Posterior and Anterior Permeability Defects? Morphologic Observations on Streptozotocin-treated Rats

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Structural abnormalities of the blood-ocular barrier were examined in streptozotocin (STZ)-treated hyperglycemic rats, after 9 days, 6 months, and 10 months' duration of "diabetes," and in normoglycemic control animals using the horseradish peroxidase tracer technique combined with light and electron microscopy. The most frequent abnormalities consisted of small areas of diffuse dense staining by the tracer of (1) the retinal pigment epithelium and (2) the nonpigmented ciliary epithelium. Pigment epithelium abnormalities occurred occasionally in both groups of animals with approximately equal frequency and extent. Ciliary body abnormalities occurred also in both groups, but were frequent; statistically, the probability of these changes was not significantly different between the two groups. At the ora serrata, tracer escape was present through the retinal pigment epithelium into subretinal space and retina. Retinal vascular leakage occurred rarely and may be related to tracer toxicity rather than hyperglycemia. Thus, using the HRP method, we cannot confirm the claim that sustained STZ-induced hyperglycemia causes breakdown of the blood-retinal barrier in the rat. Invest Ophthalmol Vis Sci 24:1259-1268, 1983

Streptozotocin (STZ)-induced hyperglycemia in rats has been used as an experimental model to study alterations of the blood-retinal barrier (BRB).1-6 Vitreous fluorophotometry has been employed to measure such alterations.1-4 Increased levels of vitreous fluorescence were reported in three studies1-3 and interpreted as evidence for a breakdown of the barrier. Recovery of barrier function was ascribed to insulin administration2 and to pancreatic islet transplantation.3

Some morphologic studies have suspected the retinal pigment epithelium as a site of barrier breakdown.5,6 Fluorescein produced large areas of relatively uniform pigment epithelial staining5 while horseradish peroxidase (HRP) showed no difference between hyperglycemic and control rats in one study5 but in another, showed several types of leaky RPE lesions almost exclusively in hyperglycemic animals.6 Both studies agreed that the blood-retinal barrier to HRP remained unaltered at the level of the retinal blood vessels, yet others described increased permeability to HRP of retinal capillaries in STZ-hyperglycemic rats7 and in alloxan-diabetic dogs.8

We report here observations in the acute, intermediate and chronic stages on the distribution of blood-borne HRP in retina and ciliary body of rats made hyperglycemic with STZ, and in appropriate control animals.

Materials and Methods

Animals

A total of 35 male hooded rats were studied at one of three intervals following the induction of hyperglycemia by STZ injection: (A) 9 days (acute stage), (B) 6 months (intermediate stage), and (C) 10 months (chronic stage). Eighteen rats received the STZ injection and became hyperglycemic while 17 of the rats served as controls. Fourteen of the 17 control rats never received STZ, three received one dose of STZ, but remained normoglycemic.

STZ was administered as a freshly prepared 10% solution in citrate buffer at pH 4.5 in a dose of 65 mg/kg BW by injection into the femoral vein after a 24-hr fast.

All 18 treated rats remained consistently hyperglycemic and glucosuric. Their blood glucose was measured periodically by the Eyetone-dextrostix system with the result occasionally monitored by the hexokinase enzymatic endpoint method. Urine sugar and...
Table 1. Nonfasting blood glucose levels and weight change in STZ-treated hyperglycemic and in normoglycemic control rats after 9 days (group A), 6 months (group B), and 10 months (group C). Mean values are followed by standard deviations of the mean.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>Nonfasting blood sugar values mg/dl by</th>
<th>Weight loss* (−) or gain (+), g at end of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colorimetry Before STZ</td>
<td>After STZ</td>
</tr>
<tr>
<td>A</td>
<td>Hyperglycemic</td>
<td>6</td>
<td>140 ± 21</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>8</td>
<td>201 ± 46</td>
</tr>
<tr>
<td>B</td>
<td>Hyperglycemic</td>
<td>4</td>
<td>106 ± 15</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>4</td>
<td>100 ± 14</td>
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<tr>
<td>C</td>
<td>Hyperglycemic</td>
<td>8</td>
<td>169 ± 22</td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>5</td>
<td>180 ± 14</td>
</tr>
</tbody>
</table>

* Measured against initial body weight at time of STZ-injection/assignment to control group.

acetone were measured daily. Test and control animals in each group were housed and fed together (Table 1). Ether anesthesia was used throughout.

Group A consisted of 14 rats approximately 4 months old weighing between 390 and 450 g. Six STZ-treated animals became consistently hyperglycemic and glucosuric. They received no insulin. Eight animals served as normoglycemic controls and included one that had been ineffectually injected with STZ. This rat is treated separately in the morphologic results section (Table 2).

