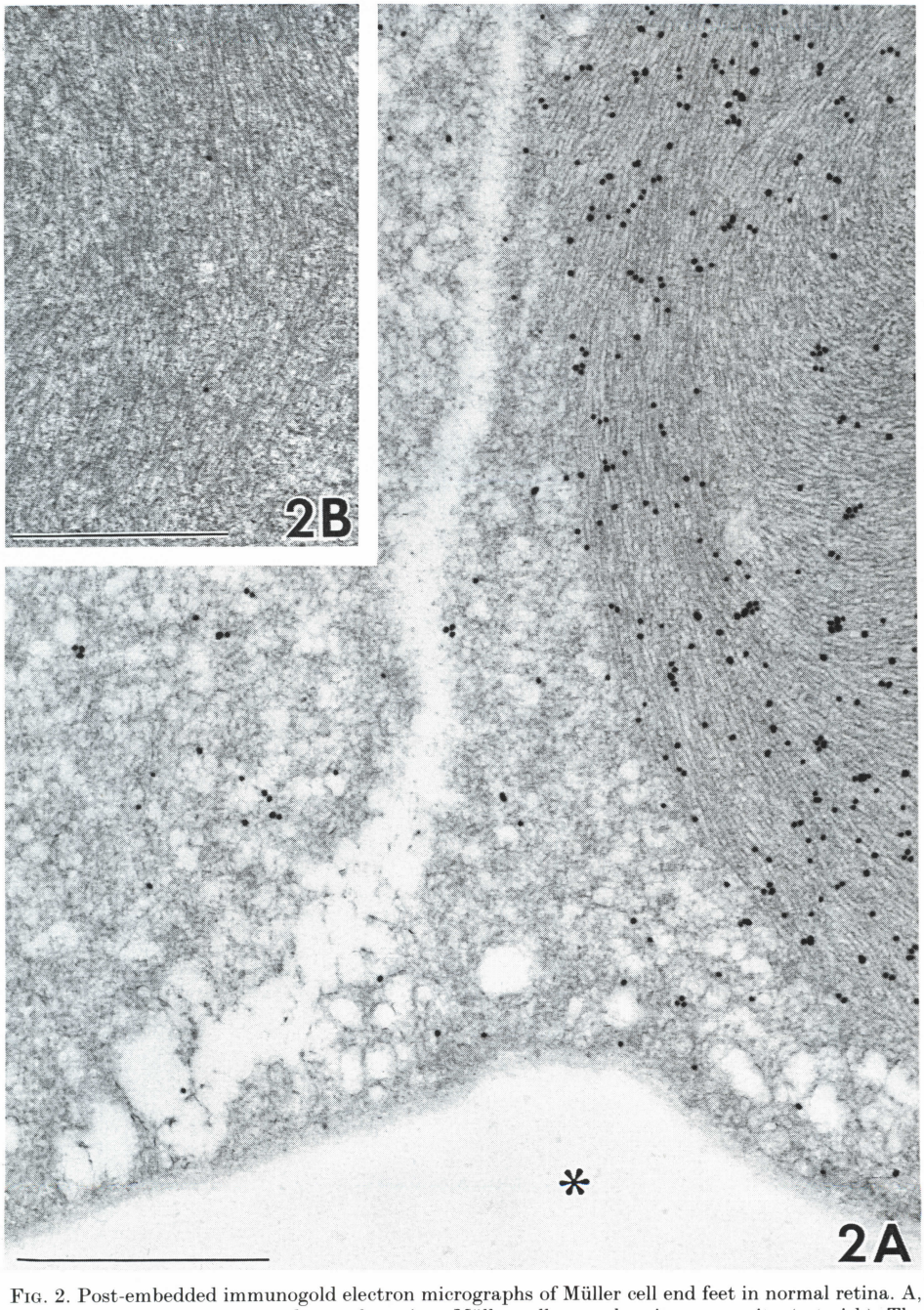

FIG. 2. Post-embedded immunogold electron micrographs of Müller cell end feet in normal retina. A, Anti-GFAP labeled filaments and cytoplasm in a Müller cell near the vitreous cavity (asterisk). The filaments are 10 nm in diameter and the gold spheres are 20 nm in diameter (bar represents 1·0 μm). B, Non-immune IgG substituted for anti-GFAP results in unlabeled filaments (bar represents 1·0 μm).

