Changes in Intermediate Filament Immunolabeling Occur in Response to Retinal Detachment and Reattachment in Primates

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The immunolabeling patterns for vimentin and glial fibrillary acidic protein (GFAP) were studied in five rhesus monkeys that had undergone retinal detachment or detachment and reattachment. Anti-vimentin and anti-GFAP labeling intensity increased in Müller cells after 2 days of detachment. Weak anti-vimentin labeling of the basal RPE cytoplasm, which was absent in control tissue, was detected 2 days after detachment. After detachment for 7 days and reattachment for 7 or 14 days, the pattern and extent of intermediate filament (IF) labeling changed. In Müller cells, the labeling, which in controls was restricted to processes near the vitreal border of the retina, was present in Müller processes spanning the entire retina. In retinal pigment epithelium (RPE) cells, prominent anti-vimentin labeling was identified in the basal and basolateral cytoplasm. The extent of RPE and Müller cell IF labeling in two animals whose retinas had been detached and then reattached for 150 days was different from that found at either the 7- or 14-day reattachment time points. This suggests that the abnormal IF distribution triggered by detachment may be attenuated after a lengthy period of reattachment. Invest Ophthalmol Vis Sci 31:1474–1482, 1990

Both vimentin and glial fibrillary acidic protein (GFAP) form 10-nm intermediate filaments (IFs) and are members of a single multigene family.[1,2] Each has a highly conserved central domain and a hypervariable end region.[1,3] Both proteins are composed of a single polypeptide chain, and on sodium dodecyl sulfate (SDS)-polyacrylamide gels, they migrate at 53 kD and 55 kD, respectively.[3] Vimentin has been identified in a variety of cell types of different embryonic origin, whereas GFAP is found apparently only in glial cells.[1] It has been proposed that IFs may serve both structural and transport functions[1,4,5] although definitive experimental evidence in that regard has not been reported.

The expression both of GFAP and of vimentin is known to change in response to factors which perturb the cells' microenvironment. Vimentin often appears in cells that do not normally express it after they have been dissociated and grown in tissue culture.[6] In the retina, GFAP has been shown to increase in response to several types of injury[7-9] and to increase as a result of at least one inherited degeneration.[10] The levels and distribution both of GFAP and of vimentin change dramatically after experimental retinal detachment in the cat.[11,12]

In this study, we report for the first time that vimentin, which is not normally found in monkey retinal pigment epithelium (RPE) cells in vivo, is expressed by these cells after retinal detachment. Secondly, we show that changes in IF immunolabeling, analogous to those described in the cat retina 30–60 days after detachment,[11,12] can be identified in primate Müller cells 2 days after detachment. These changes persist beyond the detachment interval, and can also be identified for at least 2 weeks after retinal reattachment. Finally, we present results from two animals that were allowed to recover for 5 months after reattachment. Comparisons between the IF distributions at the various detachment–reattachment time points suggest that prolonged periods of recovery may result in attenuation or partial regression of the abnormal IF changes.

Materials and Methods

Animals and Surgery

Unilateral retinal detachments were produced in five adult, domestically bred, rhesus monkeys (University of California, Davis, CA), whose average age

was more than 10 yr. Reattachments were performed in four of the animals after 7 days' detachment. Retinal detachments and reattachments were performed according to the technique published previously.[13,14] The detachment and reattachment intervals, in days, were: 2:0, 7:7, 7:14, and 7:150 (two animals). The boundaries and other characteristics of each detachment were recorded on a standard ophthalmic detachment chart at the end of each surgery. The eyes were examined periodically with an indirect ophthalmoscope. The animals were sacrificed at the conclusion of the experiment by intravenous injection of barbiturate. This was followed by intracardiac perfusion of fixative. All animals were maintained and cared for in accordance with the ARVO Resolution on the Use of Animals in Research.

Fixation

The fixation protocol and staining techniques used in this study have been published previously.[15] Briefly, animals were entrained to a 12:12 light–dark cycle and sacrificed 4 hr after light onset. Animals were fixed by intracardiac perfusion of 1% glutaraldehyde and 1% paraformaldehyde in phosphate buffer, and then the eyes were enucleated. After removal of the anterior segments, eyecups were immersed in the same fixative for 1 hr. The tissue was stained en bloc with 1% aqueous uranyl acetate, and dehydrated with a series of graded methanols. Specimens were embedded in LR White resin polymerized at 52°C.

Immunocytochemistry

We employed the indirect, postembedding technique described by Erickson and co-workers.[16] For electron microscopy, thin sections were placed on nickel grids and preincubated in normal goat serum at a dilution of 1:40 for 15 min before overnight incubation in either rabbit anti-bovine GFAP (Dako, Carpinteria, CA) or a mouse monoclonal antibody to vimentin (Dako) (both diluted 1:400 in phosphate buffered saline containing 1% bovine serum albumin (PBS/BSA)). The specificity of these antibodies in both normal and detached retina has been characterized previously by Western analysis of electrophoretically separated homogenates of retinal proteins.[12,17] In addition, immunoelectron microscopy indicated that both antibodies selectively label 10-nm-diameter IFs in retinal cells.[17] After several washes in PBS/BSA, grids were incubated in goat anti-rabbit or goat anti-mouse IgG-Au (5 nm; Janssen). After further washing, grids were stained 1–2 hr with osmium tetroxide vapors, after which they were carbon-coated and viewed by transmission electron microscopy.

