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Blood-retinal barrier breakdown in retinitis pigmentosa: light and electron microscopic immunolocalization

S.A. Vinores, M. Küchle, N.L. Derevjanik, J.D. Henderer, J. Mahlow, W.R. Green and P.A. Campochiaro
Wilmer Ophthalmologic Institute, Johns Hopkins University, School of Medicine, Baltimore, MD, USA

Summary. Macular edema can contribute to visual loss in the retinitis pigmentosa (RP), but the sites and mechanism of blood-retinal barrier (BRB) breakdown leading to macular edema are not known. An understanding of the mechanisms involved could lead to the design of effective pharmacologic therapy to prevent or minimize macular edema in RP. To investigate this problem, immunohistochemical staining for albumin was performed on paraffin sections of 22 normal and 29 RP-affected eyes. Specimens were graded for extent of albumin extravasation in different regions of the retina, optic nerve head, ciliary body, and iris. Electron microscopic immunocytochemical staining for albumin was performed on an additional 6 normal and 9 RP-affected eyes. Two-thirds of the eyes from patients with RP and no other ocular disorders demonstrated extravascular albumin in the inner portion of the posterior retina. This was evident even in the absence of cystoid macular edema (CME), but eyes that had CME showed extensive BRB failure. In some cases, passage of albumin from the choroid to the retina was prevented even in the absence of the retinal pigment epithelium (RPE). Electron microscopic immunocytochemistry revealed that albumin permeated retinal vascular endothelial cells and RPE cells that showed degenerative changes in RP.

Key words: Retinitis pigmentosa, Blood-retinal barrier, Blood-brain barrier, Cystoid macular edema

Introduction

Retinitis pigmentosa (RP) is a hereditary disease affecting about 1 in 4,000 individuals in the general population. Its symptoms include impaired dark adaptation, night blindness, constricted visual fields, and late in the disease, decreased visual acuity (Berson, 1993). Decreased visual acuity can result from loss of photoreceptors in the macula or from cystoid macular edema (CME) (Fetkenhour et al., 1977; Fishman et al., 1977; Marshall and Heckenlively, 1988). The etiology of CME in RP is not understood. The present study was conducted to determine whether CME in RP shares the characteristics of CME from other causes, such as post-surgical or inflammatory macular edema, or whether its etiology is unique to RP, possibly resulting from degeneration or loss of function of the retinal pigment epithelium (RPE) related to the photoreceptor degeneration. Immunohistochemical staining for endogenous albumin has revealed extravasation sites within the retina for a number of ocular disorders (Vinores et al., 1989, 1990a, 1994). Immunolocalization of albumin at the ultrastructural level has also provided insight into the mechanism of blood-retinal barrier (BRB) compromise in human diabetics (Vinores et al., 1993a) and experimental animals (Vinores et al., 1990b, 1992, 1993b). A small number (4) of RP eyes were previously examined by light microscopic immunohistochemical staining for extravascular albumin (Vinores et al., 1990a); however, all but one of the cases had only minimal pathologic changes and the results were inconclusive. The present study was conducted to examine a larger number of eyes with various stages of RP utilizing light and electron microscopic immunolocalization techniques to better understand the sites and mechanism of BRB compromise in RP and to examine the possible correlation between BRB breakdown and blood-aqueous barrier breakdown.

Offprint requests to: Dr. Stanley A. Vinores, 825 Maumenee, Bldg., Department of Ophthalmology, Johns Hopkins University School of Medicine, 600 N. Wolfe St., Baltimore, MD 21287-9289, USA

Materials and methods

Paraffin sections of formalin-fixed normal eyes and eyes affected by RP were obtained postmortem and immunohistochemically stained for albumin using 4-chloro-1-naphthol as the chromogen to yield a blue-violet reaction product, which contrasts with melanin, as previously described (Vinores et al., 1990a). Ages and postmortem times, all of which were less than 16 hours, were comparable for normal and RP-affected eyes. For controls, normal rabbit serum was substituted for primary antiserum or the anti-albumin antiserum was

Table 1. Specimens used for electron microscopic immunocytochemistry.

RETINITIS PEGMENTOSA FOUNDATION NUMBER	SEX	AGE (yrs)	POST-MORTEM INTERVAL (hr)	FIXATIVE	LOCATION OF TISSUE
100	M	67	3	1% formaldehyde/2% glutaraldehyde	Just inferior to macula
105	M	69	5.7	2% paraformaldehyde	Just superior to macula
106	F	39	6.5	4% formaldehyde	Perimacular
125	M	53	2.5	1% formaldehyde/2% glutaraldehyde	Just inferior to macula
133	F	67	2.5	1% formaldehyde/2% glutaraldehyde	Perimacular
193	F	17	17	4% formaldehyde	Perimacular
231	M	56	3.25	1% formaldehyde/2.5% glutaraldehyde	Perimacular to mid-periphery - nasal quadrant
320	F	31	10	4% paraformaldehyde/0.5% glutaraldehyde	Macula
324	F	67	4.5	4% paraformaldehyde/0.5% glutaraldehyde	Macula to mid-periphery - superior quadrant

pre-absorbed with normal goat serum for 1 hr at 4 ºC (Vinores et al., 1994). RP eyes used for light microscopy were categorized according to the severity of the disease as follows: mild= only the peripheral retina anterior to the equator is affected (up to 30%); moderate= peripheral and mid-peripheral retina at the equator are affected (30-60%); marked= all photoreceptors are lost except for those in the posterior pole (60-95%) and there is marked migration of RPE cells into the retina; total= all photoreceptors are lost including those in the macula. Sections were graded for the extent of extravascular albumin in the anterior and posterior retina, optic nerve head (if present in the section), iris, and ciliary body as follows: 0= no extravascular albumin; +1= weak, focal positivity; +2= moderate positivity; +3= widespread extravascular albumin. The relative intensity of extravascular albumin positivity (as in Tables 4 and 5) was calculated by determining the means of the grades assigned (0, +1, +2, or +3) at a designated site for each case representing a particular disease category. Extravasated albumin localized between the inner and external limiting membranes was considered to be suggestive of a breach in the inner BRB (retinal vascular endothelium); albumin positivity within the RPE, in the subretinal space, or around the outer segments of the photoreceptors, was considered to signify a dysfunction of the outer BRB (RPE) since the external limiting membrane appears to act as a secondary barrier to contain extravasated albumin (Vinores et al., 1993a). At the blood-aqueous barrier, albumin positivity was graded in the iris stroma, at the anterior iris border, posterior to the iris pigment epithelium, and at the ciliary epithelium internal to the nonpigmented ciliary epithelium.

