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Invited Review

Assessment of blood-retinal barrier integrity

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Summary. The blood-retinal barrier consists of two components which are comprised of the retinal vascular endothelium and the retinal pigment epithelium, respectively. Its functional integrity can be recognized by tight junctions between these cells with a paucity of endocytic vesicles within them and the presence of the molecules that regulate the ionic and metabolic gradients that constitute the barrier. The barrier is compromised in several disease processes and by a variety of agents, but in most cases the location and mechanism for barrier failure is not understood. Perfusion with a variety of radiolabeled tracer molecules, vitreous fluorophotometry, or magnetic resonance imaging can be used to quantitate blood-retinal barrier leakage. Fluorescein angiography or magnetic resonance imaging can localize sites of leakage in vivo with limited resolution. Evans blue dye can be used to visualize blood-retinal barrier failure in gross pathological specimens and immunohistochemical labeling of serum proteins such as albumin or fibrinogen can be used to localize sites of blood-retinal barrier breakdown by light microscopy. Tracers such as horseradish peroxidase, microperoxidase, or lanthanum, or the immunocytochemical demonstration of albumin can be used to reveal blood-retinal barrier breakdown at the ultrastructural level and provide insights into the mechanisms involved. This review discusses the advantages and limitations of each of these methods to aid in selection of the appropriate techniques to derive the desired information.

Key words: Blood-retinal barrier, Blood-brain barrier, Vascular endothelium, Retinal pigment epithelium, Astrocytes

Introduction

Normal retinal function requires strict control of extracellular fluid and ions. The features that enable this regulation to occur collectively comprise the blood-retinal-barrier (BRB). The BRB functions by excluding blood-borne proteins from the retina and by sustaining ionic and metabolic gradients (Dermietzel and Krause, 1991). Failure of this barrier leads to macular edema, which is a major cause of visual loss and can occur in association with diabetic retinopathy or other ocular disorders, following ocular surgery, or from other causes (Patz et al., 1973; Cunha-Vaz, 1976; Eagle, 1984). BRB dysfunction may also result in the liberation of serum-derived factors that could potentiate the ocular complications of several disease processes. In some cases, structural defects such as vascular abnormalities or cell damage reveal the site of BRB compromise, but in other situations the source of BRB leakage is unclear. Finding an appropriate means to localize the specific site of BRB failure for the various ocular disorders could provide the basis for devising effective therapeutic intervention to prevent or ameliorate macular edema.

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Anatomical correlates

The BRB consists of an inner and an outer component. The inner BRB is comprised of the retinal vascular endothelium (RVE) and the outer BRB is formed by the retinal pigment epithelium (RPE) (Cunha-Vaz, 1976; Raviola, 1977). The BRB is analogous to the blood-brain barrier (BBB) in that both are characterized by complexly arranged tight junctions between the barrier-forming cells and a paucity of endocytic vesicles within these cells (Cunha-Vaz et al., 1966; Shakib and Cunha-Vaz, 1966; Casley-Smith, 1969; Cunha-Vaz, 1976; Raviola, 1977; Essner, 1987; Sagaties et al., 1987; Brightman, 1989; Janzer, 1993). The tight junctions of the RPE, whose formation appears to be regulated by diffusible factors from the neural retina (Rizzolo and Li, 1993) are more resistant to opening due to ionic stress than are other interepithelial tight junctions (Sandig et al., 1990). They are, however, more susceptible to opening due to osmotic stress than are the tight junctions of the RVE (Laties and Rapoport, 1976).

The inner BRB, like the BBB, has secondary components. Pericytes ensheath the RVE and both pericytes and endothelial cells are surrounded by a

negatively-charged basement membrane which is composed of a combination of various types and proportions of collagens, laminin, fibronectin, glycosaminoglycans including proteoglycans (primarily heparan sulfate), and glycoproteins with unknown functions such as nidogen and entactin (Sagaties et al., 1987; Brightman, 1989; Dermietzel and Krause, 1991). It is not clear what influence pericytes have on the BRB, but pericyte loss, which occurs in human diabetic retinopathy (DR) (De Oliveira, 1966; Yanoff et al., 1969; Addison et al., 1970; Engerman et al., 1977) and experimental models of DR (Kinoshita and Nishimura, 1988; Midena et al., 1989; Robison et al., 1989) accompanies BRB breakdown (Cunha-Vaz et al., 1975; Krupin et al., 1978, 1979; Waltman et al., 1978a,b; Tso et al., 1980; Jones et al., 1982; Blair et al., 1984b; Lightman et al., 1987b; Williamson et al., 1987; Vinores et al., 1989, 1990a,b, 1993a,b). Retinal vessels have a higher density of interendothelial junctions and of endothelial vesicles than brain vessels, suggesting greater vascular permeability; however, pericytes are approximately four times as numerous in retina, probably to compensate for a more permeable endothelial barrier in retina than in brain (Stewart and Tuor, 1994).

Thickening of the retinal perivascular basement membrane does not directly correlate with increased resistance to vascular leakage since thickened perivascular basement membranes are characteristic of human DR and experimental models of the disorder (Yanoff, 1969; Ashton, 1974; Williamson and Kilo, 1977; Fischer and Gärtner, 1983; Frank et al., 1983; Robison et al., 1983; Sima et al., 1985; Vinores et al., 1988; Vinores and Campochiaro, 1989). Changes in the composition of the basement membrane at the BRB in experimental models of diabetes, however, have been reported (Caldwell et al., 1986; Das et al., 1990) and may reflect functional alterations. As with cerebral vessels forming the BBB, retinal vessels are invested by astrocytic processes (Bjorklund and Dahl, 1985; Tout et al., 1993); however, within the retina, Müller cells also participate in the ensheathment of vessels (Hogan and Feeney, 1963; Cunha-Vaz et al., 1966; Rasmussen, 1972; Büssow, 1980).