Two tissue specimens were examined from the detached or reattached regions for each of the five experimental eyes. In addition, two types of controls were included for each animal. At least one specimen was obtained from an attached area of retina adjacent to the experimentally detached-and-reattached region. Additionally, one specimen was obtained from the opposite eye that corresponded closely to the detached or reattached region in the experimental eye. For light microscopic immunocytochemistry, 1-μm-thick sections were placed on formvar-coated glass slides and surrounded with a detachable plastic well. Formvar was used to prevent LR White sections from being washed away during the incubation procedures. Approximately 5–7 mm of retinal length uncorrected for curvature was included in each tissue specimen.

Sections were incubated in normal goat serum (1:40; Vector) for 30 min. The serum was rinsed off, and primary antiserum, either anti-GFAP or anti-vimentin, (both diluted 1:400), were added and the sections were allowed to incubate overnight at room temperature in a humidified atmosphere. The next day, sections were rinsed 3 × 5 min in PBS/BSA and then incubated for 1 hr at room temperature with the appropriate secondary antibody (1:40) conjugated to 5 nm-Au (Janssen). After this incubation the sections were rinsed in PBS (without BSA) and distilled H₂O. Then the Au labeling was silver-enhanced with Enhance II (Janssen). All slides for a given antibody were processed together, and each slide was silver-enhanced for an identical amount of time. When complete, the enhancing process was stopped by rinsing with distilled H_2O. Selected slides were stained with a mixture of methylene blue, azure II, and toluidine blue in aqueous solution containing Na borate, and then counterstained with basic fuchsin. Slides were dried and coverslipped, and then photographed with a Zeiss (Thornwood, NY) photomicroscope III.