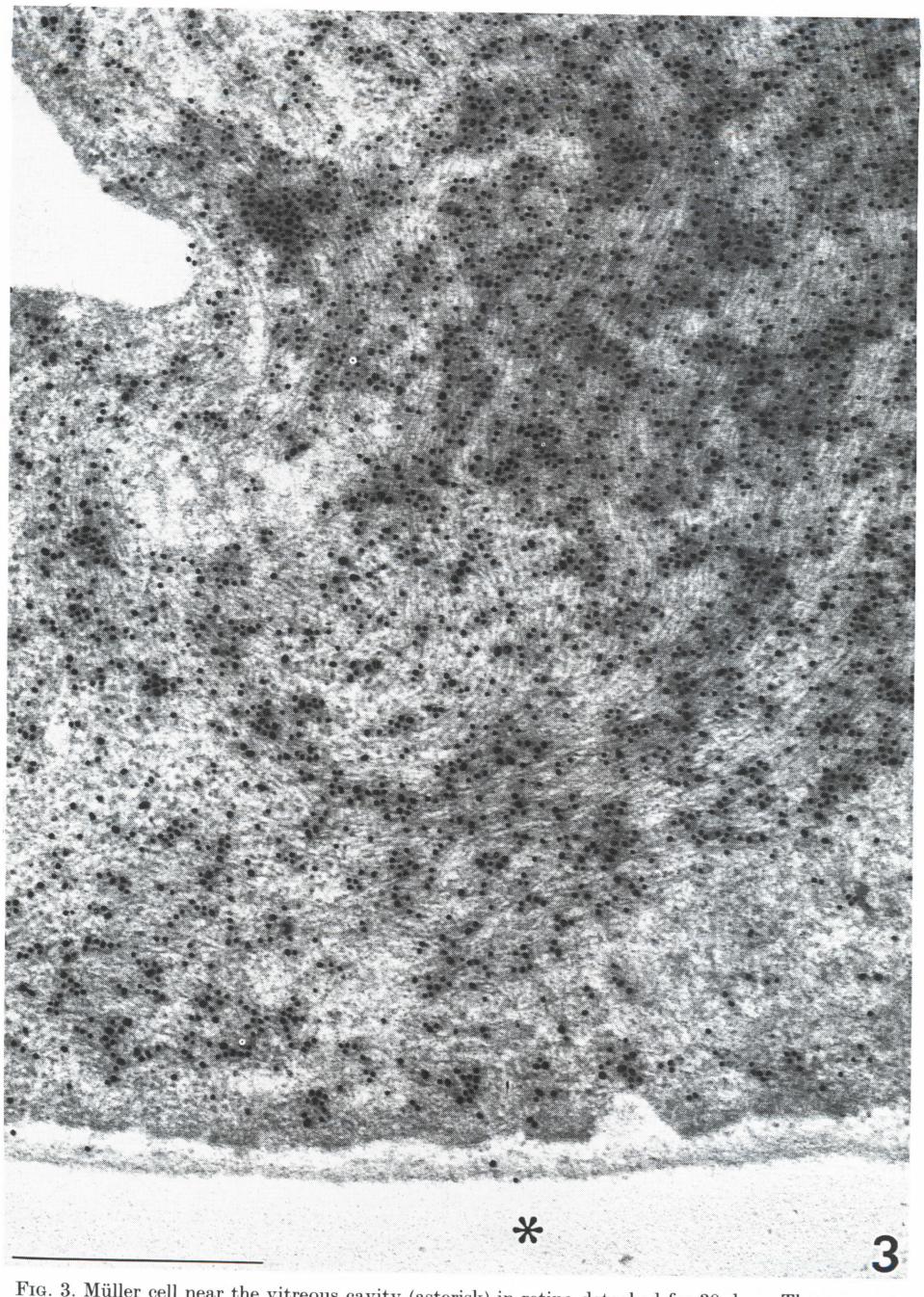

FIG. 3. Müller cell near the vitreous cavity (asterisk) in retina detached for 30 days. The numerous intermediate filaments and cytoplasm in the Müller cell are heavily labeled with anti-GFAP (post-embedded immunogold technique; bar represents 1·0 μm).