Glial regulation

Like the BBB, the development and/or maintenance of the inner BRB appears to be under the control of perivascular astrocytes. Astrocytes migrate into the retina from the optic nerve (Stone and Dreher, 1987; Ling and Stone, 1988; Watanabe and Raff, 1988; Ling et al., 1989) and their migration across the retina coincides with the spread of patent vessels (Tout et al., 1993). Astrocytes are confined to the vascularized regions of the retina, where their processes invest retinal microvessels and they are absent from avascular retinas like those of the horse and rabbit (Bjorklund and Dahl, 1985; Stone and Dreher, 1987; Schnitzer, 1987, 1988a,b,c; Tout et al., 1993). One important difference between brain and retina is that in the retina, Müller cells, as well as astrocytes, participate in the formation of the perivascular sheath (Hogan and Feeney, 1963; Cunha-Vaz et al., 1966; Rasmussen, 1972; Büssow, 1980). In vascularized retinas, such as those of the human, cat, and rat, two distinct layers of vessels exist: the inner and outer vascular plexuses. The inner vascular plexus, which resides in the nerve fiber layer and ganglion cell layer, is almost totally invested by astrocytic processes (Büssow, 1980; Stone and Dreher, 1987; Schnitzer, 1988c; Chan-Ling et al., 1990; Holländer et al., 1991; Holash and Stewart, 1993), whereas Müller cells are a major contributor to the perivascular sheaths in the outer vascular plexus (Kondo et al., 1984; Holländer et al., 1991; Holash and Stewart, 1993), which is found in the outer plexiform layer.

Embryonic neural tissue can induce non-neural vessels to develop BBB or BRB features (Stewart and Wiley, 1981), but the specific role of astrocytes and the mechanism by which this occurs remain somewhat controversial. Cell contact with astrocytes has been reported by some investigators to be essential for vascular endothelial cells to establish a barrier function (Stewart and Wiley, 1981; Bradbury, 1984; Shivers et al., 1984; Janzer and Raff, 1987; Tao-Cheng et al., 1987; Brigthman, 1989; Tontsch and Bauer, 1991); other investigators found that a secreted factor derived from astrocytes, rather than direct cell contact, was responsible (Arthur et al., 1987; Maxwell et al., 1987, 1989; Shivers et al., 1988; Neuhaus et al., 1991). Müller cells share the ability of astrocytes to induce the formation of BRB properties in the vascular endothelium (Tout et al., 1993). In the feline model of retinopathy of prematurity, hypoxia causes retinal astrocytes to degenerate with a coincident failure of the BRB. As astrocytes recolonize the retina days later, the BRB is re-established (Chan-Ling and Stone, 1992; Chan-Ling et al., 1992), suggesting that astrocytes are necessary to establish a functional BRB. Recent studies with cerebral astrocytes, however, have provided uncertainity concerning the role of astrocytes in the integrity of the structurally analogous BBB. Gliotoxin 6-aminonicotin-amide, which also causes degeneration of astrocytes, does not result in a loss of BBB integrity in rats (Krum and Rosenstein, 1993), presenting uncertainity that astrocytes are essential for maintaining a functional BBB. In chickens, BBB characteristics are evident by embryonic day 14, which is prior to perivascular ensheathment by astrocytes (Albrecht et al., 1990; Holash et al., 1993) also shedding doubt on the astrocytic control of BBB function. Finally, Holash et al. (1993) found no evidence that mature astrocytes had the capacity to induce BBB features as embryonic neural tissue did (Stewart and Wiley, 1981), suggesting that they may function in maintaining the BBB, but the initial establishment of the BBB may be more complex than initially thought.

BRB-specific proteins

A variety of proteins can be used as immunohistochemical markers to reveal functional BRB sites. Those whose functions are known fall within two categories: 1) proteins responsible for maintaining ionic or metabolic gradients and, therefore, cell polarity, and 2) proteins associated with tight junctional complexes (zonulae occludens).

Detailed reviews of the enzymes, receptors, and transporter molecules associated with the BBB can be found elsewhere (Dermietzel and Krause, 1991; Janzer, 1993). Retinal capillaries generally share the same molecular characteristics as brain capillaries with the exception of γ-glutamyl-transpeptidase, which is absent from retinal capillaries, but expressed in brain capillaries (Holash and Stewart, 1993). A 140 kDa membrane protein associated with pericytes in the central and peripheral nervous system has been localized only in regions with a functional BBB or blood-nerve barrier (BNB). Its distribution closely resembles that of γ-glutamyl-transpeptidase and it is likely to be involved in some resorptive or transport function. This protein is also expressed in retinal capillaries and the RPE (Krause et al., 1988).

The glucose transporter, GLUT1, is another molecule that is concentrated on cells that form interendothelial or interepithelial occluding junctions, thus constituting blood-tissue barriers such as the BBB and the BRB. It has been demonstrated on the capillary endothelial cells of the retina, optic nerve, and iris, and on the RPE cells, the ciliary body epithelium, and the posterior epithelium of the iris, all of which have occluding intercellular junctions and comprise the BRB, the BAB, and the BNB (Harik et al., 1990; Takata et al., 1990, 1992; Takagi et al., 1994). Although GLUT1 is localized on cells with occluding junctions in ocular tissue, there is no association of the transporter with the tight junction itself. GLUT1 is not found on the capillary endothelium of vessels in the choroid, ciliary body, sclera, or other retro-orbital tissues that do not form a functional barrier. It is also absent from large vessels, since they do not have major transport functions (Harik et al., 1990). The appearance of GLUT1 developmentally coincides with the appearance of barrier characteristics in the retinal capillaries (Dermietzel et al., 1992) and its abundance increases with age (Cornford et al., 1993; Vannucci, 1994). There is approximately a four-fold greater abundance of immunoreactive GLUT1 on the ablumenal membranes of capillary endothelial cells compared to the lumenal membranes, with about 40% of the protein contained within the cytoplasm (Gerhart et al., 1989; Farrell and Pardridge, 1991). In diabetes, there is a down-regulation of GLUT1 in brain microvessels (Pardridge et al., 1990), but within retinal microvessels, GLUT1 levels on both the lumenal and ablumenal surfaces and within the cytoplasmic compartment of the endothelium are increased more than ten-fold (Kumagai et al., 1994a,b), possibly accounting for, at least in part, the more devastating effects diabetes has on the retina than on the brain.

