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Immunohistochemical light and electron microscopy of basal laminar deposit

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Abstract The formation of basal laminar deposit (BLD) is one of the histopathologic changes in the aging human macula. BLD is assumed to be an early stage of age-related macular degeneration. The location of BLD, between the RPE plasma membrane and its basement membrane and in the outer collagenous zone of Bruch's membrane, and its ultrastructure suggest that it is composed of excessive amounts of basement membrane material. The main components of basement membranes are type IV collagen, heparan sulfate proteoglycans (HSPG) and laminin. Labeled antibodies against these components can therefore be used for the identification and localization of basement membrane material by means of immunohistochemical techniques. In this study the presence of type IV collagen, laminin and HSPG was determined in aged human maculae by immunohistochemistry and immunoelectron microscopy. Tests for the presence of type VI collagen and fibronectin were also performed. We obtained 76 eyes from 68 human subjects at autopsy or after surgical enucleation for anteriorly located choroidal melanomas. The finely granular component of BLD stained positive with antibodies against type IV collagen, HSPG and laminin, but the long-spacing collagen component of BLD did not. Neither component of BLD was stained with antibodies against type VI collagen or fibronectin. We conclude that BLD consists partly of excess basement membrane material.

Introduction

The formation of basal laminar deposit (BLD), one of the histologic changes in the aging human macula [19, 24, 25], is assumed to be an early stage in age-related macular degeneration (ARMD) [15, 18]. The presence of BLD is accompanied by a decrease in visual acuity [18]. The presence of BLD is accompanied by a decrease in visual acuity [18]. It precedes the atrophic as well as the exudative type of age-related macular degeneration [18].

The location of BLD between the RPE plasma membrane and its basement membrane and in the outer collagenous zone (OCZ) of Bruch's membrane, close to the basement membrane of the choriocapillaris, and its largely amorphous structure suggest that BLD is composed of relatively large amounts of basement membrane material. This might be produced by the RPE and the choriocapillaris endothelial cells [5, 24].

Ultrastructurally, BLD consists of two main components: banded material, called long-spacing collagen [10], and homogeneous, finely granular material with the same electron density as basement membranes [15, 23]. These two components can be intermixed in different ratios depending on the type of BLD. Ultrastructurally early-type BLD, often found in small patches, contains more finely granular material than long-spacing collagen. In the late-type BLD, which can be seen as a linear band between the RPE and Bruch's membrane,

the main component is long-spacing collagen [23]. A flocculent-type BLD, which is often seen in longstanding geographic atrophy, is also composed mainly of homogeneously staining material. It is called flocculent BLD because of the cumuliform or flocculent arrangement of this deposit when examined by light microscopy [19].

It has previously been suggested that under certain conditions long-spacing collagen, with a periodicity of 100–120 nm, can be formed through direct polymerization of basement membrane material [7, 13]. There are indications that these deposits, which accumulate in the region of the basement membranes, are a manifestation of a gradual degeneration of the associated cells [18].

The main components of the basement membranes of most tissues are type IV collagen, heparan sulfate proteoglycans (HSPG) and laminin [1, 2, 6]. Labeled antibodies against these components can therefore be used for the identification and localization of basement material by means of immunohistochemistry. Fibronectin, an important factor in cell adhesion [6, 14], is also found in basement membrane [5], and thus might also be one of the components of BLD.

Ultrastructural studies have revealed a lateral arrangement of the approximately 100-nm beaded filaments of type VI collagen [3, 4, 12]. This might be an alternative explanation for the formation of long-spacing collagen, the main component of late-type BLD.

The aim of this investigation was to determine whether or not BLD is composed of basement membrane material, as disclosed by the presence of type IV collagen, laminin or HSPG. We also tested for the presence of type VI collagen and fibronectin.

Materials and methods

We obtained 76 eyes from 68 human subjects at autopsy or after surgical enucleation for anteriorly located choroidal melanoma. Age at time of enucleation ranged from 29 to 95 years (mean 73, SD 20). Time between enucleation of the eye or death of the subject and fixation ranged from 0.5 to 10 h.