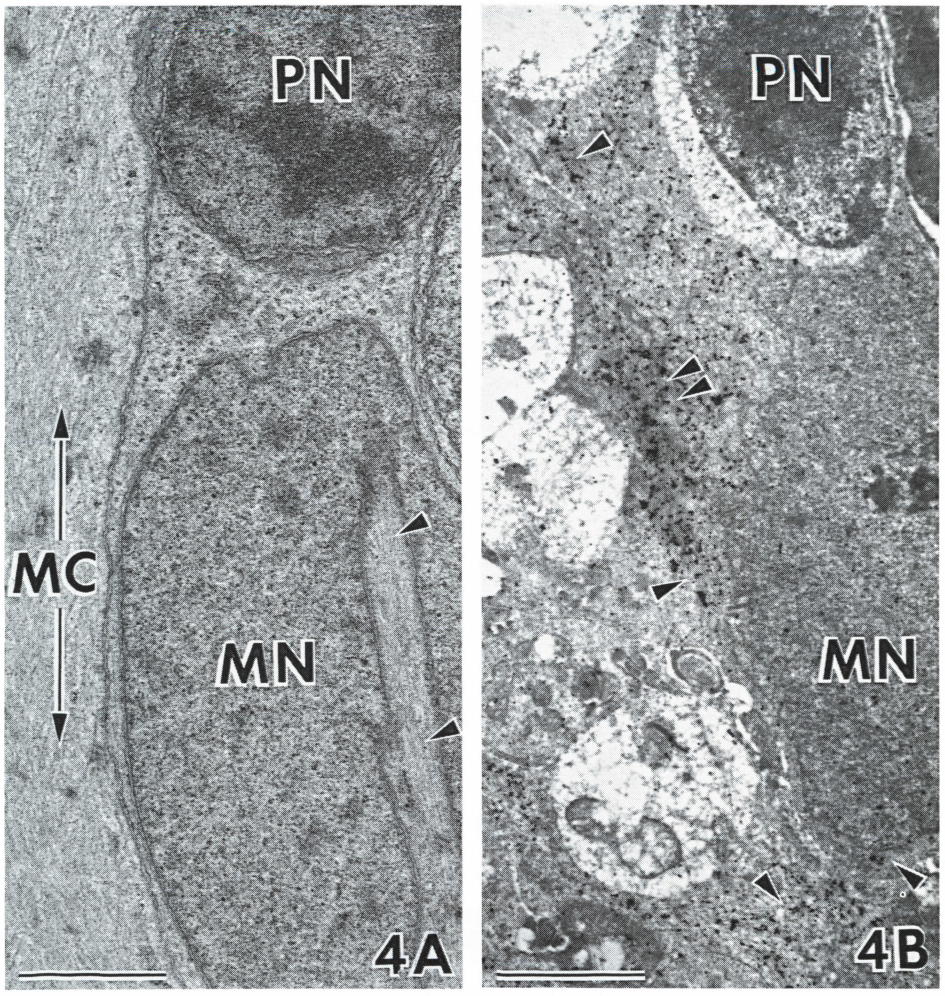

FIG. 4. The outer plexiform layer (OPL) of a retina detached for 30 days. A, Tissue processed for conventional electron microscopy reveals a Müller-cell nucleus (MN) translocated to the OPL. There are numerous intermediate filaments near the Müller-cell nucleus (arrowheads) and in the cytoplasm of an adjacent Müller cell (MC) (PN = photoreceptor nucleus; bar represents 2·0 μm). B, Post-embedded immunogold electron micrograph of a Müller-cell nucleus (MN) translocated to the OPL. The Müller-cell cytoplasm and intermediate filaments label with anti-GFAP (arrowheads; PN = photoreceptor nucleus; bar represents 2·0 μm). The area indicated by the double arrowheads is enlarged in Fig. 5.

4. Discussion

Müller cells in normal cat retina contain intermediate filaments that are 10 nm in diameter, GFAP-immunoreactive and restricted to the Müller cells' end feet near the vitreoretinal border. Our results agree with other studies showing some GFAP in this region of normal retinas (Bignami and Dahl, 1979; O'Dowd and Eng, 1979; Ohira, Oshima and Kikuchi, 1984; Bjorklund et al., 1985). Although we could not detect this pattern by light-microscopic immunocytochemistry of normal retinas, by immuno-electron microscopy anti-GFAP reactivity was found in the inner Müller cells as intermediate filaments and a diffuse component over the cytoplasm. The diffuse