The ZO-1 protein is a specific constituent of epithelial and endothelial tight junctions (Stevenson et al., 1986; Watson et al., 1991) that can serve as a structural marker for the BBB or BRB.

Other markers have shown a specific association with the BRB, but their functions are unknown. One such example is the postnatally-appearing endothelial-barrier antigen (EBA), which can be found on the luminal surface of endothelial cells in vessels forming a BRB, BBB, or BNB (Sternberger and Sternberger, 1987; Sternberger et al., 1989; Rosenstein et al., 1992; Ghabriel et al., 1994). EBA is greatly reduced or absent from fenestrated or injured vessels and from vessels associated with inflammatory cells or reactive astrocytes where the barrier function is compromised (Sternberger and Sternberger, 1987; Sternberger, 1989; Sternberger et al., 1989). Conversely, PAL-E is an antigen found in capillaries and veins throughout the body, but the antigen is absent from the endothelium at the BBB, BRB, and BAB. When these barriers break down, however, PAL-E is induced (Schlingemann et al., 1985, 1994). Immuno-electron microscopy shows the PAL-E epitope is associated with endothelial vesicles (Schlingemann et al., 1985), so its expression, coincident with barrier compromise, could indicate the induction of vesicular transport as a mechanism for barrier failure.

HT7, a highly glycosylated immunoglobulin-like surface glycoprotein, is another example of a BRB- or BBB-specific marker of unknown function that has been demonstrated in vessels with blood-tissue barrier properties and in the RPE (Risau et al., 1986; Albrecht et al., 1990; Seulberger et al., 1990). HT7 can be induced coincidently with other blood-tissue barrier characteristics in vessels that normally do not form functional barriers, such as those of the choriallantoic membrane, by embryonic brain (Risau et al., 1986; Schlosshauer and Herzog, 1990) or by soluble factors derived from astrocytes (Lobrinus et al., 1992).

Radiolabelled tracer molecules

A number of molecules have been labelled with a radioactive isotope and used as tracers to assess BRB function. Such molecules should be water soluble non-electrolytes that are not bound to plasma proteins, metabolized, or actively transported at the site of the BRB. Examples of molecules used in this fashion include sucrose, mannitol, inulin, urea, and albumin (Johanson, 1989) (Table 1). Intravenous inoculation of these labelled molecules and their subsequent assay in extravascular fluids can be used to quantitate BRB breakdown, but not to localize sites of leakage (Ennis and Betz, 1986; Lightman et al., 1987b; Lightman and Greenwood, 1992). It is an effective method for comparing permeability rates of molecules with different molecular weights or different molecular properties, or

Table 1. Tracer molecules for assessing BRB integrity.

TRACER	MOLECULAR WEIGHT	DIAMETER	APPLICATIONS	LIMITATIONS
Lanthanum	139	0.114nm (4nm in colloidal form, but it can penetrate spaces as small as 2 nm)	Localization of BRB breakdown sites at the electron microscopic level	Requires perfusion or fixed tissue; not useful clinically
Fluorescein	376	0.55nm	Clinical or experimental; can visually determine whether BRB is functional *in vivo* by fluorescein angiography; vitreous fluorophotometry can be used to quantitative leakage	Can show general areas of leakage, but not specific sites at the cellular level
^{14}C-Sucrose	344	1.04nm	Quantitation of BRB leakage in experimental animals	Cannot localize site(s) of leakage; impractical for clinical studies
Microperoxidase	1,900	2nm	Localization of BRB breakdown sites in experimental animals; can be used for electron microscopic evaluation	Requires tissue fixation and sectioning; not useful clinically; may be cost-prohibitive
Horseradish peroxidase	40,000	5nm	Localization of BRB breakdown sites in experimental animals; can be used for electron microscopic evaluation	Requires tissue fixation and sectioning; may induce artifactual BRB breakdown; not useful clinically
Albumin	60,000	7.4nm	With a radioactive label, BRB leakage can be quantitated, but not localized; sites of BRB breakdown can be immunolocalized by light or electron microscopy	Not useful clinically, but fixed clinical and experimental specimens can be evaluated immunohistochemically
Evans Blue Dye	961, but it forms a complex with serum albumin	7.8nm	Gross visualization of areas with BRB compromise	Only useful with gross pathological specimens
Fibrinogen	340,000		Immunohistochemical localization of BRB breakdown sites	Large molecular weight limits sensitivity, only detects areas of substantial leakage; use limited to pathological specimens

for comparing inward with outward BRB permeability rates (Ennis and Betz, 1986; Lightman et al., 1987a; Johanson, 1989).

Fluorescein

Vitreous fluorophotometry (VFP) can be used to quantitate and fluorescein angiography to visibly detect BRB failure in vivo in a clinical or experimental setting following intravenous injection of sodium fluorescein or carboxyfluorescein, which is 1000 times less lipid soluble than fluorescein (Grimes et al., 1982) and appears to have a lower affinity for the carrier-mediated system facilitating the transport of the molecule from the retina to the choroid (Tsuboi and Pederson, 1986; Grimes, 1988b). Both tracer molecules are comparable in molecular weight, spectral characteristics, and permeability from the choroid to the retina (Blair et al., 1984a; Blair and Rusin, 1986; Tsuboi and Pederson, 1986). The small molecular weights of these tracers enable one to detect minor breeches in the BRB that would go unrecognized with protein tracers. VFP, however, cannot distinguish between inner and outer BRB breakdown.