After enucleation the macula lutea was dissected from the globe and hemisectioned in the direction of the optic disc. One half was fixed in 4% v/v formaldehyde, pH 7.4, for 24 h at room temperature and embedded in paraffin for light microscopy and immunohistochemical studies. The other half of the macula was hemisectioned again. One part, without aldehyde fixation, was snap-frozen in isopentane at −70° C and subsequently stored in liquid nitrogen for immunohistochemical studies. The other part was fixed in 2% w/v paraformaldehyde, pH 7.4, for 1.5 h at room temperature and was either infiltrated with 2.3 *M* sucrose and frozen in liquid nitrogen for immunoelectron microscopy of ultrathin frozen sections or dehydrated with a graded series of ethanol and embedded in LR-white for immunoelectron microscopy on semithin and ultrathin plastic sections.

To determine the influence of fixation on immunoreactivity, one macula was divided into three parts. One part was fixed in 4% formaldehyde, one part in 2% paraformaldehyde and one part in a mixture of 4% formaldehyde and 1% glutaraldehyde (pH 7.4), for 2 h at 4° C.

To determine the influence of the duration of fixation on immunoreactivity, two maculae were each divided into six equal parts and all parts were simultaneously fixed in 2% paraformaldehyde at 4° C 1 h after enucleation. After 1, 1.5, 2, 2.5, 3, and 24 h, respectively, fixation was terminated and the tissue specimens were washed and stored until further processing in phosphate buffered saline (PBS), pH 7.2, at 4° C.

To determine the influence of time between enucleation of the eye or death of the subject and fixation of the macula on immunoreactivity, one macula was divided into four equal parts and stored at 4° C in a few drops of the vitreous fluid of the eye. Specimens were fixed in 2% paraformaldehyde at 4° C 1, 2, 3 and 24 h respectively, after enucleation. All tissue specimens were subsequently processed for immunohistochemical analysis.

Light microscopy

For immunohistochemical analysis of cryofixed macular tissue with alkaline phosphatase and peroxidase-labelled antibodies, cryostat sections (5 µm) were cut and mounted on glass slides coated with 3-aminopropyl triethoxy silane (AAS); Sigma, St. Louis, USA) and air dried. Cryostat sections were further treated as described for paraffin sections.

Paraffin section (6 µm) were mounted on glass slides and after deparaffinization and rehydration, endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 25 min at room temperature. After rinsing with water and PBS the sections were incubated with 0.1% pronase in PBS for 10 min at 37° C, pH 7.4. The reaction was stopped with cold PBS and the slides were washed several times with PBS at room temperature. Before treatment with pronase several sections were incubated for 30 min with either 6 *M* guanidine hydrochloride (Sigma) in 50 m*M* sodium acetate, pH 6.5, at room temperature or 0.1% sodium borohydride (Sigma) to restore immunoreactivity after aldehyde fixation [2, 11]. The slides were placed in a Sequenza immunostaining workstation (Shandon Scientific Ltd, Astmoor Runcorn, UK) and incubated with primary antibodies. These included rabbit anti-human collagen type IV (1:50 dilution; AKZO Organon Teknica, Boxtel, The Netherlands), mouse anti-human collagen type VI (1:20 dilution; Heyl, Berlin, Germany), rabbit anti-EHS mouse sarcoma laminin (1:50 dilution; AKZO Organon Teknica), rabbit anti-human fibronectin (1:1200 dilution; Dakopatts, Glostrup, Denmark) and mouse anti-human HSPG (1:2 dilution; Chemicon International, Temecula, USA). After washing and incubation with biotinylated secondary antibodies, sections were incubated with avidin-biotin-horseradish-peroxidase complex (1:1200 dilution; ABC, Dakopatts). Finally immunoreactivity was visualized with 0.02% 3,3′-diaminobenzidine (DAB) tetrahydrochloride (Fluka, Hilversum, The Netherlands) in PBS with 0.05% hydrogen peroxide. Other sections were used to visualize immunoreactivity with alkaline phosphatase-labeled secondary antibodies and a phosphatase substrate (Vector Laboratories Inc. Burlingame, USA). Sections from 24 maculae were stained with peroxidase and those from 44 maculae with alkaline phosphatase-labeled secondary antibodies. The sections were counterstained with Mayer's hematoxylin for 1 min. For negative controls. normal rabbit or mouse serum replaced the primary antibodies. Basement membranes of the capillaries of the retina and choriocapillaris served as internal positive controls. Antigen retrieval methods for formalin-fixed paraffin-embedded tissue [20] were tested, but were not suitable for macular tissue.