Specimens from eyes with advanced RP which were used for electron microscopic immunocytochemical staining for albumin and their corresponding pathology reports were provided by the RP Foundation Fighting Blindness (Baltimore, MD). Details of each specimen are provided in Table 1. Normal eyes (n=6), provided by the Old dominion Eye Bank (Richmond, VA) were also used for immunocytochemical staining for albumin for comparison. Immunocytochemical staining was performed after a 1 hr treatment with sodium borohydride to facilitate antibody penetration as previously described (Vinores et al., 1993a). Specimens were viewed with or without a uranyl acetate counterstain on a JEOL-100 CX electron microscope at 60 kV and photographed with 4489 Electron Microscope Film (Kodak, Rochester, NY).

Results

Light microscopy

In most sections of normal eyes, immunohistochemical staining for albumin was confined to the

Fig. 1. Immunohistochemical staining for albumin visualized by blue-violet reaction product. **a.** In normal retina, albumin immunoreactivity is limited to vascular lumens (arrowhead). **b.** A «stream» of albumin (arrowheads) appears to be emanating from the positively stained choroid (bottom) and entering the retina in a RP-affected eye (case 19). The inner retina is diffusely stained. **c.** Albumin appears to be contained within the choroid (bottom) despite the absence of the retinal pigment epithelium in this RP-affected eye (case 17). This patient did not present with cystoid macular edema. **d.** Diffuse albumin positivity was demonstrated throughout the inner retina, but appeared to be contained by the external limiting membrane (arrowheads) in this mildly affected eye from a patient with Cockayne's syndrome (case 15). Cystoid macular edema was not evident in this patient. **e.** Albumin positivity is visualized from the inner limiting membrane (arrow) to the external limiting membrane (arrowhead) and in the choroid (bottom) from an eye with a moderate degree of RP (case 12), but no staining is seen from the external limiting membrane to Bruch's membrane, suggesting that only the inner BRB has been compromised in this region. **f.** In another area of retina from the same case, diffuse albumin positivity is again seen throughout the inner retina and focal positivity is also seen within RPE cells (arrowheads), in the subretinal space, and surrounding the photoreceptor outer segments (arrow). **g.** Immunoreactive albumin is demonstrated within the vessel wall (arrowhead) in a case of marked RP (case 22). **h.** Albumin appears to be leaking from retinal vessels, that are partially surrounded by RPE cells, and permeating the inner retina in a RP-affected eye (case 19). **i.** Albumin appears to have leaked from this vessel in the optic nerve head of a RP-affected eye (case 4), even though no extra-vascular albumin positivity was demonstrated in the macula and the patient had no evidence of cystoid macular edema, optic nerve lies to the left. Immunoperoxidase using 4-chloro-1-napthol without counterstain. a,b,c,f,h, x 140; d,e,i, x 70; g, x 190

lumens of vessels (Fig. 1a) and to the choroid, where there is no blood-tissue barrier due to the fenestrated vascular endothelium. In 32% of the normal eyes, however, some relatively minor foci of extravascular albumin, which usually were associated with what appeared to be artifactual retinal detachments, were found in the subretinal space (Table 4). Sections from eyes of patients with RP with no other ocular disorders showed a comparable number of eyes with albumin positivity between the external limiting membrane and Bruch's membrane in the anterior and posterior retina, but the relative extent of positive staining in this area in the RP-affected eyes was greater (Tables 2, 4). When outer BRB compromise occurred, albumin could be immunolocalized in the subretinal space, surrounding the photoreceptor outer segments, or within the