Group B consisted of eight rats also about 4 months old and weighing between 350 and 500 g. Four STZ-treated animals became consistently hyperglycemic and glucosuric. They received 4–5 units of NPH insulin weekly, which was enough to insure sustained

Table 2. Frequency of tissue blocks with ocular barrier abnormalities per number of tissue blocks studied by light microscopy in different locations of the eyes of STZ-treated and control rats at 9 days (A), 6 months (B), and 10 months' duration (C) of experimental condition.

<table>
<thead>
<tr>
<th>Location of tissue blocks</th>
<th>RPE*</th>
<th>Retina</th>
<th>Ora†</th>
<th>Ciliary body†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Posterior + midperiph.</td>
<td>Anterior</td>
<td>Posterior + midperiph.</td>
<td>Anterior</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ</td>
<td>0/50 (1/50)</td>
<td>0/45</td>
<td>0/26</td>
<td>3.8</td>
</tr>
<tr>
<td>Control†</td>
<td>4/87</td>
<td>4.5</td>
<td>0/54</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STZ</td>
<td>2/47 (2/47)</td>
<td>1/43</td>
<td>2.3</td>
<td>0/17</td>
</tr>
<tr>
<td>Control†</td>
<td>0/33</td>
<td>0</td>
<td>0/11</td>
<td>0</td>
</tr>
<tr>
<td>STZ</td>
<td>1/63 (6/63)</td>
<td>0/29</td>
<td>0</td>
<td>0/60</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control§</td>
<td>5/35</td>
<td>14.3</td>
<td>2/16</td>
<td>12.5</td>
</tr>
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</table>

* RPE abnormalities listed here are first those that consisted of diffuse cytoplasmic staining by the tracer HRP. Listed in brackets are changes of focal RPE proliferation ("group 3 lesions").
† Ora and ciliary body were processed within the same tissue blocks so that favorably oriented sections from these showed both areas.
‡ This group of 8 "control" animals contains 1 STZ-treated but normoglycemic rat, which showed no RPE or retinal vascular barrier abnormalities, but frequent ora and ciliary body changes, as the other controls.
§ This group of 5 "control" animals contains 2 STZ-treated but normoglycemic rats, which showed no RPE or retinal vascular barrier abnormalities, but frequent ora and ciliary body changes, as the other controls.
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Fig. 1. Retina of rat, 9 days following streptozotocin (STZ) treatment. Horseradish peroxidase reaction product diffusely and densely stains cytoplasm of individual or groups of RPE cells (arrows). Tracer does not extend into subretinal space around photoreceptor outer segments (OS); ONL = outer nuclear layer (original magnification X1100).

hyperglycemia while preventing hyperglycemic or diabetic death. Four animals, not injected with STZ, served as normoglycemic controls.

Group C consisted of 13 rats approximately 2 months old weighing between 200 to 340 g. Eight STZ-treated animals became consistently hypoglycemic and glucosuric. They received NPH insulin approximately every 12 days. Five animals served as normoglycemic controls and included two that had been ineffectually injected with STZ. These two are treated separately in the morphologic results section (Table 2). In groups B and C the last insulin injection given was 4 and 5 units, respectively, 2 days prior to killing.

Tracer Technique and Tissue Processing

The permeability of the BRB to the protein tracer, HRP, was studied in one eye of each of the animals of group A, B, and C, at 9 days, 6 months and 10 months, respectively, following STZ injection. In both normal and “diabetic” animals occasional vascular leakage was present in groups A and B (see Table 2) and was attributed to an HRP-induced increase of blood vessel permeability as previously observed in rodents by others. Thus, in group C, 5 min prior to HRP injection, diphenhydramine hydrochloride and methysergide maleate, each in a dose of 1 mg/kg were given intravenously in order to prevent an HRP-induced increase of blood vessel permeability. For all groups, HRP type VI was freshly dissolved in normal saline in a concentration of 180 mg/ml. The femoral vein was surgically prepared and 180 mg/kg of tracer were injected. The preparation site was then sutured. From 10 to 20 min after injection one eye of each animal was enucleated, promptly fixed in 4% glutaraldehyde in cacodylate buffer at pH 7.4 and opened coronally at the limbus. Fixation was continued for 2 hrs at 4 C.

Comparable tissue samples were taken from all eyes, consisting of posterior retina (adjacent to the optic nerve), intermediate retina (midway between nerve and ora), ora serrata, and ciliary body. These were embedded in agar, sectioned into 50–100 μm thick slices, and processed for peroxidase activity.