Electron microscopy

Macular tissue from three eyes was fixed with 2%, paraformaldehyde, pH 7.4, for 1.5 h at 4° C; the tissue was dehydrated with

50% ethanol for 2 × 1 h followed by 70% ethanol twice for 1 h and embedded in LR-White (TAAB, Berkshire, UK). The LR-White was polymerized at 50° C for 24 h [17, 21]. A piece of the similarly fixed and dehydrated maculae from five eyes were embedded in Lowicryl-K_4M (Balzers, Maarssen, The Netherlands). Polymerization was carried out under ultraviolet radiation at −35° C for 24 h and at room temperature for an additional 48 h. Semithin sections (1 μm) were cut with a glass knife and mounted on AAS-coated glass slides.

Colloidal gold immunolabelling on semithin plastic scections was performed as described below for ultrathin plastic sections, with an additional silver enhancement reaction (15 min, room temperature, in darkness) (Aurion, Wageningen, The Netherlands). These semithin sections were examined by reflection-contrast microscopy (Axioplan, Zeiss, Oberkochen, Germany) which allows detection of very delicate immunogold-silver staining.

Ultrathin sections (50–60 nm) were cut with a diamond knife and mounted on unfilmed 200-mesh copper grids. Immunostaining for electron microscopy was performed as previously described [8]. Sections were incubated with primary antibodies, diluted in PBS/BSA/glycine for 2 h at room temperature. Dilutions of primary antibodies of 1:10 and 1:20 were used for type IV collagen, laminin, HSPG and fibronectin. Transmission electron microscopy was performed (Zeiss TEM 902) and electron micrographs were made on sheet film (Kodak SO 163, Rochester, New York, USA).

For immunoelectron microscopy of frozen sections, tissue was first fixed with 2% w/v paraformaldehyde, pH 7.4, for 1.5 h at 4° C and subsequently infiltrated with 2.3 *M* sucrose for 1 h. Ultrathin frozen sections were prepared on a cryostat (Cryo-Nova, LKB, Stockholm, Sweden) with a glass knife and mounted on formvar-filmed, 200-mesh copper grids. Immunoreactions were performed as previously described [8], although incubation times were shortened to 1 h and blocking of background staining with normal goat serum was omitted. The basement membranes of the choriocapillaris and the capillaries in the retina served as positive internal controls.

Results

The results of the immunohistochemical analyses are summarized in Table 1.

The light microscopy results of immunostaining cryostat sections with antibodies against type IV collagen, laminin, fibronectin and HSPG revealed specific staining of the capillaries of the retina and choriocapillaris and the choroidal vasculature. Bruch's membrane, the RPE basement membrane and drusen were not stained. In places BLD was faintly positive for type IV collagen and laminin. Treatment of the cryostat sections with pronase prior to immunostaining did not affect the results.

Immunohistochemical studies on paraffin sections, after pretreatment with pronase, showed that the basement membranes of the RPE and the choriocapillaris, as well as the retinal and choroidal vessels were intensely positive for type IV collagen and laminin (Fig. 1). BLD was not positive for either type IV collagen or laminin except for a thin layer directly adjacent to the RPE plasma membrane. This positive immunoreaction was exhibited by both flocculent BLD (Fig. 2a) and a thin layer of BLD (Fig. 2b). Hard and soft drusen were not positive (Figs. 1 and 2).