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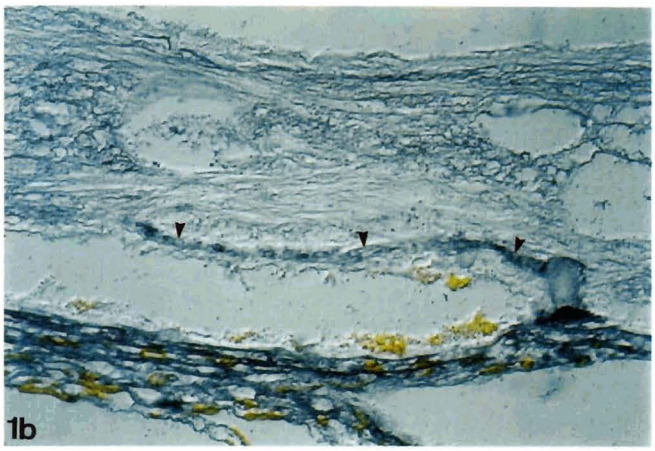
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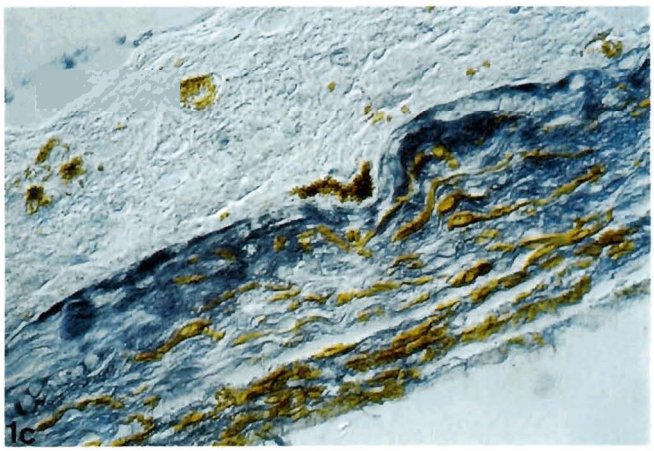
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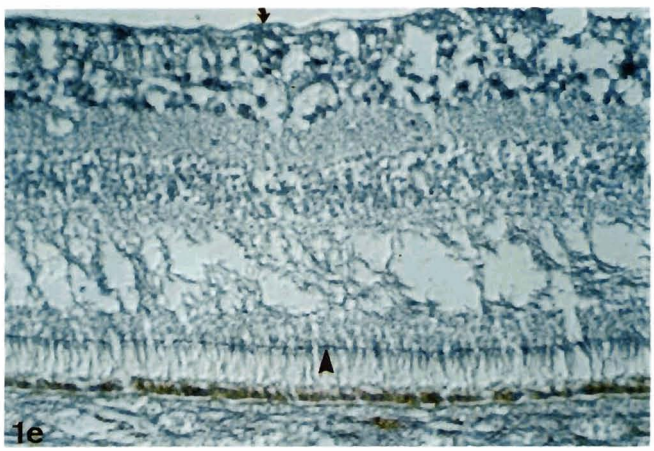
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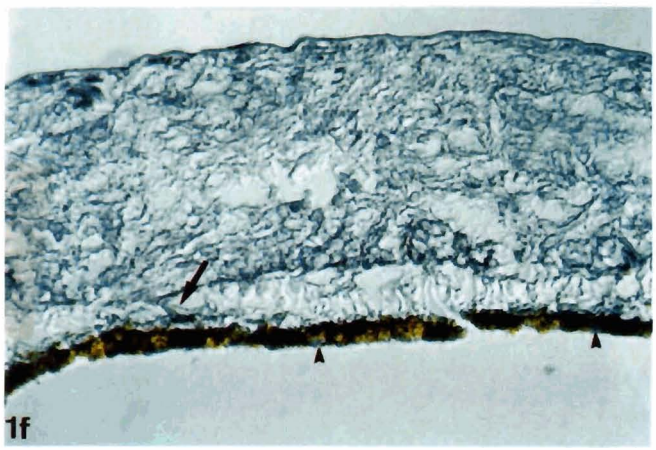
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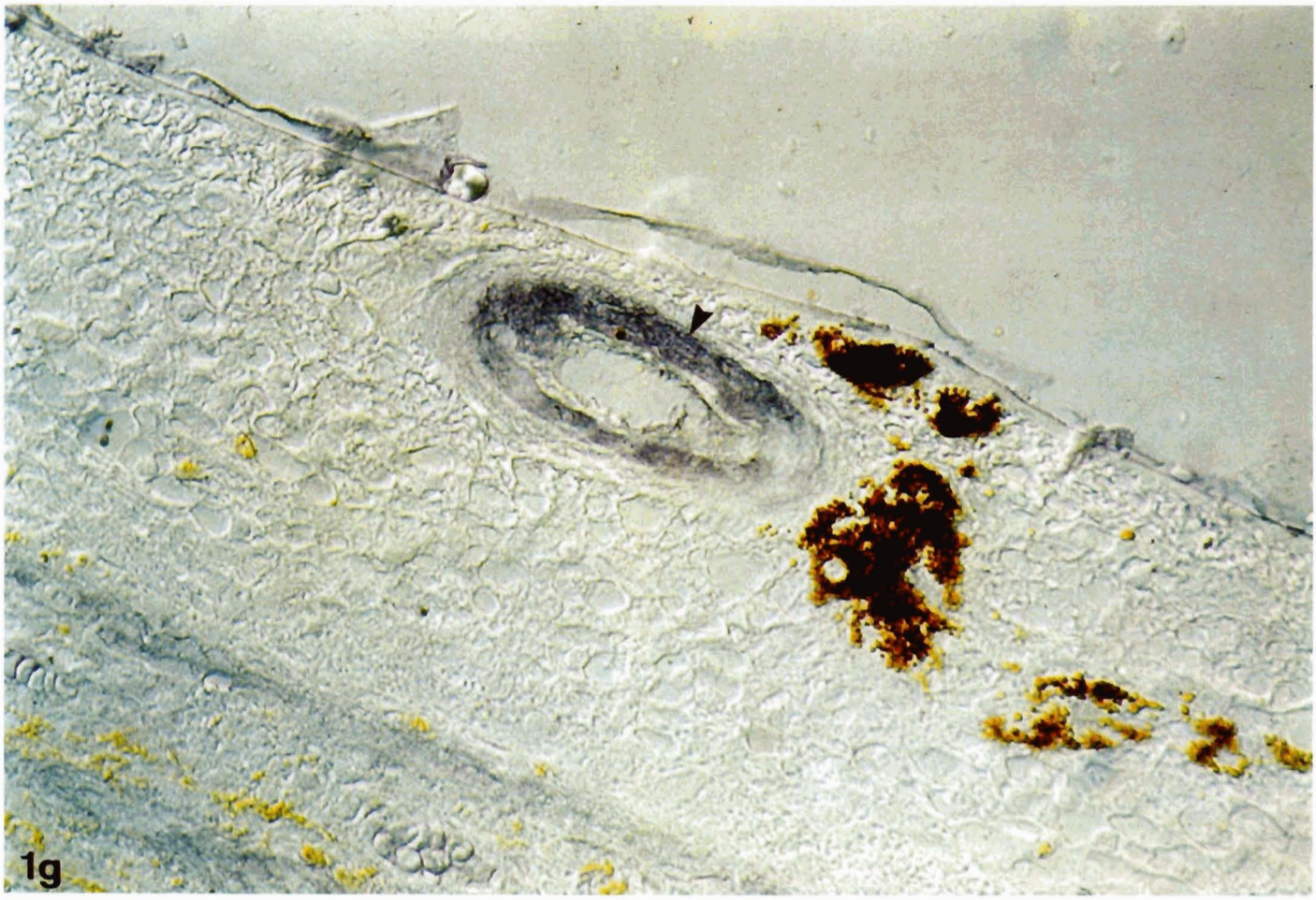
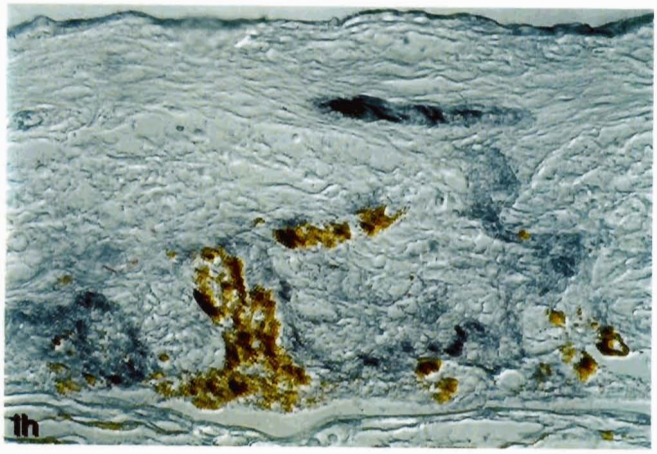
cytoplasm of the RPE cells. When other ocular disorders (described in Table 3) were involved in conjunction with RP, albumin positivity between the external limiting membrane and Bruch's membrane was demonstrated with greater incidence and extent in both the anterior and posterior retina (Tables 3, 4). In a few cases, albumin immunoreactivity suggestive of outer BRB compromise was widespread, but usually it suggested focal breaches in the BRB (Fig. 1b). Occasionally, albumin appeared to be contained within the choroid even in the absence of the RPE (Fig. 1c).

