Pathologic Studies of the Blood–Retinal Barrier in the Spontaneously Diabetic BB Rat

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The BB rat spontaneously develops a diabetic state that closely resembles human type I diabetes. The authors studied the pathologic changes of the retina and retinal pigment epithelium of four normal and nine diabetic BB rats using (1) light and electron microscopy with the horseradish peroxidase tracer technique, and (2) trypsin digest preparations of the retinal vessels. They observed a retinal pigment epitheliopathy characterized by (1) derangement of the plasmalemma infoldings; (2) patchy organdie degeneration leading to focal necrosis; (3) increased permeability to horseradish peroxidase; and (4) repair of the pigment epithelium. Focal thickening of the retinal vascular basement membrane was seen occasionally, but the trypsin digest preparations were unremarkable. These studies suggest that diabetic retinal pigment epitheliopathy may be one of the early changes of diabetic retinopathy and may provide a pathogenetic mechanism for early disruption of the blood–retinal barrier. Invest Ophthalmol Vis Sci 25:302–311, 1984

Breakdown of the blood–retinal barrier (BRB) has been observed by vitreous fluorophotometry in the absence of other signs of retinopathy. This has been reported in diabetic patients,1,2 in rats made diabetic by streptozotocin (STZ)3–6 or alloxan,7 and in monkeys made diabetic with STZ or pancreatectomy.8 The site of this breakdown has been sought in morphologic studies on streptozotocin diabetic rats. Tso et al found multifocal cellular damage and disruption of the blood–retinal barrier to horseradish peroxidase (HRP) in the retinal pigment epithelium (RPE).9 Kirber et al observed diffuse penetration of fluorescein into the RPE cells.10 These investigators found no abnormal leakage from the retinal vasculature. However, Ishibashi et al noted increased pinocytosis of HRP into the retinal capillary endothelial cells and penetration of HRP into the retinal capillary basement membrane and the retinal extracellular space.11 Wallow and Engerman reported opening of tight junctions of the retinal vessels to HRP in alloxan-diabetic dogs.12

While there is fairly good evidence that the changes observed in experimentally induced diabetes in animals relates to the diabetic state itself,3 the possibility that some of these abnormalities might be due to toxic effects of diabetogenic drugs has not been excluded conclusively.9

A syndrome that closely resembles human type I diabetes develops spontaneously in some BB rats (a Wistar-derived rat from the Bio Breeding Laboratories of Canada Ltd.).13 It is characterized by hyperglycemia, glycosuria, hyperketonemia, ketonuria, hypoinsulinemia, and rapid death from ketoacidosis without insulin therapy. There is marked mononuclear cell infiltration in the pancreas with selective beta cell destruction. Mounting evidence suggests that the syndrome has an autoimmune cause.14 In this report, we describe the pathologic changes of the retina and RPE of normal and diabetic BB rats by light and electron microscopic techniques.

Materials and Methods

Eyes from six diabetic and three normal BB rats were studied by light and electron microscopy using the HRP tracer technique. Trypsin digest studies also were done on three additional diabetic BB rats and on one additional normal BB rat. The normal rats consisted of three females and one male, and the diabetic rats consisted of six females and three males. (The BB rats were obtained through the courtesy of A. A. Like, MD, of the University of Massachusetts School of Medicine.)

When killed, the age of the normal rats was 5.9 ± 0.6 months and that of the diabetic rats was 7.7 ± 1.5 months. The normal rats weighed 252 ± 17 g when killed, and the diabetic rats weighed 291 ± 58
The rats had been diabetic for 4.2 ± 1.2 months and had received single daily injections of 1.34 ± 0.50 units of PZI insulin. The dose for each rat was adjusted daily based on a urine sugar determination with Tes-Tape (Lilly) or on a measurement of the glucose concentration in the plasma. When killed, the mean blood sugar among the normal rats was 132 ± 57 mg/100 ml; it was 429 ± 93 mg/100 ml among the diabetic rats.

Except for albinism (a trait of all BB rats), clinical examination by slit lamp and indirect ophthalmoscopy revealed no abnormalities. Vitreous fluorophotometry was attempted on some rats. Interpretation of the results was difficult, however, because of excessive artifact due to marked reflection of light within these albinotic eyes.

For morphologic tracer study, HRP was used as previously described. Each rat received diphenhydramine intravenously in a dose of 1 mg/kg. Five minutes later, they received a dose of HRP of 200 mg/kg. Thirty minutes later, the eyes were removed. The eyes were opened along the pars plana and placed in Smith’s fixative for 6 hr. They then were washed overnight in phosphate buffer at 4°C. The posterior segments were divided into quadrants, and multiple 100 μm sections were cut with a Smith-Farquhar tissue sectioner, reacted with diaminobenzidine and hydrogen peroxide, and embedded in Durcupan epoxy resin. One-micron thick sections were cut and examined by light microscopy either with or without staining with Mallory’s azure II methylene blue. Thin sections were examined with an electron microscope either unstained or prepared with uranyl acetate and lead citrate.