Immunostaining for type VI collagen on frozen as well as on paraffin sections revealed no immunoreactivity in BLD, the basement membrane of the RPE, hard drusen or Bruch's membrane (Fig. 1c). For fibronectin, immunoreactivity was diffuse in Bruch's membrane (Fig. 1d), and negative in BLD and hard drusen. Pretreatment of the deparaffinized and rehydrated sections with guanidin hydrochloride or sodium borohydride did not further enhance the immunoreactivity.

Semithin sections of LR-White-embedded tissue examined by the immunogold-silver technique using reflection-contrast light microscopy showed that the BLD and the basement membranes of the RPE and the choriocapillaris were positive for type IV collagen and laminin (Fig. 3). Drusen did not stain.

Immunoelectron microscopy of ultrathin frozen sections and ultrathin sections of LR-White-embedded tissue showed intense type IV collagen specific staining of the basement membrane of the choriocapillaris and only very weak staining of the basement membrane of the RPE in the same section (Fig. 4). Non-specific background staining was virtually absent. The staining pat-

Table 1 Results of immunohistochemical analysis of human maculae examined by light and electron microscopy (*LM/EM*) *BLD* basal laminar deposit, *B-RPE* basement membrane of the retinal pigment epithelium, *B-CC* basement membrane of the choriocapillaris, *Ch* choroid, *BM* Bruch's membrane, ret.cap. retinal capillaries

	BLD LM/EM	B-RPE LM/EM	B-CC LM/EM	drusen LM/EM	ret.cap. LM/EM	BM LM/EM	Ch LM/EM
Type IV collagen	++[a]/+[b]	++/±	++/++	−/×	+/×	−/−	++/×
Laminin	+[a]/±	++/±	++/+	−/×	+/×	−/−	++/×
HSPG	++[a]/×	−/×	±/×	−/×	++/×	−/×	+/×
Fibronectin	−/−	±/−	±/−	−/×	× /×	++/+	+/×
Type VI collagen	−/×	−/×	±/×	−/×	×/×	−/×	±/×

[a] Early type and flocculent BLD
[b] Homogeneous component only
++, strongly positive; + positive; ±, faintly positive; –, negative; ×, no results available