The most conspicuous difference in extravascular albumin positivity encountered when comparing normal eyes to RP eyes was in the inner retina. Among 22 normal eyes, only a single focus of extravascular albumin within the inner portion of the anterior retina





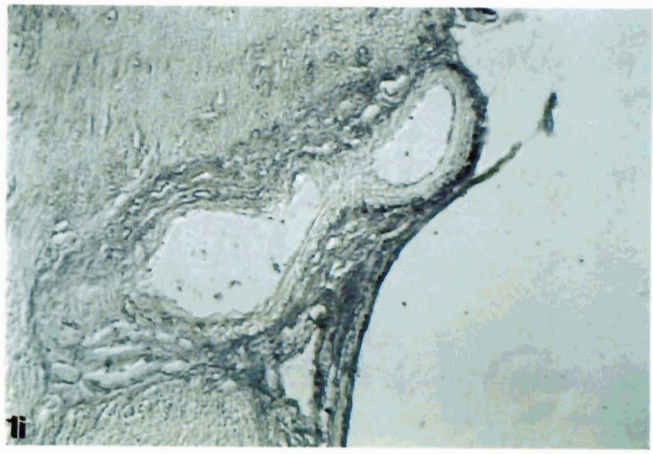


Table 2. Extravascular albuminn in retinitis pigmentosa with no ocular complications.

EYE #	SEVERITY OF DISEASE	CYSTOID MACULA EDEMA	POSTERIOR RETINA		ANTERIOR RETINA	
			Inner BRB	Outer BRB	Inner BRB	Outer BRB
1	Total	None	+2	+2	0	0
2	Total	Moderate	+3	0	+3	+3
3	Total	None	0	+2	0	+1
4	Total	None	0	0	+2	+1
5	Total	Mild	+2	+1	0	0
6	Marked	None	+2	0	0	0
7	Marked	None	+2	0	0	0
8	Moderate	None	+1	+1	0	0
9	Moderate	None	0	0	0	0
10	Moderate	None	0	0	0	0
11	Moderate	None	0	0	0	0
12	Moderate	Mild	+1	0	+2	+2
13	Moderate	Marked	+3	+3	+3	+2
14	Mild	None	+3	0	+3	0
15	Mild	None	+3	0	+3	0

Inner BRB designates extravascular albumin positivity between the inner limiting membrane and the external limiting membrane; outer BRB designates albumin positivity between the external limiting membrane and Bruch's membrane. Extravascular albumin staining: 0, negative; +1, weak, focal positivity; +2, moderate positivity; +3, widespread positivity.

Table 3. Extravascular albumin in retinitis pigmentosa with ocular complications.

EYE #	SEVERITY OF RETINITIS PIGMENTOSA	COMPLICATIONS	POSTERIOR RETINA		ANTERIOR RETINA	
			Inner BRB	Outer BRB	Inner BRB	Outer BRB
16	Total	Angle closure glaucoma, ischemic iris, necrosis, preretinal membrane	+1	+1	+1	+2
17	Total	Aphakia, glaucoma	+1	+1	+1	+1
18	Total	Aphakia, glaucoma	+2	+3	NA	NA
19	Total	Aphakia	+3	+3	+3	+3
20	Total	Aphakia, angle closure glaucoma	0	0	0	+1
21	Total	Aphakia	0	0	0	+1
22	Marked	Aphakia, intracapsular cataract extraction OU	+2	+1	+2	+1
23	Marked	Aphakia	+1	0	0	+2
24	Marked	Acute monocytic leukemia	+1	+1	NA	NA
25	Marked	Acute monocytic leukemia	+2	+2	NA	NA
26	Moderate	Autolysis OU, RP only in anterior retina	0	0	+1	0
27	Moderate	Pseudophakia, extracapsular cataract extraction/PCL	+2	+2	0	+2
28	Moderate	Aphakia, extracapsular cataract extraction, keratoplasty	+1	+1	+1	0
29	Mild	Autolysis OU, RP only in anterior retina	0	0	+1	+2

Inner BRB designates extravascular albumin positivity between the inner limiting membrane and the external limiting membrane; outer BRB designates albumin positivity between the external limiting membrane and Bruch's membrane. Extravascular albumin staining: 0, negative; +1, weak, focal positivity; +2, moderate positivity; +3, widespread positivity; NA, tissue was not available.

was seen in a single eye. In contrast, RP-affected eyes with no other ocular disorders demonstrated inner retinal positivity in the posterior retina of 67% and in the anterior retina of 40% of eyes; the positivity was sometimes widespread even in the absence of CME (Figs. 1d-f, Tables 2, 4). Some eyes appeared to show a simultaneous breakdown of the inner and outer BRB (Fig. 1f). There was no correlation between extent of photoreceptor degeneration and extent of albumin extravasation, but cases with CME had extensive albumin leakage. The involvement of other ocular complications increased the percentage of RP eyes with extravasated albumin. In some cases, albumin could be visualized within the walls of retinal vessels, demonstrating failure of the inner BRB (Fig. 1g). Some retinal vessels were partially surrounded by RPE cells

Table 4. Extravascular albumin in normal and retitnitis pigmentosa eyes. Percent showing positivity (relative extent of positivity).