VFP was used to show abnormally high concentrations of fluorescein in the vitreous of all retinitis pigmentosa (RP) patients evaluated, which correlated with the extent of RPE and photoreceptor damage and with capillary leakage (Fishman et al., 1981; Cunha-Vaz and Travassos, 1984). Fluorescein leakage was even detected in RP patients with no ophthalmoscopically apparent abnormalities and only minor changes in the electroretinogram (ERG) and in carriers of the X-linked recessive gene for RP even though their fundus examination and ERG were normal (Fishman et al., 1981).

VFP and fluorescein angiography have been used extensively to study BRB breakdown in diabetic retinopathy in humans (Cunha-Vaz et al., 1967, 1975, 1993; Krupin et al., 1978; Waltman et al., 1978b; Kernell and Ludvigsson, 1985; Krogsaa et al., 1987; Engler et al., 1991) and in animal models (Waltman et al., 1978a; Jones et al., 1979, 1982; Tso et al., 1980; Blair et al., 1984a; Vine et al., 1984). Most investigators have shown that fluorescein leakage is directly correlated with the progression of the disease; however, Grimes (1988a), using quantative fluorescence microscopy with carboxyfluorescein failed to show a greater accumulation of carboxyfluorescein in the

retinas of diabetic rats compared with those of normal rats. Fluorescein-based methods have also been used to evaluate BRB compromise mediated by adenosine agonists, prostaglandins E_1, and other stimulators of cyclic adenosine monophosphate (Michels and Maumenee, 1975; Sen and Campochiaro, 1989, 1991), as well as in experimental proliferative vitreoretinopathy (Sen et al., 1988) and associated with pars planitis and aphakia (Cunha-Vaz and Travassos, 1984).

Magnetic resonance imaging

Magnetic resonance imaging (MRI) enhanced by the paramagnetic contrast agent, gandoliniumdiethylenetriaminetetraacetic acid has been used to localize and quantify BRB breakdown (Berkowitz et al., 1991, 1992; Sato et al., 1992; Sen et al., 1992; Wilson et al., 1992; Ando et al., 1994). The results obtained with MRI correlate with those using fluorescein-based methods and with immunocytochemical staining for albumin, but

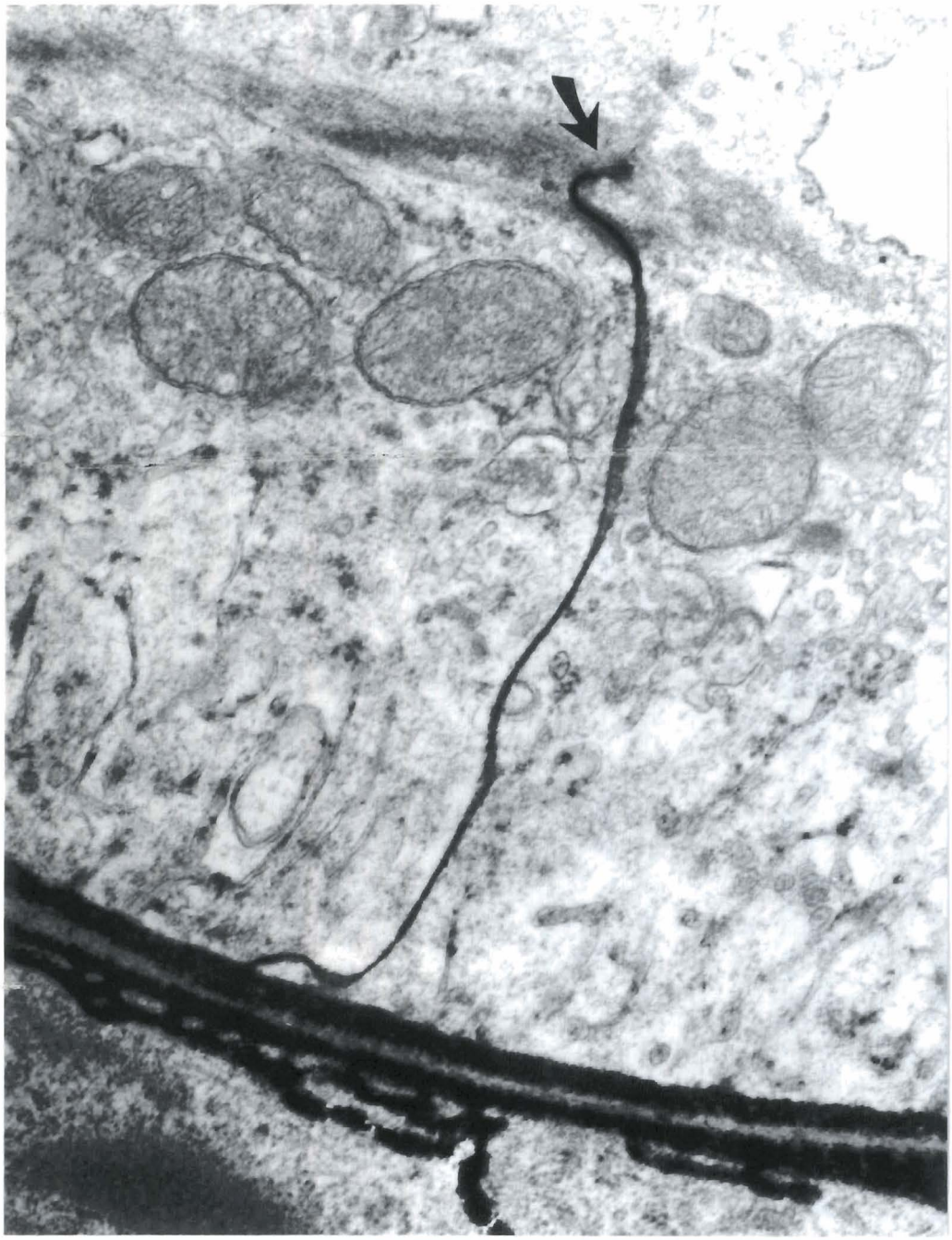

Fig. 1. Colloidal lanthanum used as a tracer in a normal rat. Lanthanum is prominent in Bruch's membrane (bottom) and can be found in the basal infoldings of the RPE. It can be visualized in the intercellular space between two RPE cells up to the level of the tight junction (arrow), but it does not penetrate the tight junction showing that the outer BRB is intact. Uranyl acetate counterstain. x 42,500