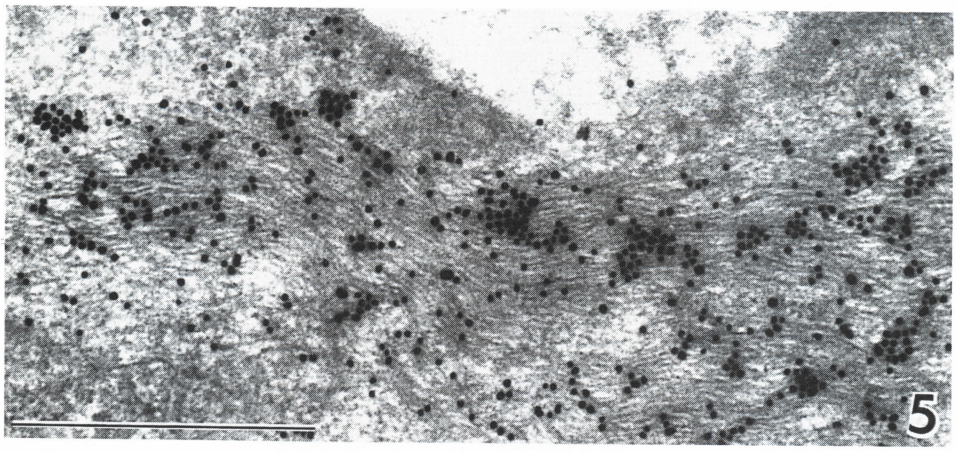

FIG. 5. Post-embedded immunogold electron micrograph of Müller-cell cytoplasm in the OPL of a retina detached for 30 days. High magnification of the area in Fig. 4B indicated by double arrowheads reveals numerous anti-GFAP labeled filaments (bar represents 1·0 μm).

component could either be non-filamentous GFAP or cross-sections of individual filaments. Other researchers have reported similar GFAP localization in cerebellar astrocytes (Schachner et al., 1977).

Following retinal detachment, cat Müller cells greatly increase the amount of GFAP in their cytoplasm. Thirty days after detachment this additional GFAP is located throughout the cell as intermediate filaments and possibly as a diffuse cytoplasmic form. It is significant that this increase in Müller-cell GFAP occurs only in the region of retinal detachment. Müller cells located in attached, normal-appearing regions of the eyes with a detachment express similar low levels of GFAP as do Müller cells in normal retinas. An increase in the expression of GFAP has been reported to occur in Müller cells throughout the retina of rats after multiple penetrating ocular wounds (Bignami and Dahl, 1979). From our data it appears that penetration of the eye, per se, does not lead to an increase in GFAP. The eye is penetrated three times as part of our detachment procedure, yet Müller cells in normal-appearing (attached) regions have levels of GFAP similar to Müller cells in normal retina. Bignami and Dahl (1979) detected granulation tissue that filled the vitreous cavity and described the retina as raised in thick folds and severely disorganized. Consequently, it appears likely that their procedure produced proliferative vitreoretinopathy and a massive retinal detachment, and this, rather than penetration of the eye, led to the increased GFAP.

The function of intermediate filaments is not yet known, but they may serve as structural coordinators of various cytoplasmic organelles, including the nucleus (Lazarides, 1980). Indeed, the intimate association of the GFAP-containing filaments and translocated Müller-cell nuclei suggests a correlation between the filaments and nuclear relocation. Müller-cell nuclear migration is apparent as early as 2 days after retinal detachment (Erickson et al., 1983) and may be a preliminary event to mitosis (Erickson, unpubl. data). Müller-cell nuclei first label with tritiated thymidine 2 days post-detachment and mitotic figures are observed 24 hr later in the inner nuclear, outer plexiform and outer nuclear layers (Erickson et al., 1983). We are currently examining Müller cells at these early detachment times to determine the relationship between GFAP-containing filaments, nuclear location and mitosis.