DIAGNOSIS	n	POSTERIOR RETINA		ANTERIOR RETINA	
		Inner BRB	Outer BRB	Inner BRB	Outer BRB
Normal	22	0% (0)	32%(0.41)	4%(0.04)	32%(0.45)
Retinitis pigmentosa with no ocular complications	15	67% (1.47)	33% (0.60)	40% (1.07)	33% (0.60)
Retinitis pigmentosa with other ocular complications	14	79% (1.14)	64% (1.07)	67% (0.83)	75% (1.25)

Inner BRB designates extravascular albumin positivity between the inner limiting membrane and the external limiting membrane; outer BRB designates albumin positivity between the external limiting membrane and Bruch's membrane. Ocular complications are described in Table 3. The percent showing positivity indicates the percentage of eyes with extravascular albumin in a particular area. The relative extent of positivity is the mean of the values assigned to each case describing the extent of albumin extravasation in a particular area (individual values for retinitis pigmentosa-affected eyes are listed in Tables 2 and 3).

(Fig. 1h). Extravasation of albumin could also be demonstrated through the walls of large vessels in the optic nerve head, even in cases without CME and in which no extravascular albumin was detected in the macula (Fig. 1i). No significant compromise of the blood-aqueous barrier at the level of the iris or the ciliary body was noted in RP-affected eyes.

Electron Microscopy

Specimens from 9 cases of advanced RP were examined using electron microscopic immunocytochemical staining for albumin to localize sites of BRB breakdown. Six of these cases (100, 105, 106, 125, 133, and 324) demonstrated extravascular albumin in the inner retina, suggestive of inner BRB breakdown. Immunocytochemical staining was visualized in the intercellular spaces (Fig. 2a), within cell processes in the nerve fiber layer (Fig. 2b), and within the extracellular matrix surrounding retinal vessels, but in cases 100, 133 and 324 it was not clear how the albumin reached the abluminal side of the retinal vessels. In vessels with a functional BRB, albumin was demonstrated on the luminal surface, but not within the vascular endothelial cells or on the abluminal side of the vessels (Fig. 3a). In cases 105, 125, and 231, however, diffuse cytoplasmic positivity for albumin was demonstrated in the vascular endothelial cells of large (Fig. 3b) and small (Fig. 4) retinal vessels. Cytoplasmic positivity for albumin was also seen in RPE cells that showed degenerative changes in cases 105, 106, and 231 (Fig. 5). Some positivity was also seen within gliotic processes that bordered the RPE in case 231. In normal eyes, small foci of extravascular albumin were detected in the inner retina of 2 of 6 eyes and in the subretinal space of 1 of 6 eyes, but not within the retinal vascular endothelial cells or the RPE cells which comprise the BRB.

Table 5. Albumin extravasation relative to severity of retinitis pigmentosa with no ocular complications. Percent showing positivity (relative extent of positivity).

SEVERITY OF DISEASE	n	POSTERIOR RETINA		ANTERIOR RETINA	
		Inner BRB	Outer BRB	Inner BRB	Outer BRB
Marked to total	7	71% (1.57)	43% (0.71)	29% (0.71)	43% (0.71)
Mild to moderate	8	62% (1.38)	25% (0.50)	50% (1.38)	25% (0.50)

Inner BRB designates extravascular albumin positivity between the inner limiting membrane and the external limiting membrane; outer BRB designates albumin positivity between the external limiting membrane and Bruch's membrane. The percent showing positivity indicates the percentage of eyes with extravascular albumin in a particular area. The relative extent of positivity is the mean of the values assigned to each case describing the extent of albumin extravasation in a particular area (individual values for retinitis pigmentosa-affected eyes are listed in Tables 2 and 3).

Discussion

RP is sometimes accompanied by CME (Fetkenhour et al., 1977; Fishman et al., 1977), but the location and mechanism of BRB failure leading to CME is not understood. Immunolocalization of endogenous albumin has proven to be a useful technique for addressing this problem (Vinores et al., 1989, 1990a,b, 1992, 1993a,b, 1994). Vitreous fluorophotometry has shown a loss of BRB function even in the early stages of RP (Fishman et al., 1981; Prager et al., 1982). Fluorescein angiography has revealed defects in the RPE accompanied by diffuse leakage (Geltzer and Berson, 1969; Newsome, 1986; Spalton et al., 1987). Fluorescein leakage from perifoveal capillaries was detected in only a few cases (Newsome, 1986), but retinal vessels showed atrophy with loss of endothelium and invasion of the basement membrane by macrophages and glia in RP (Marshall and Heckenlively, 1988), which should account for some vascular leakage of proteins. The present study has revealed with greater resolution that can be achieved with fluorescein-based methods, that the predominant site of BRB failure in RP is the retinal vasculature (inner BRB) and that albumin extravasation occurs by permeation of both damaged RPE cells (outer BRB) and vascular endothelial cells. Since the primary source of CME in RP appears to be the inner BRB, as was found for CME due to other causes (Vinores et al., 1989, 1990a, 1994), and is not simply due to irreparable damage to the RPE, RP patients may be amenable to pharmacologic therapy to prevent or reduce CME.

Our findings support the observations of Fishman et al. (1989), who using fluorescein angiography in RP patients found leakage from both the retinal capillaries and the RPE. In five of six patients with angiographic

macular edema present for at least a decade, leakage through the RPE predominated. We do not have any information concerning the duration of CME in the patients in our study, but patients with more extensive RP changes tended to have more leakage through the RPE compared to patients with less extensive RP changes, while both groups had prominent leakage from the retinal capillaries. Interestingly, Fishman et al. (1994) found that patients treated with acetozolamide had improvement in leakage from the retinal capillaries, but little change in leakage from the RPE.