MRI has distinct advantages. It is not subject to the optical limitations of VFP and allows one to distinguish between inner and outer BRB failure in the rabbit (Sato et al., 1992; Ando et al., 1994). It's resolution is not as great as that resulting from the microscopic evaluation of exogenous or endogenous tracers, but MRI allows in vivo analysis, thus enabling the investigator to monitor progressive changes in BRB integrity within the same animal.

Evans blue dye

Evans blue dye can be used for visualization of areas with BRB breakdown in gross pathological specimens, but it is not used clinically or microscopically. It forms a complex with albumin and the relatively high molecular weight of this complex limits its sensitivity. It is not frequently used on ocular tissues, but may provide a quick assessment of BRB integrity as an adjunct to more detailed studies (Laties and Rapoport, 1976; de Bara et al., 1989).

Lanthanum

Lanthanum forms an electron dense colloid when the pH of a solution containing ionic lanthanum is raised (Revel and Karnovsky, 1967). This can be used as tracer for the electron microscopic localization of BRB breakdown sites (Pederson, 1979) (Fig. 1). It can be used prior to, concurrent with, or following fixation, but lanthanum salts may have toxic effects on unfixed cells (Martinez-Palomo et al., 1973). In colloidal form, lanthanum has a particle size of 40 Å, but can penetrate spaces as small as 20 Å (Revel and Karnovsky, 1967), enabling one to detect sites of leakage that larger molecules would not reveal. Using lanthanum as a tracer, Caldwell et al. have demonstrated that BRB failure in diabetic rats is mediated by alterations in membrane permeability and increased vesicle formation in both the RVE (Caldwell and Slapnick., 1992) and the RPE (Caldwell et al., 1985), while the tight junctions remain unaltered, a finding confirmed using immunocyto-chemical staining for albumin (Vinores et al., 1990b). Lanthanum has also been used to show that prostaglandin E_1-induced BRB failure occurs by an opening of the interendothelial tight junctions (Pederson, 1979), which has also been demonstrated using immuno-cytochemical staining for endogenous albumin (Vinores et al., 1992). Permeable interepithelial cell junctions and an increase in the number of pinocytotic vesicles have also been demonstrated in dystrophic rat RPE using this technique (Caldwell and McLaughlin et al., 1983).

Horseradish peroxidase

Horseradish peroxidase (HRP) is a tracer that has frequently been used to assess BRB integrity. Following intravenous injection or perfusion with the enzyme,

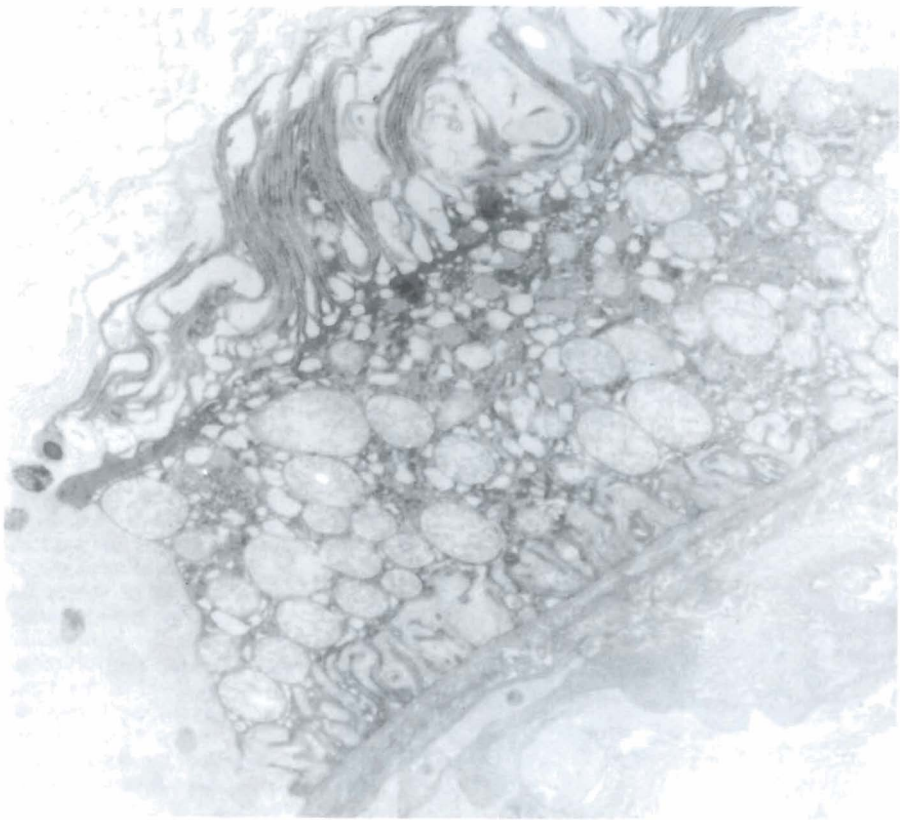

Fig. 2. Horseradish peroxidase (HRP) used as a tracer in a 1 month spontaneously diabetic BB rat shows an RPE cell that has become permeable to the enzyme, indicative of outer BRB breakdown. HRP is found within the cytoplasm, but not the organelles of one RPE cell; an adjacent RPE cell (lower left) remains impermeable to HRP. 3,3'-diaminobenzidine without counterstain. x 15,000

reaction with 3,3'-diaminobenzidine yields a granular reaction product that can reveal BRB breakdown sites at the electron microscopic level (Fig. 2). This technique has been used to evaluate the BRB in dystrophic rats (Caldwell and Mc Laughlin, 1983), following lens extraction (Tso and Shih, 1977) or prolonged hypotony (Tso and Shih, 1976) in monkeys, and after light damage in rabbits (Putting et al., 1992; Zweypfenning et al., 1992). The use of HRP, however, has often produced conflicting results. For example, some investigators have demonstrated BRB breakdown associated with diabetes using HRP as a tracer (Wallow and Engerman, 1977;