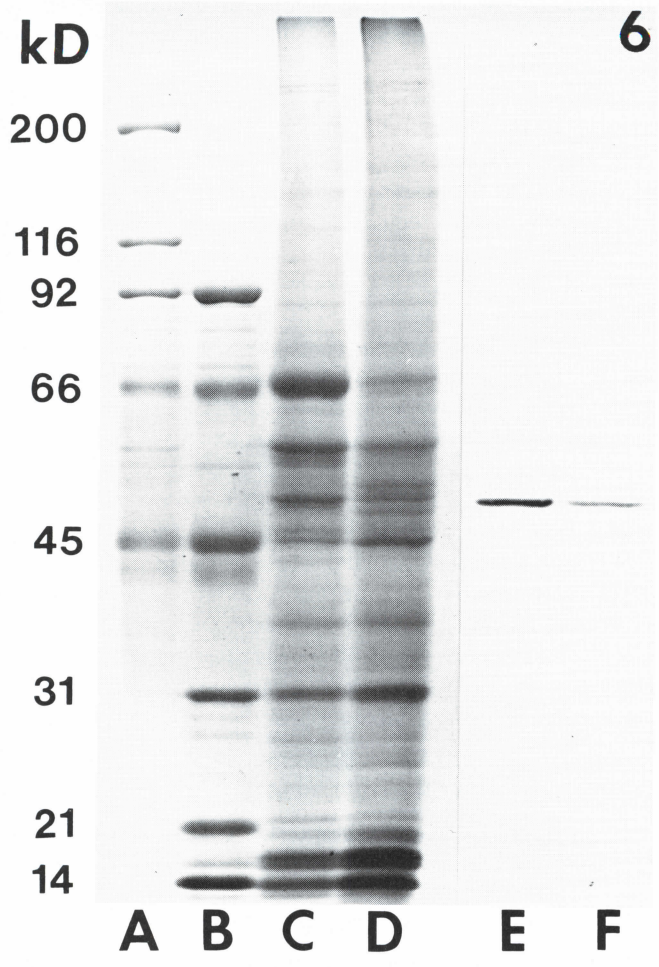

FIG. 6. Coomassie Blue-stained SDS–PAGE showing proteins of normal and 30-day detached retina, and a corresponding immunoperoxidase blot with anti-GFAP. Samples of retinal homogenates containing 50 μg protein were analysed by SDS–PAGE, or the proteins transferred to nitrocellulose paper. Lanes A and B: molecular-weight standards. Lane C, 30-day detached retina; lane D, normal retina; lane E, anti-GFAP immunoblot of 30-day detached retina; lane F, anti-GFAP immunblot of normal retina. The 51000 MW band in the detached retina is more intense in the Coomassie-stained SDS–PAGE (Lane C) and in the anti-GFAP labeled immunoblot (Lane E). There is a faint second band at 47000 MW that probably represents a GFAP degradation product (see text).

The similarities between Müller cells in detached retinas and reactive astrocytes elsewhere in the central nervous system (CNS) provides additional understanding of their individual reponses to various pathological conditions. For instance, reactive astrocytes show an increase in the number of GFAP filaments and can form glial 'scars' (Kerns and Hinsman, 1973; Kao and Chang, 1977; Kao, 1980). An analogous response, associated with detachment of the retina, is the formation of multicellular, periretinal membranes that are connected to the neural retina (Laqua and Machemer, 1975; Laqua, 1975; Clarkson, Green and Massof, 1977; Erickson et al., 1983). Some cells in these membranes are GFAP-immunoreactive and contain 10-nm diameter filaments (Rodrigues, Newsome and Machemer, 1981; Hiscott et al., 1984). Since

Müller cells in detached cat retina form similar membranes (Erickson et al., 1983), and express GFAP in filamentous form, Müller cells may be precursors of GFAP-containing cells in periretinal membranes.

We showed previously that separation of the neural retina and RPE results in morphological changes in both cellular layers (Anderson et al., 1983; Erickson et al., 1983). Here we show that separation results in enhanced expression of a specific protein by the Müller cells. A major question raised by these studies is, what factor(s) in the retinal microenvironment regulate(s) these specific cellular changes? In the case of Müller cells, detachment results in a complex series of events, including cellular proliferation, hypertrophy of existing processes, growth of new processes, migration of the cell nucleus and cell body, increased RNA synthesis, and increased 10-nm GFAP filaments (Erickson et al., 1983; Erickson, Fisher, Guerin and Anderson, 1984). These responses must be mediated indirectly, since Müller cells do not border the RPE. Because glial cells are known to be sensitive to changes in their microenvironment caused by neuronal activity (Henn, Haljamae and Hamberger, 1972; Ripps and Witkovsky, 1985), and if it is assumed that the activity of retinal neurons is altered by detachment, it is possible that changes in Müller-cell metabolism may be mediated by factors arising from damaged retinal neurons or by changes in their electrical activity. Alternatively, since the detached retina is separated from the choroidal blood supply, and the blood–retina barrier is probably disrupted, serum-derived components or a change in normal levels of metabolites may be causative.

The increase in GFAP within the Müller cells after retinal detachment may prove useful for studying control of GFAP gene expression after CNS injury and also the function of this enigmatic cytoskeletal protein.

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