Albumin extravasation is not as pronounced in eyes with RP as it is in eyes with diabetic retinopathy, ocular infections, or ocular melanomas (Vinores et al., 1989, 1990a, 1993a, 1994), but it was demonstrated in all cases that had CME. Some eyes revealed little or no extravascular albumin even though they were in advanced stages of the disease, showing that photoreceptor loss did not correlate with BRB breakdown. Extravascular albumin was visualized in the retina and optic nerve head in some patients without any evidence of CME, indicating that this technique is capable of

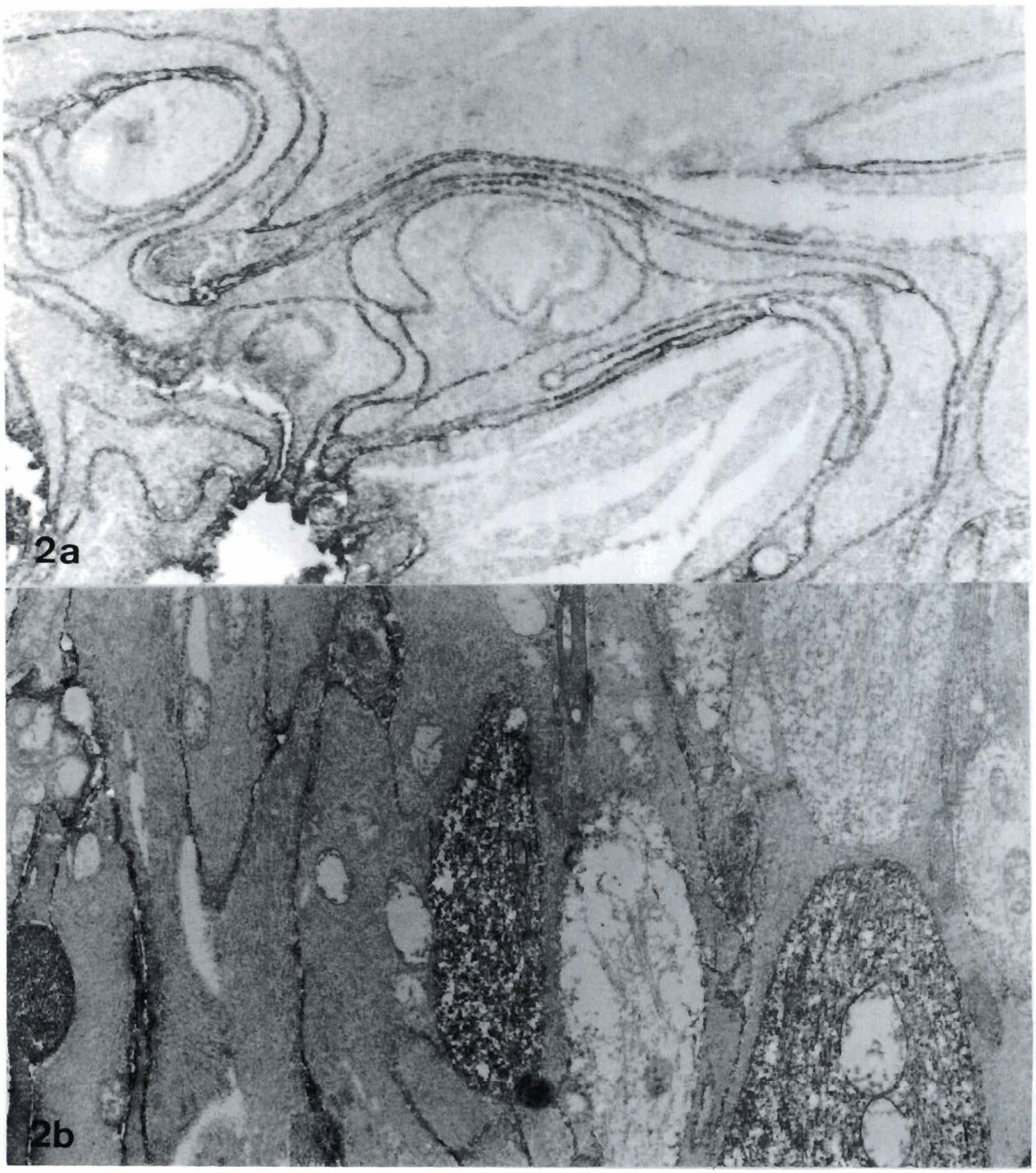


Fig. 2. Immunocytochemical staining for albumin. **a.** Immunoreactive albumin is demonstrated in the intercellular spaces between cell processes in the inner retina of case 231. Immunoperoxidase without counterstain. x 16,750 **b.** Albumin-positivity is demonstrated within cell processes (arrowheads), as well as in intercellular spaces in the nerve fiber layer of case 125. Immunoperoxidase counterstained with uranyl acetate. x 8,750

demonstrating breaches in the BRB in the absence of or prior to the development of CME.

RPE cells migrate into the retina in RP where they may surround vessels and re-establish polarity and junctional complexes (Li et al., 1995). It appears that these perivascular RPE cells may form a secondary barrier around leaky vessels and vessels not completely encircled by RPE cells may still leak on the sides unopposed by RPE cells. The external limiting membrane also acts as a secondary barrier to help contain albumin extravasated through either the inner BRB or the outer BRB in that respective portion of the retina, as was previously demonstrated (Vinores et al., 1993a).

In some areas there appeared to be a barrier impeding albumin from entering the retina from the choroid, even though the RPE (which normally constitutes the outer BRB) was absent in these areas. It is not clear whether the flow of albumin at these sites was restricted by changes that occur within the choriocapillaris or by gliotic processes in the absence of photoreceptors.

Aqueous flare measurements showed that the blood-aqueous barrier was also impaired in RP (Küchle et al..