For trypsin digest studies,18 eyes were removed and fixed in 10% neutral buffered formalin for 24 hr and then washed overnight in running tap water. The eyes were opened and the retinas were removed and placed in a 3% solution of trypsin in TRIS buffer at 37°C for ½–1½ hr. Under a dissecting microscope, the partially digested retinas were lifted in and out of distilled water with a cyclodiasis spatula. This caused the friable, digested retinal parenchyma to separate from the vessels. The vascular tree was floated on a slide, air dried, stained with PAS-hematoxylin, and examined by light microscopy.

Flat mounts of the vasculature of trypsin digested retinas showed capillaries containing endothelial cells and pericytes. Nuclei were identified as being those of endothelial cells by their large size, light, vesicular staining, and ellipsoid shape. Pericytes had small, round, darkly staining nuclei. The pericyte nuclei seldom protruded when the nucleus was at the side of the capillary, as in human preparations. Differentiating between the two cell types was difficult in this situation, since all nuclei appeared quite dark when viewed on end. Such cells were considered to be endothelial cells if the nuclei were long and pericytes if they were relatively short. One thousand capillary cell nuclei were counted from a normal rat, and 500 capillary cell nuclei were counted in each of three diabetic rats.

Results

The pathologic alterations in the retina of the diabetic BB rat were confined largely to the RPE and retinal vasculature.

Retinal Pigment Epitheliopathy

Plasmalemma infolding changes: The normal BB rats had prominent, uniformly organized, convoluted basal infoldings in the RPE cells at the ultrastructural level (Fig. 1). In contrast, the basal infoldings of the RPE of the diabetic rats showed varying derangement in most of the blocks studied (Fig. 2). In some areas, no basal infoldings were present for substantial lengths along the base of a RPE cell (Fig. 3). In other areas, bizarre focal exaggerations of the basal infoldings were seen, usually where the adjacent basal plasmalemmas were infolded minimally. These exaggerated infoldings had large cisterns and/or elaborate reduplications of the basal infoldings that usually contained HRP (Fig. 2). In addition, similar infoldings were seen occasionally in the lateral plasmalemma of the RPE cells, an area that had few infoldings in the normal rats. By light microscopy, many RPE cells had focal accumulation of HRP reaction-products adjacent to the basal or lateral plasma membrane. These correlated with the focal exaggerations of the basal infoldings seen by electron microscopy. Despite these exaggerated infoldings, the predominant derangement of the basal plasmalemma of the RPE was a dramatic reduction of infolding (compare Figs. 1–3).

Cytoplasmic organelle and nuclear degeneration: Foci of degenerating RPE cells in the diabetic rats exhibited a spectrum of organelle degenerative changes that included dilation of the smooth endoplasmic reticulum (Fig. 4), swelling of the mitochondria, and widening of the cleft between the leaflets of the nuclear membrane. Some of the cytoplasmic changes were extensive and associated with dissolution of the chromatin pattern of the nuclei, indicating frank necrosis (Fig. 5). Adjacent cells usually showed milder degenerative abnormalities such as dilation of the endoplasmic reticulum. Increased permeability to HRP: Multifocal areas of diffuse infiltration of HRP into the cytoplasm of RPE cells were observed by both light and electron microscopy. Such RPE cells stood out conspicuously in unstained sections by light microscopy (Fig. 6A). These cells were observed in 12 of 150 blocks from diabetic...
BB rats and often multiple adjacent cells showed this feature. In contrast, only one isolated cell with this cytoplasmic alteration was seen in 39 blocks from the normal BB rats. In some areas, there was a varying degree of diffuse penetration of HRP from cell to cell, which suggested the possibility of a gradient in severity.
of cellular injury. These affected cells tended to be flattened and sometimes were more heavily stained with Mallory's azure II methylene blue. Some of these cells had pyknotic nuclei, indicating necrosis.

By electron microscopy, these cells showed severe degenerative changes with or without necrosis. Occasionally, HRP was observed in the subretinal space between the photoreceptor elements (Fig. 6B). On electron microscopy we did not observe damage to the tight junctions except as part of generalized cell de-
generation. When HRP was seen to penetrate into the subretinal space, it appeared to have done so across necrotic cells rather than between cells having incompetent tight junctions.