Results

In control sections taken from either the unoperated eye or attached retinal regions of the experimental eye, anti-vimentin labeling occurred in Müller cell end-feet and Müller cell processes surrounding the ganglion cell bodies; some labeling of distal processes extended into the inner plexiform layer (IPL). At the light microscopic level, labeling was rarely observed sclerad to the inner nuclear layer (INL) (Fig. 1A). The absence of IF labeling in the outer retina was most evident in unstained sections, as illustrated in Figure 1B. Anti-GFAP labeling was similar to the anti-vimentin pattern except that with anti-GFAP fewer labeled processes were observed within the IPL (Fig. 1B). Neither antibody showed immunolabeling in the RPE cytoplasm of normal control tissue; however,
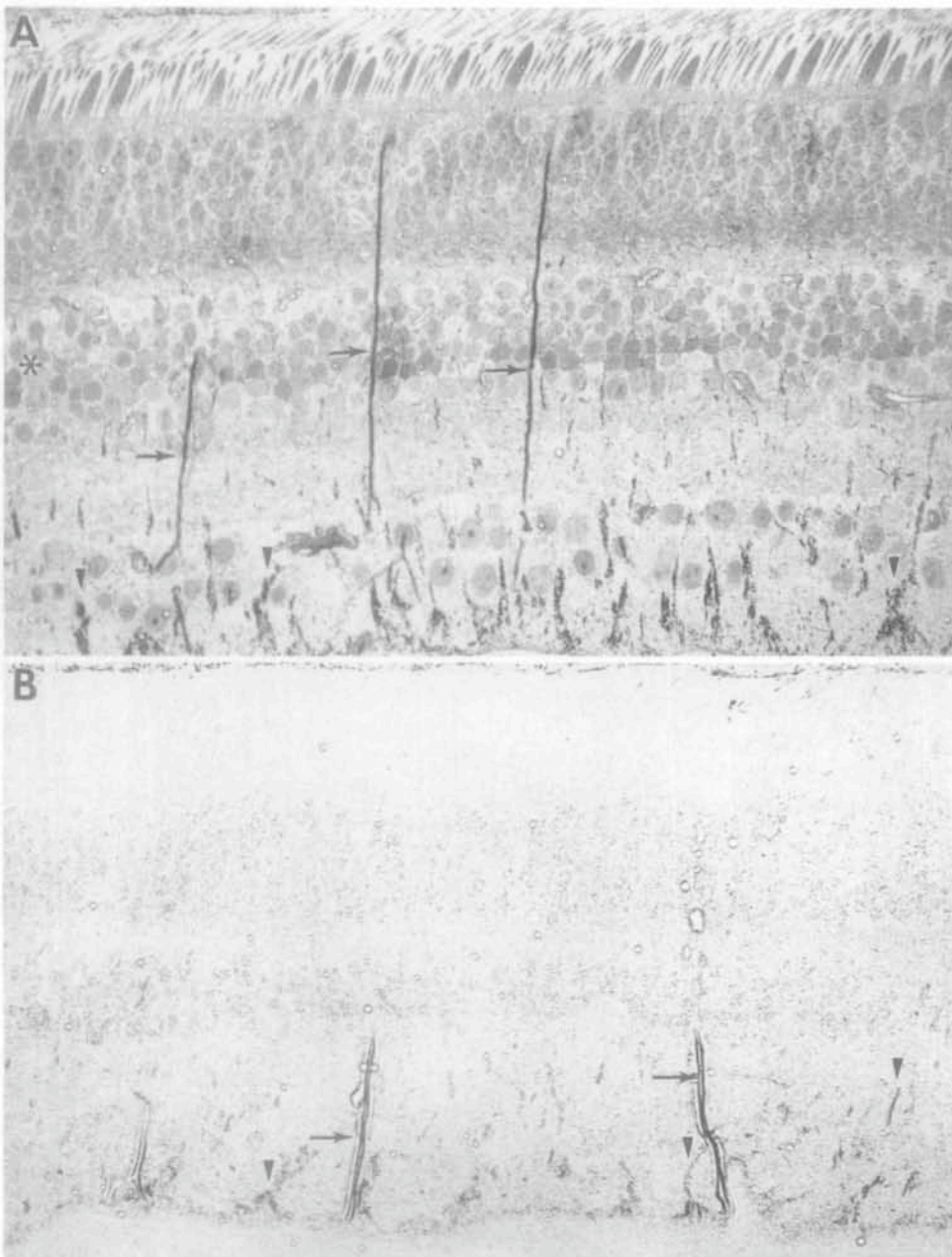
Fig. 1. Light micrographs of anti-vimentin and anti-GFAP immunolabeling in stained section of normal monkey retina. (A) Vimentin immunoreactivity is seen most prominently in the region of the Müller cell endfeet (arrowheads), although some lightly labeled processes also appear in the IPL. The black vertical lines (arrows) are folds in the formvar substrate; such folds are common artifacts of the procedure and do not signify immunolabeling. Asterisk, INL. (B) Unstained section showing anti-GFAP labeling is restricted to the Müller cell endfeet (arrowheads) and ganglion cell layer (GCL). The absence of anti-GFAP labeling in the outer retina is best illustrated in unstained tissue sections. (×300).