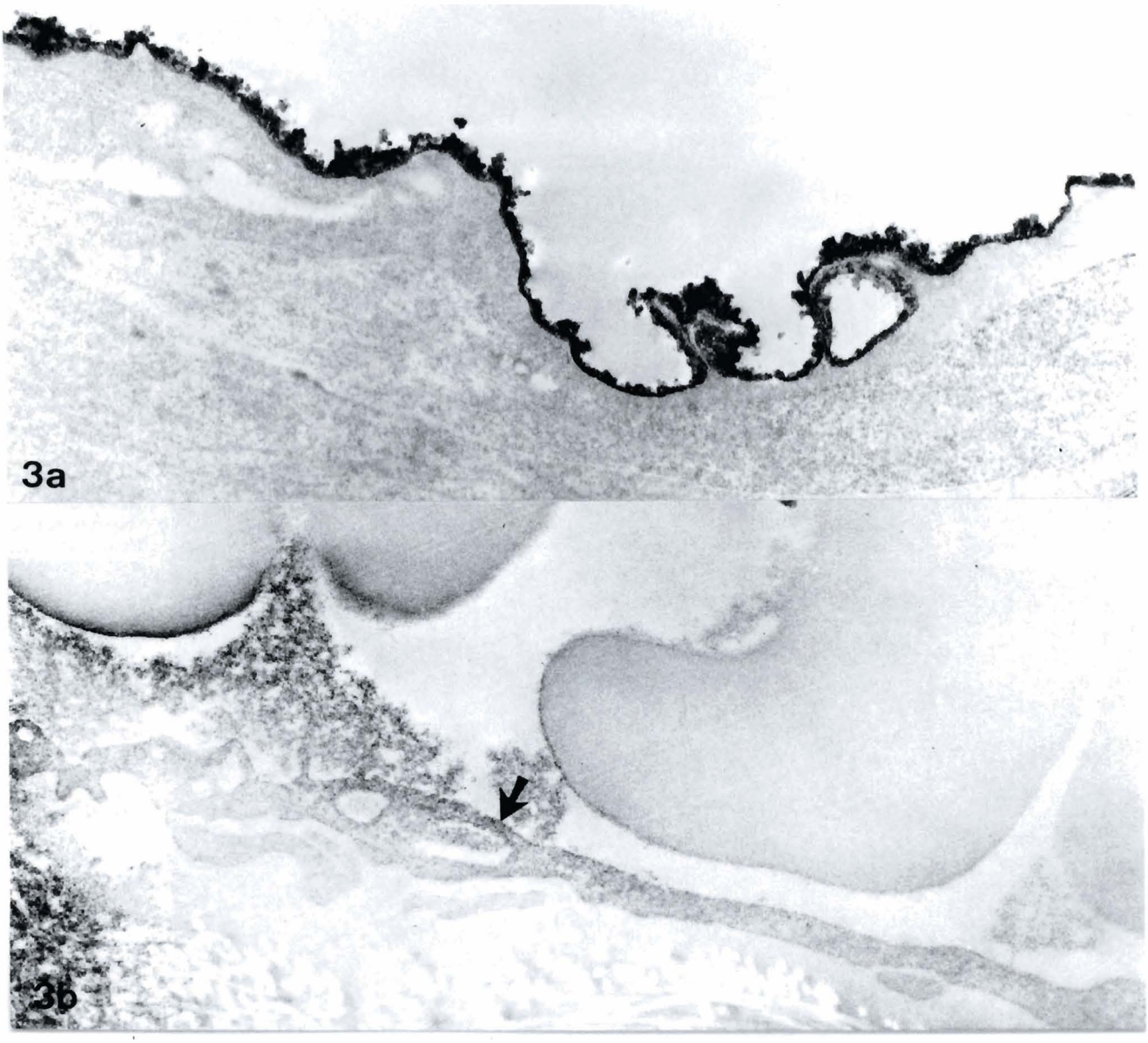


Fig. 3. Immunocytochemical staining for albumin. **a.** Albumin immunoreactivity is confined to the luminal surface of this retinal vessel from case 231, indicating an intact inner BRB at this site. Immunoperoxidase counterstained with uranyl acetate. x 17,500. **b.** In another retinal vessel from the same case, albumin-positivity is shown within the vessel lumen and in the cytoplasm of the vascular endothelial cells (arrow), indicating permeability of the vascular cell membrane to albumin. Immunoperoxidase without counterstain. x 20,000

1994), but significant difference in blood-aqueous barrier integrity from normal eyes was not demonstrated in the present study. The difference in the results of these two studies could be accounted for by differences in sensitivity of the two techniques, or possibly because only 4 of 15 cases of RP without other ocular disorders in the current study presented with CME. Alternatively, the source of the increased aqueous protein concentration in RP-affected eyes that was demonstrated by measurements of aqueous flare might be due to leakage from the peripheral retina rather than the iris or ciliary body.

In conclusion, BRB breakdown in RP is not entirely due to damage to the RPE. In fact, the predominant site of BRB compromise is not the RPE, but the retinal vasculature, as is true for post-surgical and inflammatory CME. Therefore, like post-surgical CME, the CME associated with RP may be amenable to pharmacologic therapy, as suggested by a beneficial effect from acetazolamide (Fishman et al., 1989) and methazol-