**Repair of RPE:** In areas adjacent to necrotic RPE cells, evidence of repair was seen. It appeared that the surrounding RPE cells attempted to slide (Fig. 7) or send long cytoplasmic processes over the necrotic cells (Fig. 8). As a result, the lateral plasmalemmas, which in the normal rats were roughly perpendicular to Bruch's membrane, assumed a very oblique orientation. Occasionally, macrophages were seen between the photoreceptors and the RPE (Fig. 3).

Although the RPE of both normal and diabetic rats contained lipid inclusions, these appeared to be more numerous in the diabetic animals. We observed no mitotic figures in the RPE.

**Retinal Blood Vessels**

Electron microscopy showed HRP to be excluded from the cytoplasm of the endothelial cells of the normal rats. In contrast, HRP diffusely penetrated into the cytoplasm of some retinal capillary endothelial cells of the diabetic rats (Fig. 9). We found no evidence that the HRP entered the cytoplasm by micropinocytosis. The endothelial cells otherwise appeared normal, including their tight junctions and the number of vesicles.

We did not observe evidence of penetration of HRP into the capillary basement membrane, pericytes, or extracellular space of the retina. Some pericytes had numerous glycogen granules. The pericytes occasionally showed swollen mitochondria and watery cytoplasm. No mitoses were noted in either the endothelial cells or in the pericytes.

The retinal vascular basement membrane showed focal thickening in some areas. Discrete, often nodular, foci of electron-lucent material within the basement membrane were noted in both the normal and diabetic eyes (Fig. 9). Another less frequent type of focal basement thickening was observed only in the diabetic rats (Fig. 10). These foci were composed of granular material, although they sometimes had a faint, coarsely reticular pattern.

No occlusions of the vascular lumens were seen. Neither platelet-fibrin thrombi nor glial processes were observed in the capillary lumens.

On examination of the trypsin digest preparation, the percent of endothelial cells was 81% in the normal rat and 84%, 83%, and 79% in the diabetic rats. No microaneurysms, acellular capillaries, or pericyte ghosts were observed.

**Discussion**

Our morphologic studies of the diabetic BB rat revealed a distinct retinal pigment epitheliopathy, abnormalities of the retinal vasculature and breakdown of the blood-retinal barrier. These results are of particular interest because they occurred in an animal that spontaneously develops diabetes without exposure to diabetogenic drugs. This precludes the possibility that the changes are related to diabetogenic agents as opposed to the diabetic state, a possibility difficult to
Fig. 6. Retinal pigment epithelium of a diabetic BB rat showing increased permeability to HRP. A, HRP reaction product is seen infiltrating some of the pigment epithelial cells (arrows) but does not extend into the subretinal space. (Unstained plastic section X900). B, The HRP has infiltrated the necrotic pigment epithelial cells (arrows) to the left and is seen in the subretinal space (arrowheads) extending between the inner and outer photoreceptor segments up to the external limiting membrane. The pigment epithelial cells to the right are free of reaction product. (Unstained plastic section X900).

Fig. 7. Sliding of the retinal pigment epithelial cells of a diabetic BB rat adjacent to an area of necrosis. The intercellular space containing HRP (arrows) between the pigment epithelial cells assumes an oblique orientation. Note also the dilation of the rough endoplasmic reticulum, the relatively healthy appearance of the mitochondria, and the numerous ribosomes in these cells. (X11,300).
Fig. 8. A long cell process (P) of a sliding RPE cell (N) extends to the left over a severely damaged cell (D). (X8,000).

Fig. 9. Retinal capillary from a diabetic BB rat. There is marked infiltration of HRP into the cytoplasm of the endothelial cells (arrows). However, no reaction product of HRP is seen in the basement membrane or pericyte cytoplasm. Multiple nodular foci of basement membrane thickening (arrowheads) are also present. (X18,300). Inset shows an electron-lucent nodular basement membrane thickening in higher magnification. (X30,000).
exclude conclusively if such agents are used to induce the disease.

The retinal pigment epitheliopathy that we observed was characterized by several features. First, diffuse derangement of the RPE basal plasmalemma infoldings was noted. In some areas, the basal infoldings were completely absent, whereas in other areas, bizarre exaggerations of the infoldings were seen. Such deranged infoldings also were noted on the lateral aspect of the RPE cells basal to the junctional complexes, an area that normally does not have any infoldings. Grimes et al observed large, bizarre, basal infoldings of RPE cells in STZ diabetic rats similar to those we observed in diabetic BB rats. They also showed doubling of the basal infolding by morphometric analysis. In the present study, the overall decrease in basal infoldings was so dramatic that we concluded it was a characteristic feature of the diabetic rats (compare Figs. 1-3). Differences in the causes of the diabetic state and/or its duration (1 month in their group vs over 4 months in our group) may account for the dissimilarities in the results. Grimes et al speculated that the increase in plasmalemma infoldings correlated with increased transport function by the pigment epithelial cells. In our study, there appeared to be an overall reduction of the plasmalemma infoldings, which suggests that decompensation of the RPE may occur in the more advanced diabetic state.