anti-vimentin labeling was identified in choroidal endothelial cells, choroidal melanocytes and occasional macrophages. The intensity of the IF labeling within a given tissue block was consistent from field to field across the retina. This was the case in normal as well as in detached or reattached specimens.

We have documented previously that, after experimental retinal detachments of several days duration, the most obvious histologic abnormalities are disruption of photoreceptor outer segments and mounding of the RPE apical surface.[15] In this study, comparisons between control sections and those from the 2-day detachment revealed significant changes in both Müller and RPE cells. Anti-vimentin labeling of Müller cells was heavier in the end-foot region and extended distally into the Müller cell cytoplasm within the outer nuclear layer (ONL) (Fig. 2A). Additionally, weak anti-vimentin labeling was present in the basal and lateral cytoplasm of the RPE (not shown). Anti-GFAP labeling in Müller cells was more extensive than in control specimens in cell processes at the end-foot, in the GCL, and throughout the INL. No labeling at more distal locations was identified (Fig. 2B).

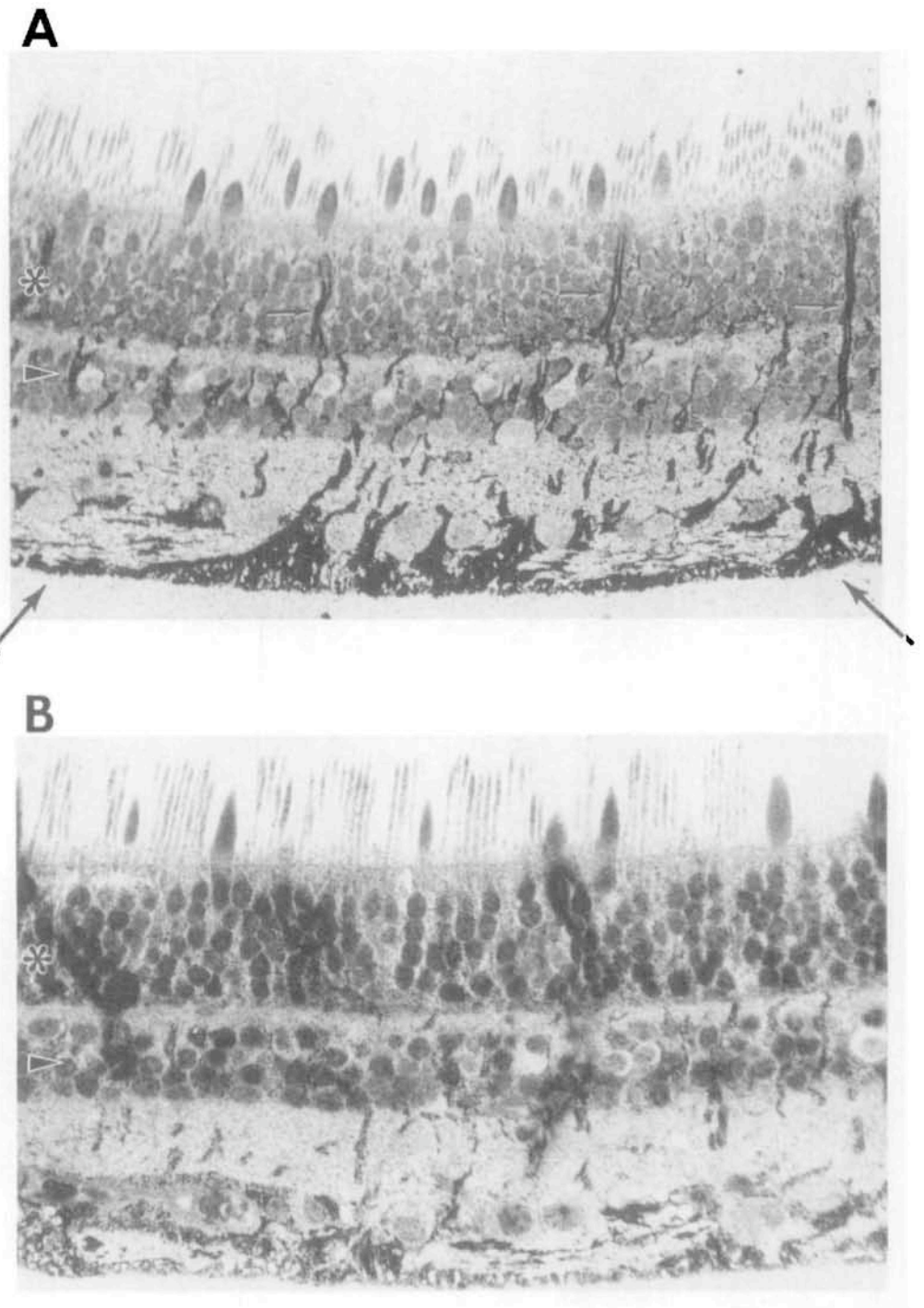In the retina detached for 7 days and then reattached for 7 days, regenerating photoreceptor outer

Fig. 2. Light micrographs of anti-vimentin and anti-GFAP immunolabeling in a monkey retina that had been detached 2 days previously. (**A**) Prominent anti-vimentin labeling is apparent in the region of the Müller cell endfeet (large arrows); somewhat lighter labeling appears throughout the INL (arrowhead) and in the ONL (asterisk). Formvar artifacts (small arrows). (**B**) The anti-GFAP pattern is similar, but the labeling intensity is less than it is with anti-vimentin. Arrowhead, INL; asterisk, ONL (×360).

segments regained about one third of their normal length and were reapposed to the RPE apical surface.[14] Heavy anti-GFAP and anti-vimentin labeling was present in Müller cell processes, extending from the inner limiting membrane through the IPL and occasionally into the OPL (Fig. 3A, B). In the retina detached for 7 days and reattached for 14 days, both anti-GFAP and anti-vimentin labeling extended throughout the Müller cell cytoplasm (Fig. 4A, B).

Immunoelectron microscopy showed that labeling occurred over dense clusters of 10-nm filaments (data not shown).