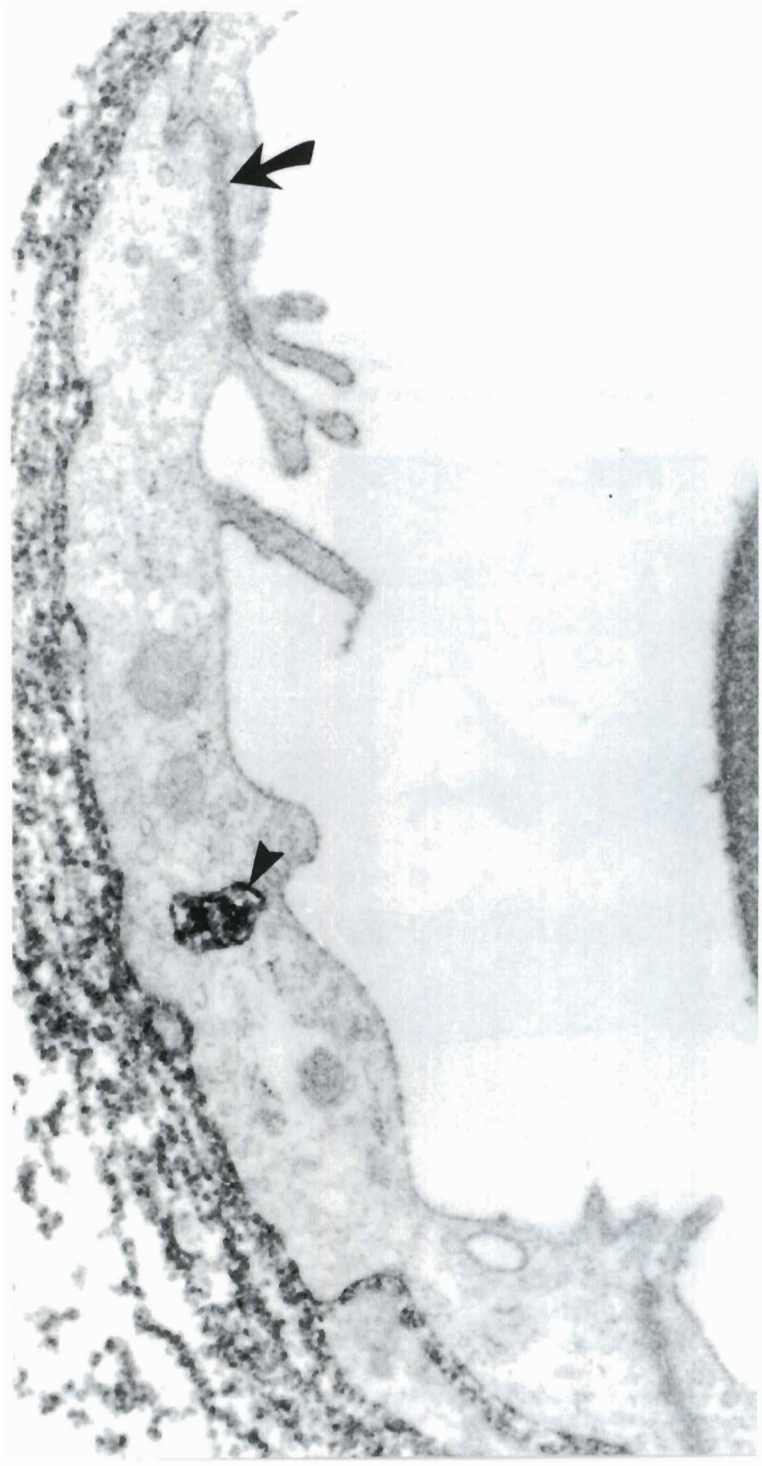

Fig. 3. Immunoperoxidase staining for albumin in a retinal vessel from a rabbit 48 hrs after treatment with prostaglandin E_1. Positivity within the perivascular extracellular matrix indicates BRB failure. Reaction product within the inter-endothelial cell junction (arrow) suggests the junction may be «open», accounting for the BRB failure. An albumin-filled vesicle (arrowhead) is found within a vascular endothelial cell, suggesting that transcytosis may also play a role in mediating BRB breakdown. Uranyl acetate counterstain. x 42,500

Ishibashi et al., 1980; Chakrabarti et al., 1990), while others, using the same technique have not seen any BRB compromise associated with diabetes (Kirber et al., 1980; Wallow, 1983; Fitzgerald and Caldwell, 1990). To provide further confusion, the RPE cells (Kirber et al., 1980) and RVE cells (Lin and Essner, 1986) of control rats have been reported to be permeable to HRP. Most of the studies cited above were performed with type II HRP. HRP, and particularly the type II isoform, has been shown to be capable of inducing pronounced vascular leakage by endocytosis and damage to the vascular endothelium (Cotran and Karnovsky, 1967; Houthoff, 1982; Balin et al., 1986; Chau et al., 1991), which is mediated, at least in part, by histamine and serotonin (Cotran and Karnovsky, 1967; Fitzgerald and Caldwell, 1990), each of which can induce capillary permeability (Westergaard, 1978; Gross et al., 1982). HRP type VI, although being more expensive, is preferred to the type II isoform as a tracer (Westergaard and Brightman, 1973; Balin et al., 1986), but some leakage has also been reported using this isoform (Cotran and Karnovsky, 1967; Lin and Essner, 1986). One possible alternative is the smaller molecular weight microperoxidase (Feder, 1971; Smith and Rudt, 1975), which appears to be nontoxic, but is also quite expensive.