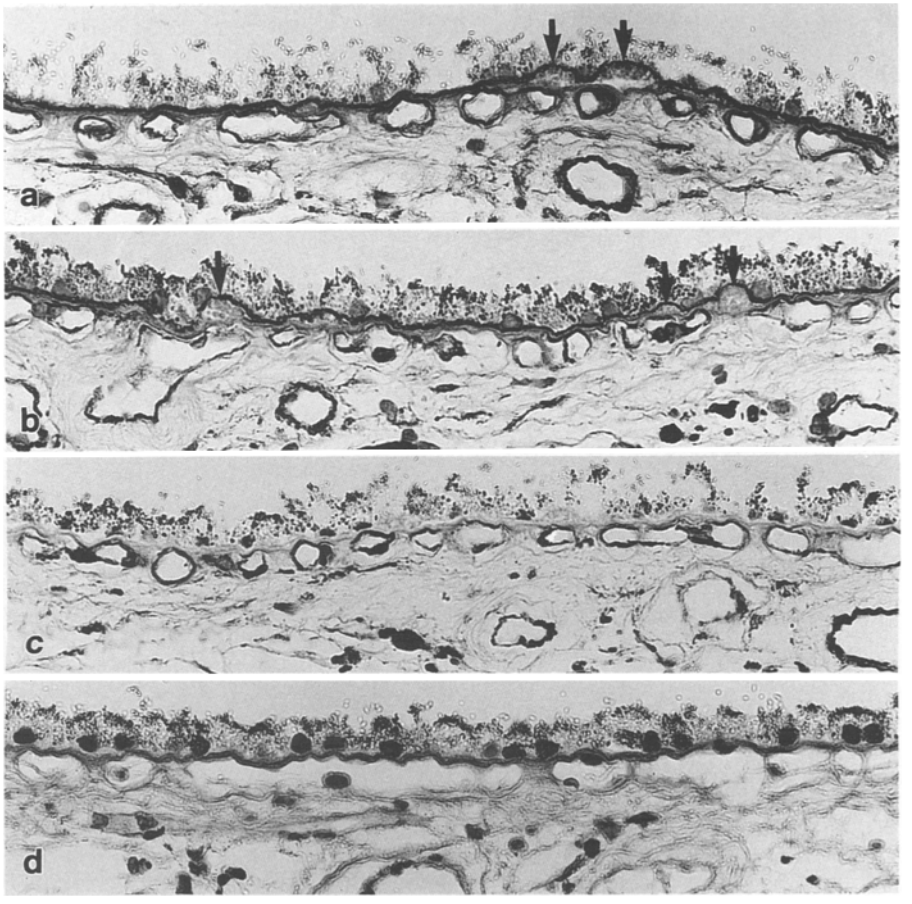

Fig. 1a–d Immunohistochemical analysis of paraffin sections of a human macula. **a** antibodies against type IV collagen. The basement membranes of the RPE and the vessels of the choriocapillaris and choroid are stained. Notice the hard drusen between the basement membrane of the RPE and the inner collagenous zone of Bruch's membrane, which show no immunoreactivity (*arrows*). **b** Antibodies against laminin. Positive staining of the vascular and RPE basement membranes can be seen. Several unstained hard drusen are present (*arrows*). **c** Antibodies against type VI collagen. Only the basement membranes of the capillaries are positive. **d** Antibodies against fibronectin. The inner and outer collagenous zones of Bruch's membrane are strongly positive. Basement membranes are faintly positive. (Immunoperoxidase staining; ×400)

tern for laminin was similar but less intense. Antibodies against fibronectin produced only diffuse staining of Bruch's membrane. The long-spacing collagen component of BLD between the RPE plasma membrane and the RPE basement membrane and in the outer collagenous zone of Bruch's membrane did not exhibit positivity for type IV collagen, laminin (Figs. 4 and 5) or fibronectin. However, the homogeneous material close to the basement membrane of the choriocapillaris was positive for type IV collagen and laminin (Figs. 4 and 5).

Fixation in 2% paraformaldehyde preserved immunoreactivity best, but this mild fixation resulted in a loss of preservation of the ultrastructure. A fixation time of 1.5–2 h ensured an acceptable ultrastructure visualization for 1 mm^3 cubes of tissue and optimum immunoreactivity. With increasing time between enucleation of the eye and fixation of the macula only a slight reduction in immunoreactivity was seen. Short fixation delays yielded the best results, but even after storage for 24 h at 4° C, immunoreactivity was still acceptable.

Discussion

The localization and ultrastructure of BLD suggest that it is composed of basement membrane material, which is probably produced by the RPE and/or endothelial cells from the choriocapillaris due to either an increase in age or degenerative changes in the macula [23]. However, definite proof that basement membrane components are involved has not been reported. It is known that various basement membranes differ in composition [2, 16], but all basement membranes contain type IV collagen, HSPG and laminin [1, 22]. Therefore positive staining for these components would be expected in the BLD. Immunoreactivity was best with short prefixation times (a maximum of about 4–6 h) and mild fixation such as 2% paraformaldehyde or 4% formaldehyde for short periods, the optimum being 1.5–2 h.

Demasking techniques, such as antigen retrieval with guanidine hydrochloride or sodium borohydride, were not successful, probably owing to the use of very mild fixation. Partial digestion of proteinaceous material in paraffin sections with pronase gave a remarkable improvement in specific immunostaining without an increase in background staining.