Although anti-vimentin labeling was absent in the normal RPE (Fig. 5A), the 7- and 14-day reattachments showed significant immunoreactivity, with a prominent concentration of Au particles in the basal (Fig. 5B) and basolateral (Fig. 5C) RPE cytoplasm at the 14-day time point. No labeling was present in the
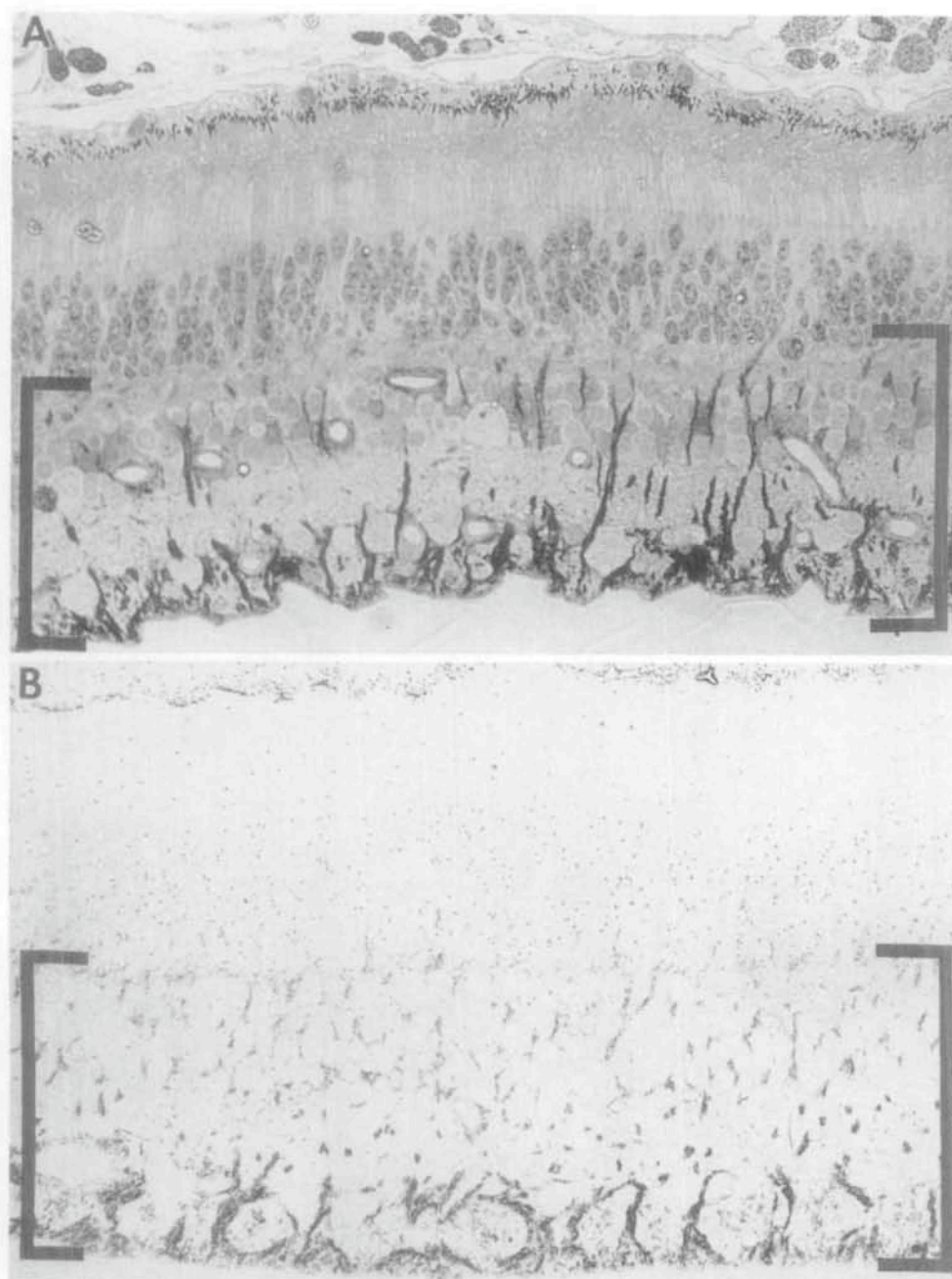
Fig. 3. Anti-vimentin and anti-GFAP labeling in a monkey whose retina was detached for 7 days, and subsequently reattached for 7 additional days. (A) In this stained section, anti-GFAP labeling is heavy from the Müller cell endfect to the OPL (brackets). (B) Anti-vimentin labeling in this unstained section is virtually identical to that seen in (A) (×300).

apical cytoplasm or in the cytoplasm of apical microvilli in any of the retinas.

In two animals whose retinas had been detached for 7 days and reattached for 150 days, anti-GFAP and anti-vimentin labeling was clearly less extensive in the outer retina than at either the 7- or 14-day reattachment time points. Fewer labeled processes were present in the ONL (Figs. 6A, B). In comparison to the normal pattern, however, differences still were apparent (Figs. 1A, B; 6A, B). No anti-vimentin labeling was detected in the RPE cytoplasm after long-term reattachment (Fig. 5D).