Immunohistochemical localization of extravasated serum proteins

The use of exogenous tracer molecules for the localization of BRB breakdown sites has been criticized because the introduction of the tracer may adversely affect the BRB either by direct or indirect action of the foreign substance on the barrier such as by cytotoxicity, histamine and serotonin induction, osmotic changes, or other mechanisms, or due to pressure fluctuations during perfusion. The immunohistochemical demonstration of endogenous serum proteins is an alternative that eliminates the need to introduce any foreign substance and can be used on fixed pathological specimens, thereby providing access to large numbers of cases, even when uncommon disorders are being evaluated. Retrospective

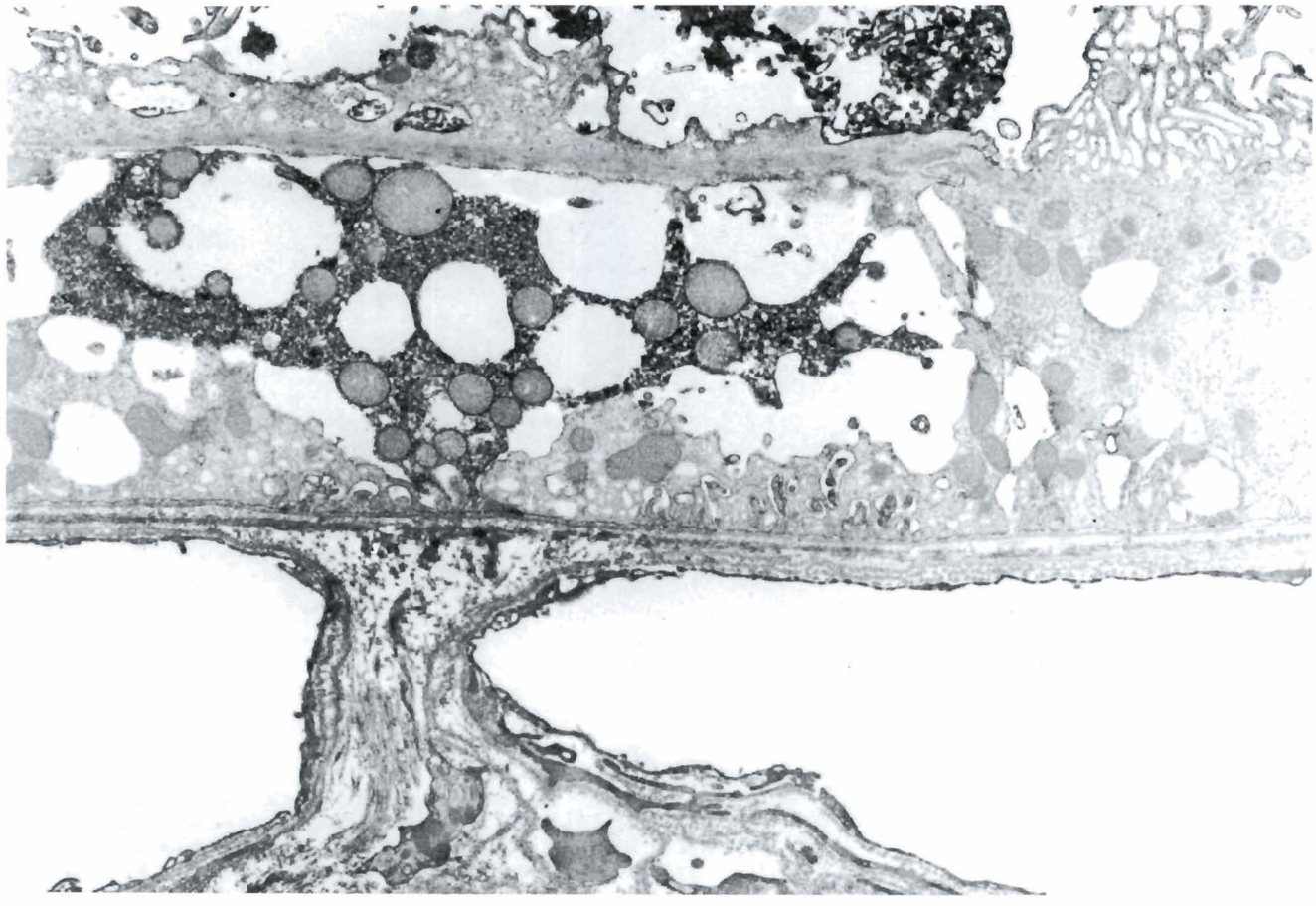

Fig. 4. Immunoperoxidase staining for albumin reveals reaction product within the cytoplasm of a damaged RPE cell from a galactosemic rat that has been maintained on a 50% galactose diet for 7 months. This indicates that the surface membrane has undergone degenerative changes that render it permeable to albumin. The choroid is at the bottom of the figure. Uranyl acetate counterstain. x 7,500