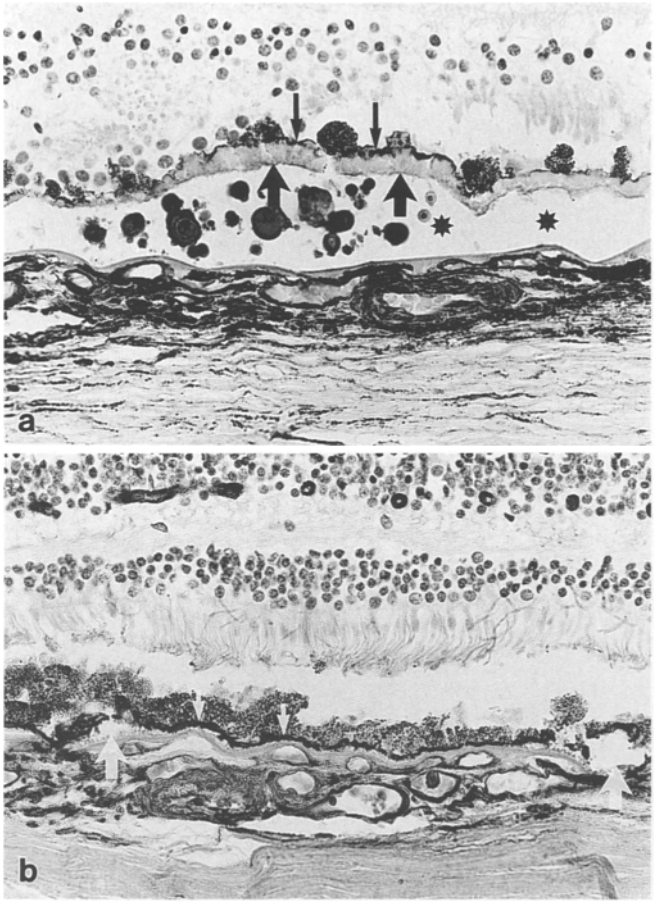

Fig. 2a, b Paraffin section of a human macula. **a** with a flocculent BLD (*large arrows*) overlying soft drusen (*asterisks*) with calcifications, stained with antibodies against type IV collagen. Note that only a small rim of the BLD adjacent to the RPE cells is stained (*small arrows*). **b** Same section of the macula, just outside the fovea, stained with antibodies against laminin. A thin layer of a late type BLD is stained (*small white arrows*). Two hard drusen (*large white arrows*) are not stained. (Immunoperoxidase; ×284