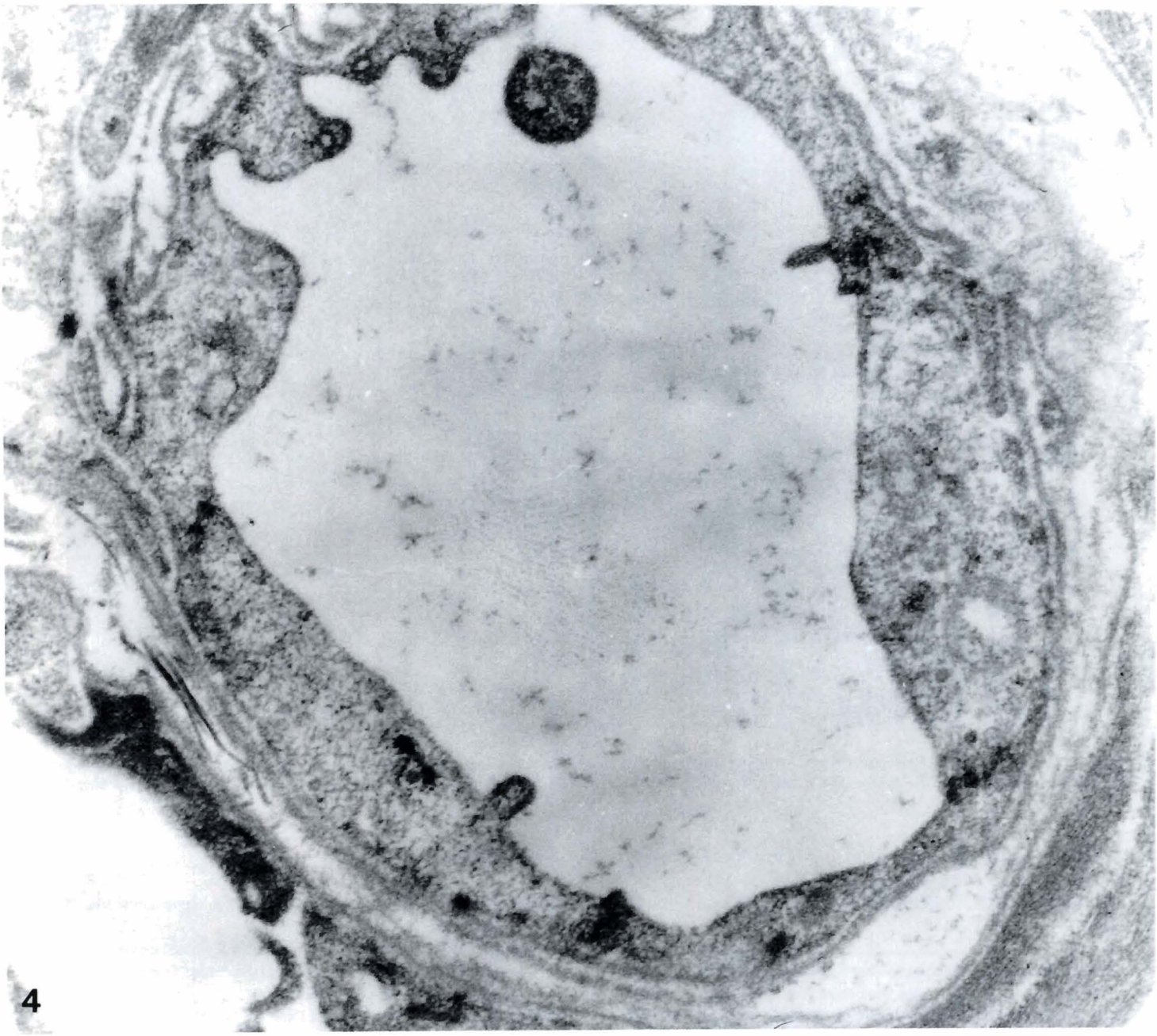


Fig. 4. Albumin immunoreactivity (granular reaction product) is demonstrated within the endothelial cells of this retinal capillary from case 105, indicating that the vascular endothelium has become permeable to albumin. Immunoperoxidase with uranyl acetate counterstain. x 35,000

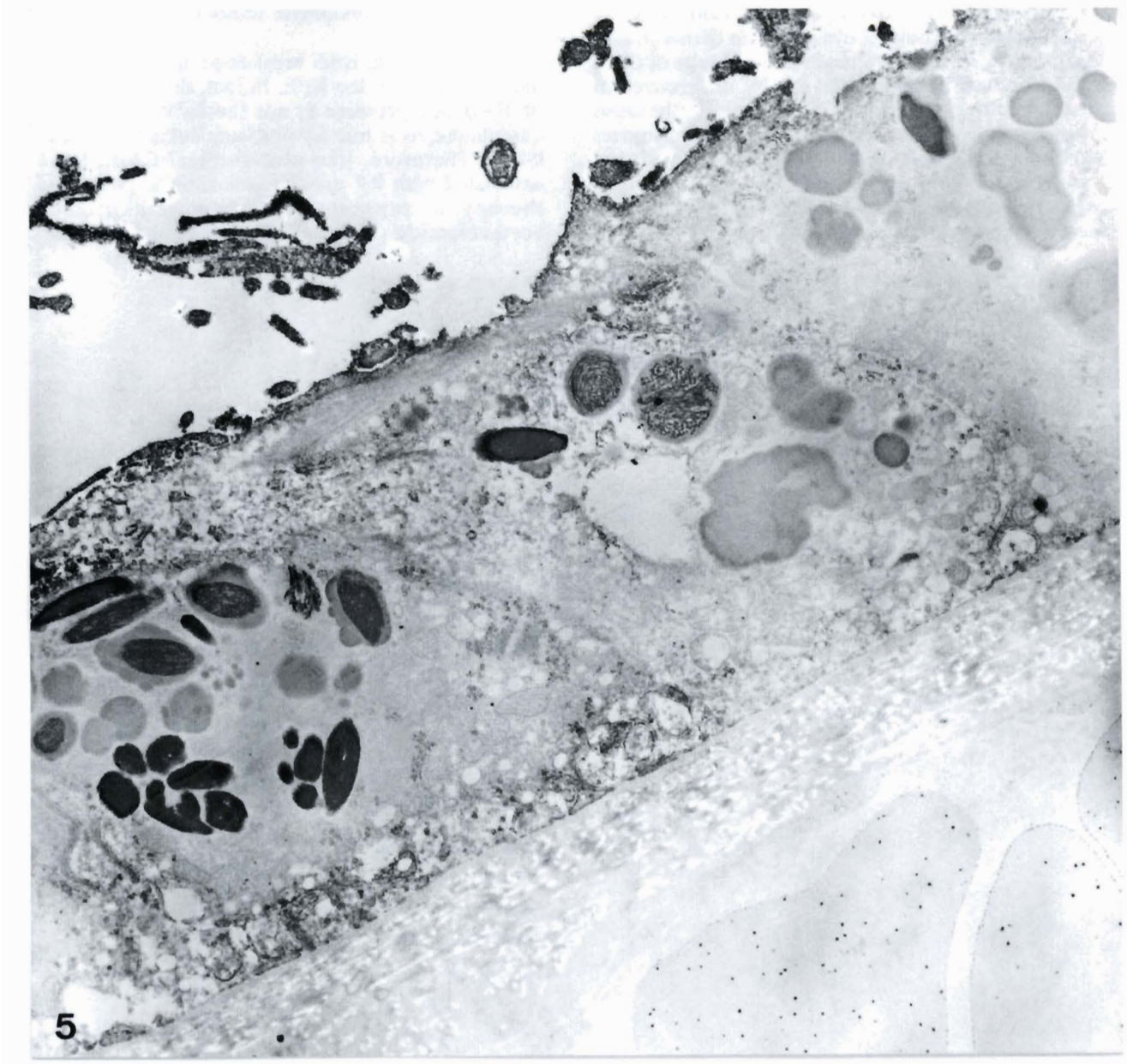


Fig. 5. Immunocytochemical staining for albumin (granular reaction product) shows that the protein permeates an RPE cell with degenerative changes (asterisk) from case 106. The adjacent RPE cell (lower right) remains impermeable to albumin. Immunoperoxidase counterstained with uranyl acetate. x 10,000

amide (Fishman et al., 1994). Future studies should be aimed at identifying possible soluble factors mediating BRB breakdown in RP to guide novel drug therapy.

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