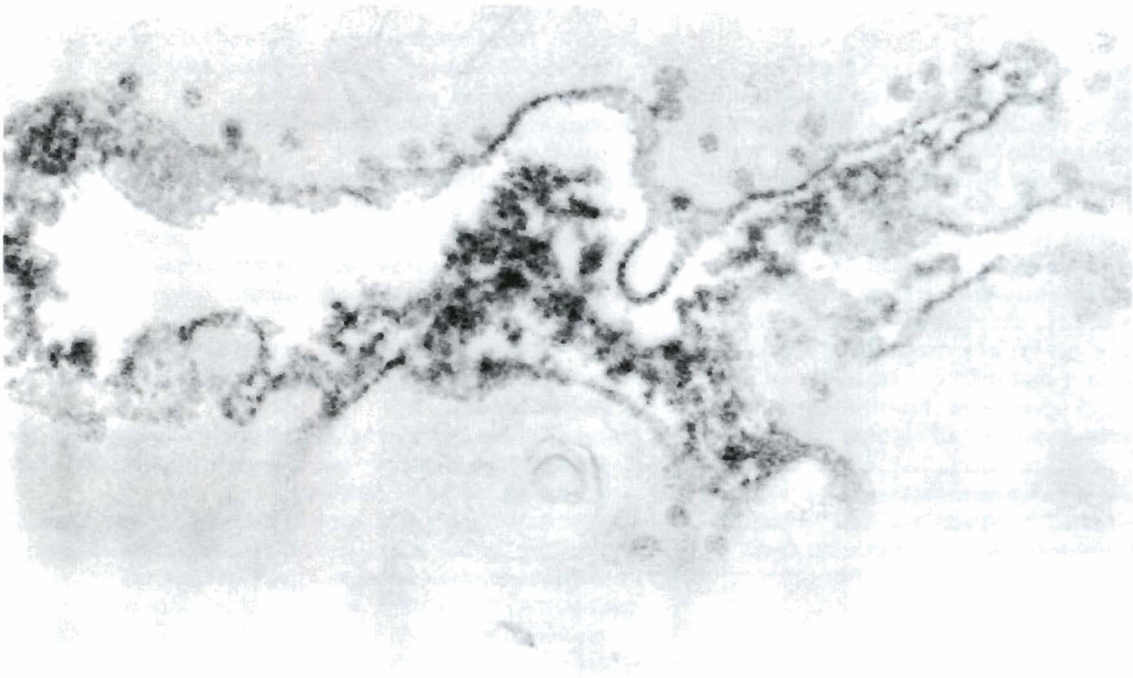

Fig. 5. Immunoperoxidase staining for albumin in a retinal vessel from a rat on a 50% galactose diet for 18 months. Albumin is demonstrated within the vessel lumen and in numerous vesicles in the RVE suggesting that BRB breakdown may occur by vesicular transport. No counterstain. x 50,000

studies can be performed on surgical, autopsy, or experimental tissues after years of storage and the technique can be used at the light microscopical level to localize the specific areas of BRB compromise and visualize the extent of BRB failure by overviewing entire sections of retina.

In normal eyes, albumin can be demonstrated only within vascular lumens and throughout the choroid, where there is no functional barrier due to the fenestrated vessels. The demonstration of extravascular albumin within the retina or RPE is indicative of BRB failure and its visualization may reveal the source of macular edema in cases where structural defects are not apparent. The immunohistochemical localization of extravasated albumin has been used to localize BRB breakdown sites in paraffin sections of eyes affected by diabetic retinopathy, retinal vascular disease, ocular inflammatory disease, ocular infections, neoplastic disease, retinitis pigmentosa, post-surgical macular edema, or other ocular disorders (Vinores et al., 1989, 1990a, 1994a,b). It is also useful for assessing the integrity of the blood-aqueous barrier (Küchle et al., 1994).

At the electron microscopic level, immunocytochemical labeling for albumin is useful to ascertain the mechanisms by which the BRB-forming cells are transgressed by seeing if extravascular albumin is found: 1) within the intercellular junctions of the RVE and RPE cells, suggesting that the tight junctions have opened (Fig. 3), 2) diffusely within the cytoplasm of RVE and RPE cells, suggesting that membrane permeability has been altered (possibly due to cell damage) (Fig. 4), or 3) contained within vesicles, suggesting that vesicular transport (transcytosis) may be operative (Fig. 5).

The immunocytochemical localization of albumin at the ultrastructural level has suggested that BRB compromise associated with diabetes or galactosemia in rats (Vinores et al., 1990b, 1993b) and diabetes in humans (Vinores et al., 1993a) is mediated by increased membrane permeability of RVE cells and RPE cells, and increased vesicular transport. In retinitis pigmentosa, albumin extravasation occurs through damaged RVE and RPE cells (Vinores et al., 1994b). This technique has also shown that prostaglandin E_1 and adenosine agonists cause BRB breakdown by opening the tight junctions between RVE cells (Vinores et al., 1992). Once the barrier-forming cells have been transgressed, extravasated protein can readily disperse through basement membranes and extracellular spaces and it is not excluded from cells that lack the capacity to form a functional barrier, such as pericytes, neurons, and glia (Vinores et al., 1990b, 1993a,b). Similar findings have been reported following BBB failure (Henkind et al., 1980; Kitagawa et al., 1991).

Fibrinogen is another serum protein whose immunohistochemical demonstration reveals sites of BRB breakdown. Due to its high molecular weight, it cannot detect minor areas of leakage, but its immunolocalization is useful for examining hard exudates, where the protein may be more resistant to degradation than albumin, and for localizing sites of hemorrhage (Murata et al., 1992, 1993).

Concluding remarks

BRB breakdown occurs in several pathological conditions and can be mediated by diverse agents. A variety of methods are available to assess BRB function quantitatively and qualitatively (Table 1) to better understand the mechanism of BRB failure from different causes. The choice of methods depends on the type of data desired, the setting (clinical or experimental), and the condition of the tissue to be evaluated (in vivo, fixed tissue, etc). Each method has its own particular limitations and sensitivity, which must be considered in its selection. Since no single method can provide quantitative data with a qualitative assessment of BRB compromise with high resolution, multiple approaches may be required to obtain an overall picture.

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