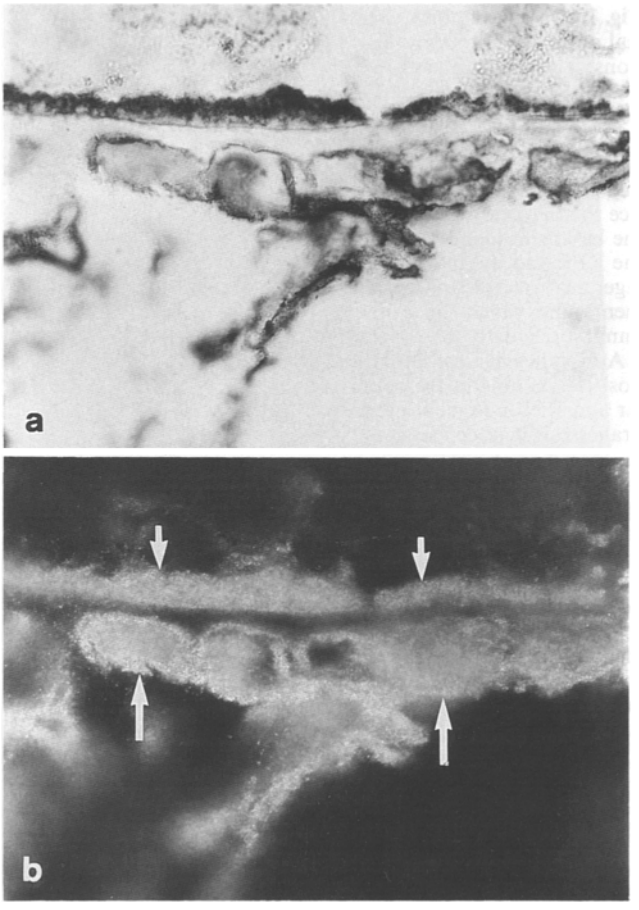

Fig. 3a, b LR-White (1 μm) section of a human macula, stained with antibodies against type IV collagen labelled with 10 nm colloidal gold particles with silver enhancement. **a** Normal light microscopy. The part of the BLD closest to the RPE cells is the most densely stained (*arrows*). **b** Reflection-contrast microscopy of same section shows staining of a large portion of a late type BLD (*small white arrows*) and the choriocapillaris (*large white arrows*). (×630)

The results of our study indicate that the basement membranes of the choriocapillaris and the RPE differ in composition, because staining for type IV collagen and laminin was intensely positive in the basement membrane of the choriocapillaris and relatively weak in the RPE basement membrane in the same sections [2, 8].

Staining of BLD for type IV collagen and laminin seemed to vary with the type of BLD and with the technique used to detect the antigen-antibody reaction. The results of immunostaining of cryosections and paraffin sections prepared for light microscopy were variable for type IV collagen and laminin but after a prior demasking treatment of paraffin sections with pronase, small portions of an early-type BLD, as well as that part of a flocculent BLD in close contact with the RPE cells, were clearly positively. Morphologically both early-type BLD and flocculent BLD were composed mainly of homogeneously stained material, which ultrastructurally resembled basement membrane material [19]. If it is assumed that BLD is deposited by the RPE cells, then the immunoreactive edge of flocculent BLD adjacent to the RPE cells suggests that configurational changes in the antigens, which occur after deposition, reduce or abolish immunoreactivity. The late-type BLD, which ultrastructurally consists mainly of long-spacing collagen, was positive only when the immunogold-silver technique was used. This suggests that in this material a lower concentration of type IV collagen-reactive material was present or that the antigens were partially not recognized by antibodies against type IV collagen.

With immunoelectron microscopy positive staining for type IV collagen of the homogeneous component of