Second, we observed that scattered foci of RPE cells underwent organelle degenerative changes that ranged from mild alteration to advanced necrosis. Cells adjacent to the severely affected cells displayed similar, albeit milder, changes. Attempts at repair consisted of debridement by subretinal macrophages and sliding of RPE cells over or under the necrotic cellular debris. These findings were similar to those observed in this laboratory in rats with diabetes induced by STZ.

Third, the foregoing RPE degenerative and necrotic changes allowed the diffuse penetration of HRP into the cytoplasm of the cells and sometimes into the adjacent subretinal space. We were unable to document convincingly the opening of the zonulae occludentes at the ultrastructural level, despite a diligent search. There appeared to be no predilection for involvement of the tight junctions in the degenerative process that occurred in the RPE cells. Rather, the junctional complexes appeared to remain intact unless the cells became necrotic. Thus, we believe that the predominant way
by which HRP traversed the RPE into the subretinal space was across severely and diffusely damaged cells, rather than between relatively normal cells with incompetent tight junctions. Similar observations were made in STZ diabetic rats.9

Kirber et al also observed diffuse penetration of HRP into some RPE cells (but not into the subretinal space) of both normal and STZ diabetic rats,10 which they attributed to an artifact of the technique. When unaccompanied by other pathologic changes, it may be difficult to know whether this finding indicates disease or is merely an artifact. However, we believe this finding in our study was not artifactitious for the following reasons: (1) penetration of HRP into the cytoplasm of RPE cells occurred only in cells that also showed advanced plasmalemma and organelle degeneration or frank necrosis; (2) penetration of HRP into the subretinal space occurred in areas where HRP infiltrated the cytoplasm of RPE cells; (3) host tissue reparative responses, such as cell sliding and removal of cellular debris by macrophages, were evident in many areas showing penetration of HRP into the RPE cells; and (4) the animals were pretreated with an antihistamine to block any presumed pharmacologic effects of HRP induced by histamine.

The retinal vasculature was studied by light and electron microscopy of sections of retina processed to localize HRP and by trypsin digest flat mounts of the retinal vessels. Penetration of HRP into the cytoplasm of some retinal endothelial cells in diabetics, but not normal rats, was seen. This has been observed by other investigators and was interpreted as a nonspecific change in endothelial cells.10,22 Focal thickening of the vascular basement membrane was observed occasionally.

Wallow and Engerman12 published electron micrographs that appeared to show the opening of the zonulae occludentes joining adjacent retinal endothelial cells in alloxan diabetic dogs. We did not observe this in our study.

Ishibashi et al observed penetration of HRP into the retinal capillary basement membrane and pericapillary retinal interstitial space of rats 2–6 months after the onset of STZ diabetes.11 They presented evidence that HRP had crossed the intercellular tight junctions. They found an increase in the number of endothelial cell vesicles that contained HRP in diabetic rats, as compared to normal rats. Our findings were similar to those of Leuenberger and Babel who observed no passage of HRP across the retinal endothelium in rats diabetic for up to 8 months after STZ.21

We have demonstrated abnormalities of the RPE that lead to breakdown of the blood–retinal barrier in the spontaneously diabetic BB rat. This finding suggests that these changes are a manifestation of the diabetic state itself. In addition, the RPE changes were more prominent than the retinal vascular changes that were present at the stage of the disease that we examined. Indeed, the retinal vascular changes seen did not include the classic pathologic findings of diabetic retinopathy. This has several potentially important implications. First, retinal pigment epitheliopathy may play an important role in the early stages of diabetic retinopathy. This is bolstered by similar findings in the RPE of monkeys made diabetic by STZ or pancreatectomy.8 While not generally considered a prominent feature of diabetic retinopathy in humans, some recent workers have recognized that RPE changes in diabetics are frequent.22 Second, the RPE rather than the retinal vasculature may be the major source of abnormal fluorescein accumulation in the vitreous, as measured by vitreous fluorophotometry that has been reported in diabetic rats and in diabetic humans with minimal or no visible retinopathy. Our results suggest that further studies are needed to determine the presence of retinal pigment epitheliopathy in human diabetics, and if it exists, how it relates to the development of classic diabetic retinopathy.

Keywords: diabetes, BB rat, retinal pigment epitheliopathy, blood–retinal barrier, diabetic retinopathy

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References