Discussion

In RPE cells, the amount and type of IFs show considerable interspecies variability. Cytokeratins—one class of IFs common in many epithelial cell types—are the most common class in mammalian RPE cells.[18] Cytokeratin and vimentin-containing IFs have been reported in guinea pig and bovine RPE in vivo,[18] whereas only vimentin-containing IFs have been identified in the chick.[18,19] Anti-vimentin immunoreactivity has been reported also in the RPE from human eyes with retinoblastoma or mela-
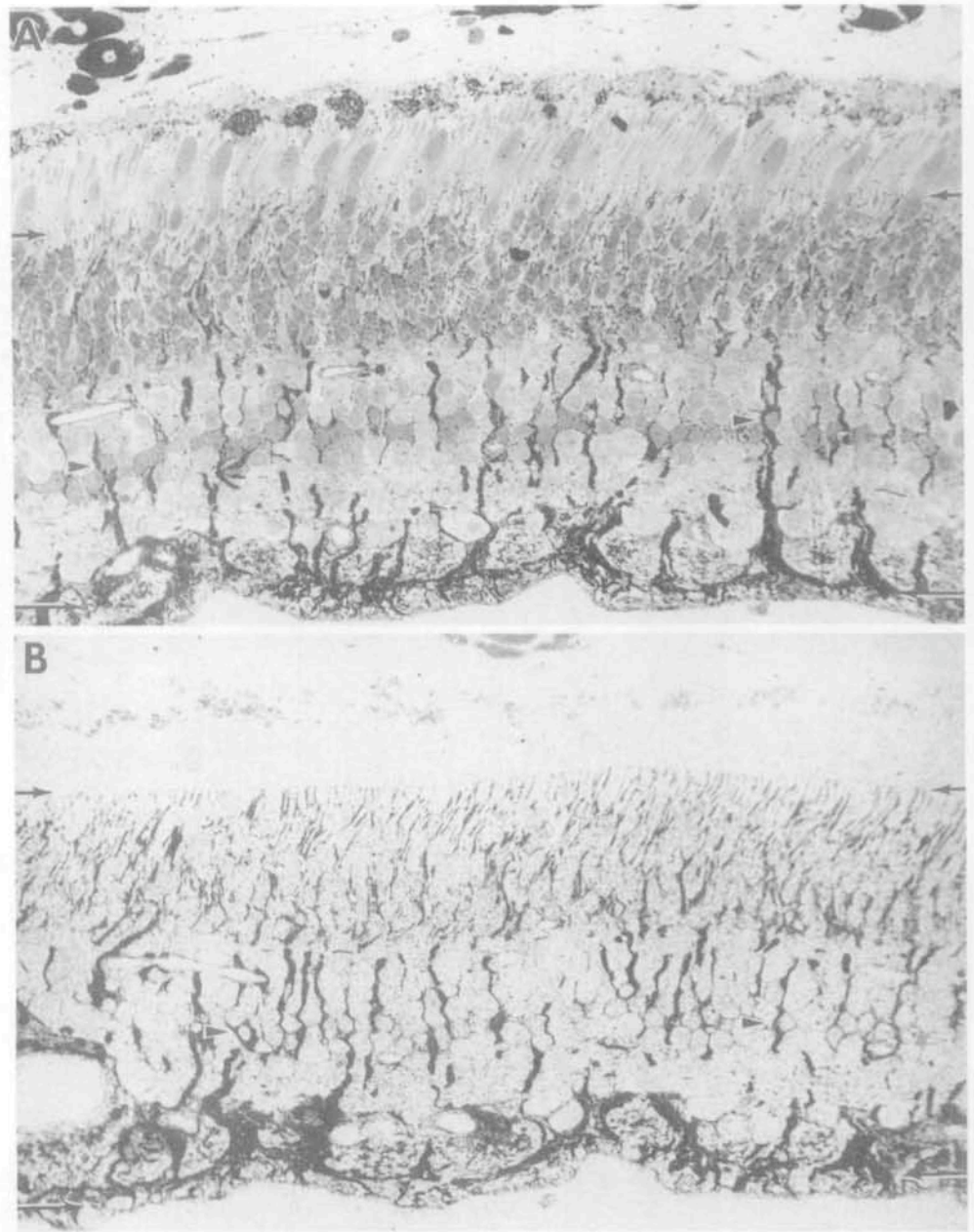
Fig. 4. Anti-vimentin and anti-GFAP labeling in a stained section (**A**) and unstaind section (**B**) of monkey retina detached for 7 days and then reattached for 14 additional days. Intense anti-vimentin (**A**) and anti-GFAP (**B**) labeling occurs throughout the Müller cell cytoplasm from the endfeet (long arrows) to the outer limiting membrane (short arrows). A number of Müller cell nuclei are outlined by the Müller cell processes (arrowheads) (×300).

noma.[20] This report presents the first evidence that vimentin, which is expressed by RPE cells of many mammalian species only in culture,[18] can also be expressed by rhesus monkey RPE cells in situ after retinal detachment. Thus, in retinal detachment, and perhaps in other types of retinal injuries or diseases, the RPE cytoskeleton apparently undergoes a modification(s) in IF expression that is similar to that in cultured RPE cells.[18,21,22] The functional significance of these modifications remains unknown. However, Owaribe et al[22] have noted that the same morphology, and presumably the same function(s), can be achieved by RPE cells when different combinations of IF and desmosomal proteins are used.