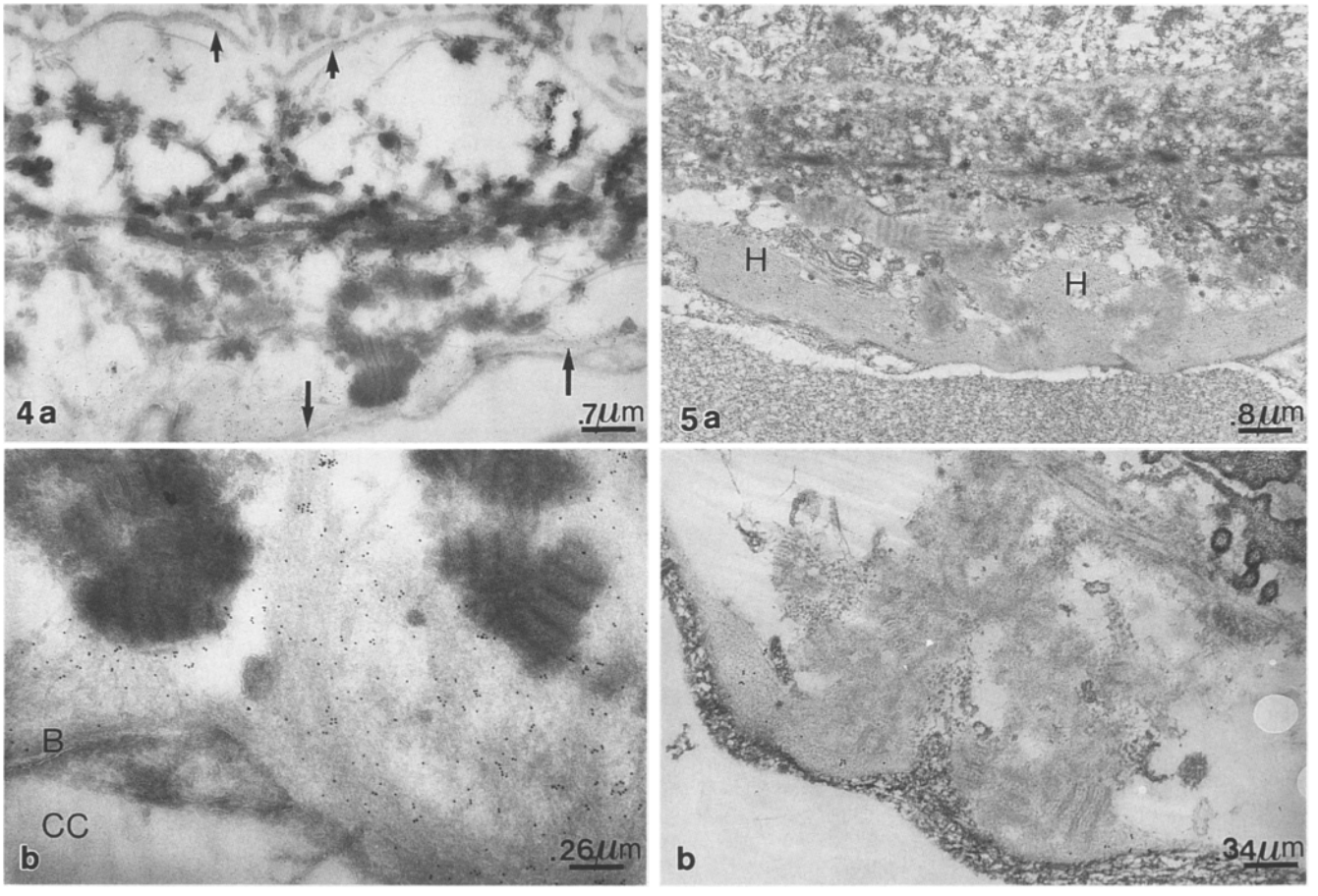

Fig. 4a, b Electron micrograph of ultrathin frozen sections of a human macula, stained with antibodies against type IV collagen labeled with 10 nm colloidal gold particles. **a** The RPE basement membrane is lightly stained (*short arrows*) in contrast to the choriocapillaris basement membrane (*long arrows*). The long-spacing collagen component of BLD in the outer collagenous zone of Bruch's membrane is not stained. **b** Higher magnification of the basement membrane (*B*) of the choriocapillaris (*CC*) and the outer collagenous zone of Bruch's membrane with BLD. Notice the labeling of the homogeneous material. The long-spacing collagen is not stained

Fig. 5a, b LR White (50 nm) sections of a human macula with BLD in the outer collagenous zone of Bruch's membrane. **a** The homogeneous materials (*H*) is positive for type IV collagen, but the long-spacing collagen is not stained. **b** Similar labeling is seen with antibodies against laminin

BLD, close to the basement membrane of the choriocapillaris, was confirmed. The long-spacing collagen component remained unstained, as has been described previously [9]. This could explain the diffuse weak staining of late-type BLD achieved with the immunogold-silver technique, as seen by light microscopy, as being due to the relatively small amount of homogeneous component dispersed between the long-spacing collagen.

Immunostaining of all types of BLD for type VI collagen was negative. Thus the hypothesis that BLD could be composed of laterally arranged 100-nm beaded type VI collagen filaments, which would then form long-spacing collagen, is unlikely. Fibronectin, a substance which is important in cell adhesion, also was not a component of BLD:

We can conclude that the homogeneously stained component of BLD, which ultrastructurally resembles basement membrane material, contains type IV collagen, HSPG and laminin and is thus probably composed of excess basement membrane material derived from the RPE and the choriocapillaris. The long-spacing collagen component of BLD did not stain. This is probably due to a difference in composition or an altered antigenicity resulting from polymerization of the material into long-spacing collagen. Type VI collagen and fibronectin were not present in BLD.

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