In the normal monkey retina, anti-GFAP and anti-vimentin immunoreactivity is restricted to Müller cell processes near the end-feet, within the GCL and the IPL. These results concur with those of other investigators, who have reported a similar labeling pattern in a number of different species.[4,11,12,17] In other systems, the appearance of vimentin-containing IFs coincides with the onset of mitotic events. For example, isolated metaphase spindles have been shown to be encased by a network of vimentin-containing IFs.[1] Vimentin mRNA levels have been shown to peak in the mid-G_1 portion of the cell cycle,[23] suggesting that an increase in vimentin expression may precede a cell's entry into S phase. In
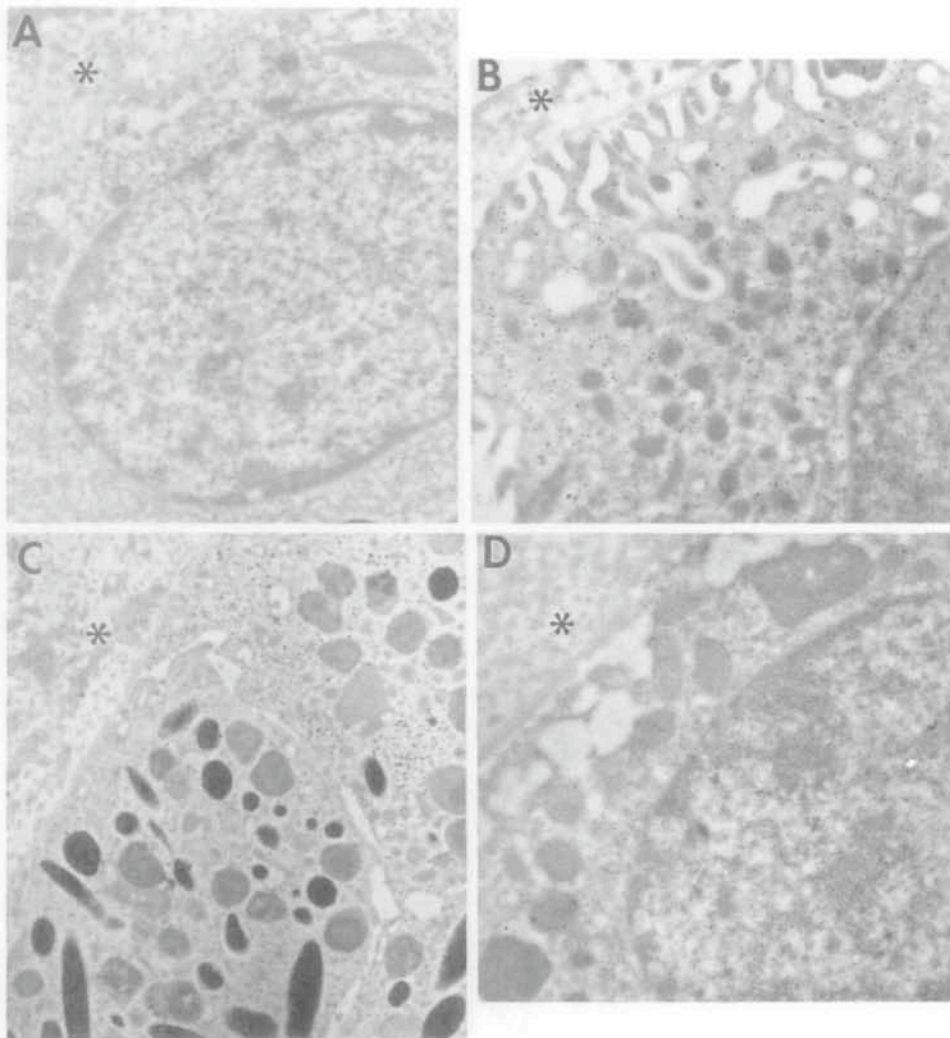
Fig. 5. Electron microscopic immunogold labeling of RPE cells with anti-vimentin. **(A)** Normal monkey RPE. No labeling above background is seen in the RPE cell cytoplasm (×10,500). **(B, C)** Monkey retina detached for 7 days and reattached for 14 days. Heavy labeling is present in the cytoplasm adjoining the basal **(B, C)** and lateral **(C)** cell surfaces **(B,** ×7,800; **C,** ×6,100) **(D)** Monkey RPE in a retina detached for 7 days and reattached for 150 days. Note the absence of labeling in the basal cytoplasm (×7,800). Asterisks, Bruch's membrane.

the feline retina, some Müller and RPE cells enter S phase within 24 hr after production of an experimental retinal detachment[15,24,25] but it is not known whether this proliferative response is preceded by increases in IF biosynthesis. GFAP levels and immunoreactivity in feline Müller cells are known to change after long-term retinal detachment (ie, 30–60 days).[11,12] In the current study we found similar changes in IF immunoreactivity in primate Müller and RPE cells within only 2 days after detachment. This data strongly suggests that corresponding increases in IF levels also occur in the primate retina within this time frame. In the absence of quantitative data, however, it remains possible that some of these changes reflect a redistribution of existing IFs rather than increased biosynthesis.

Experimental retinal detachment initiates a complex series of cellular changes in both RPE and Müller cells that includes proliferation, hypertrophy, nuclear translocation, and migration.[15,25] It is likely that the IF changes we have identified form one component of a complex array of molecular changes associated with one or more of these major cellular events. These changes may not be specific to detachment per se, but rather represent the cells' characteristic response to some alteration of their microenvironment. Perturbation of the retina, whether in response to experimental retinal detachment[11,12] or as a result of a penetrating ocular injury,[7] carcinoma,[20] light damage,[10] or inherited degeneration,[10] apparently is sufficient to trigger the onset of IF changes in RPE and Müller cells. Since vimentin mRNA levels are inducible by both platelet-derived growth factor and serum,[23] it is possible that these or other unidentified factors introduced from the circulation or secreted by ocular cells, rather than the injury or disease itself, may be the causative factor in triggering the IF response.

The major structural changes that occur after experimental retinal detachment have now been identified,[14,15,25,27] as have a few of their molecular correlates.[11-12] At least one of these changes, photoreceptor

Fig. 6. Anti-vimentin and anti-GFAP labeling in a monkey retina detached for 7 days and then reattached for 150 days. Anti-vimentin (A) and anti-GFAP (B) labeling intensity appears to be somewhat higher than in normal monkey retina (Fig. 1), but the number of labeled processes, particularly in the OPL, is lower than in the retinas reattached for 7 or 14 days (Figs. 3, 4). Labeling is heaviest in the region of the Müller cell endfeet (arrows) and is less intense in the INL (arrowheads) and OPL (asterisks) (×400).
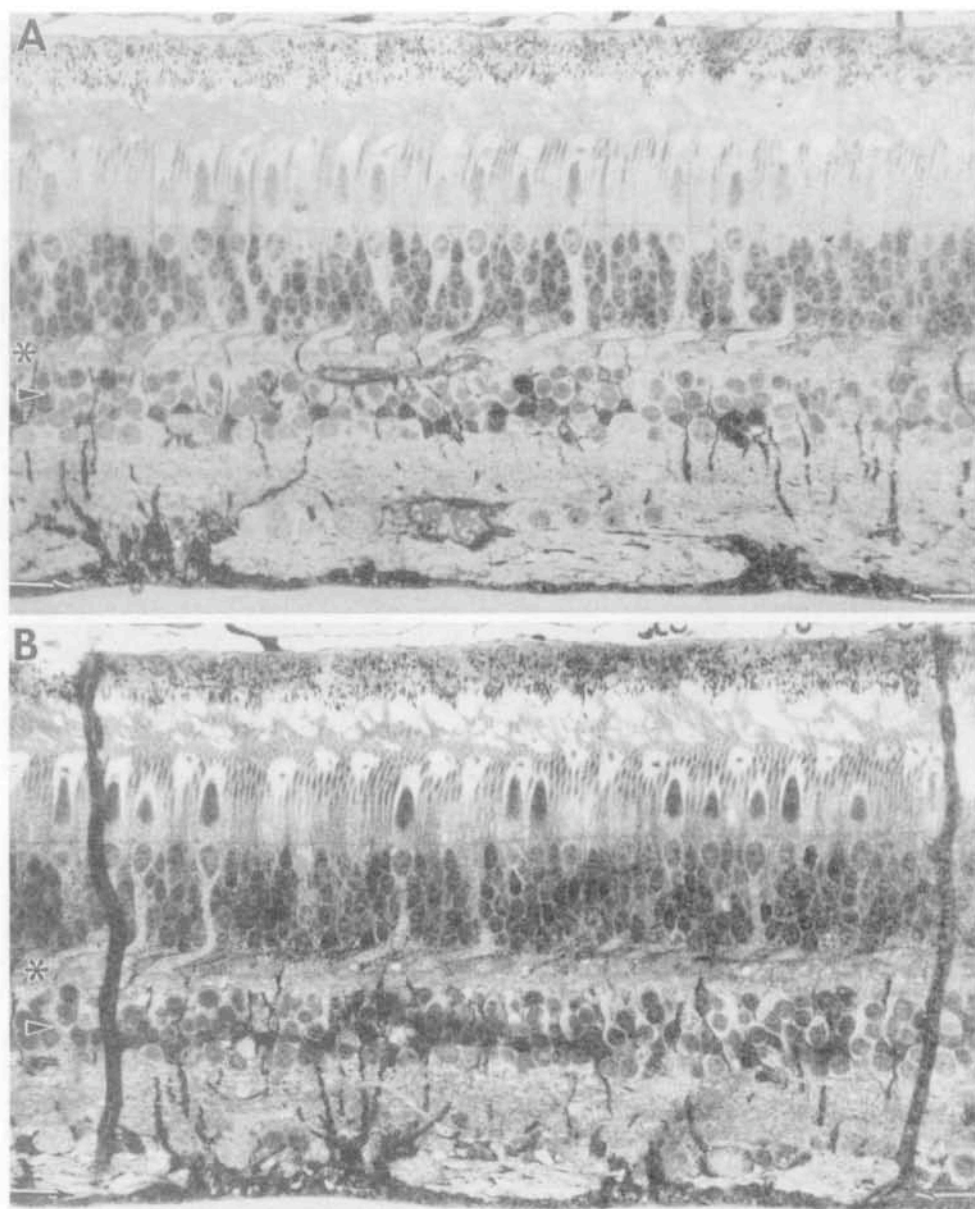
outer segment degeneration, can be halted and partially reversed if the retina is reattached promptly;[13,14,28] this is accompanied by at least partial visual recovery in many cases. However, virtually nothing is known about any other cellular or molecular aspects of the recovery process. In the two animals that were allowed to recover for 150 days after reattachment, the pattern of Müller IF immunolabeling remained abnormal. However, in comparison to earlier reattachment time points, the extent of labeling was less pronounced principally in the outer retina. Furthermore, in RPE cells anti-vimentin labeling was not detected at all. These results raise the possibility that the IF changes that occur in Müller and RPE cells after detachment are not sustained indefinitely, but may be attenuated by a prolonged period of re-

covery. Further clarification of this issue will provide additional insight into the retina's overall capacity for repair and recovery after injury.

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