Subsequently, the tissue was postfixed for 1 hr in 1% OSO4 solution in S-collidine buffer, stained in the block with 2% uranyl acetate for 1 hr, dehydrated in graded alcohols and embedded in an epoxy resin. Sections were cut 1.5 μm thick; half were stained with toluidine blue, the other half left unstained. They were examined by light microscopy at 400–1000 X magnification for evidence of tracer leakage. From four to seven tissue blocks were made from each tissue sample and four to five sections were cut and evaluated from each block. More than 3,000 sections were reviewed. Representative areas showing leaks (specified under Results) were also thin sectioned, stained with lead citrate and examined in an electron microscope.

Results

The most interesting abnormality found was diffuse dense cytoplasmic staining by the tracer HRP of a single or group of adjacent cells in the retinal pigment epithelium (RPE) (Figs. 1, 2) or nonpigmented ciliary epithelium (NPCE) (Figs. 3, 4).

Table 2 shows that no consistent differences in frequency, location or extent of these abnormalities were found between normal and hyperglycemic animals regardless of the initial age (4 months in group A and
Fig. 2. Retina of control rat, 9 days following entry into experiment. Tracer reaction product is diffusely present within cytoplasm of RPE cell at center. APV = apical vill; OS = outer segments. Adjacent normal RPE cells show tracer only within a few vesicles (arrow) near the prominent basal infoldings of the plasma membrane (original magnification X6400).

Fig. 3. Pars plicata of ciliary body of rat, 6 months following STZ treatment. One cell (arrow) of the nonpigmented ciliary epithelium is diffusely stained by dark tracer reaction product (X1100).
Fig. 4. Electron micrograph of another tissue block of the same animal shows comparable area. Tracer has leaked out of the central vascular core (black) and stained basal, lateral and apical spaces around the pigmented ciliary epithelium (PE). At the level of the nonpigmented ciliary epithelium (NPE) tracer is seen within (a) numerous vesicles (lower arrow) of normal appearing cells, and (b) as diffuse dense cytoplasmic staining of several other cells (upper arrows) (×7000).
Fig. 5. STZ-treated rat, 10 months in experiment. Two cell layers are seen, hypopigmented RPE (A) in contact with Bruch’s membrane and an irregularly hyperpigmented group of cells (B) extending away from Bruch’s membrane into the subretinal space. Tracer did not penetrate the basal hypopigmented layer. This was the most extensive lesion of its kind (original magnification ×100).

B vs. 2 months in group C) or weight (350–500 g in groups A and B vs. 200–340 g in group C). Also no difference was recognized between insulin-treated (groups B and C) and noninsulin-treated hyperglycemic animals (group A), or between streptozotocin (STZ)-treated hyperglycemic animals and a few STZ-treated animals that remained normoglycemic. Staining of the RPE occurred occasionally, staining of the NPCE was very frequent.

At times another RPE change was encountered consisting of one or several hyperpigmented cells located on top or in between hypopigmented RPE cells that were connected laterally to unaltered RPE (Fig. 5). Tracer did not stain the RPE cells or the subretinal space. Such changes were similar to the “group 3 lesions” described by others.6 Table 2 shows that they were present in nine blocks of six STZ-treated animals. They were not observed in control animals.

At the ora serrata tracer extended into the subretinal space and into the intercellular spaces of the neurosensory retina (Figs. 6, 7). Frequently, a few adjacent cells of the nonpigmented ciliary epithelium were diffusely stained. Tracer leaks were seen in every animal, hyperglycemic or control, with satisfactory tissue sections (Table 2). In groups A and B, but not in group C, several retinal blood vessels showed diffuse impregnation of their walls by tracer reaction product with extension into the paravascular tissue spaces. One STZ-treated and two control animals were involved (Tables 2 and 3).

The probability of barrier abnormalities at ora and ciliary body was not significantly different between STZ-treated and control animals when tested in the following manner: For each of the three groups of rats (A, B, and C) a chi-square statistic with Yates continuity correction was calculated, and the respective $P$ values were determined. For the ora the $P$ values for group A, B, and C were 0.95, 0.19, and 0.12, for the ciliary body they were 0.07, 0.67, and 0.29.

Electron microscopy confirmed our light microscopic observations in five blocks with diffuse, dense RPE staining, in two blocks with “group 3 lesions,” in four blocks each with ora and ciliary body changes and three blocks with tracer escape around retinal blood vessels. The accuracy of light microscopic examination was tested in the following way: four areas of diffuse, dense RPE staining in four different blocks from three different animals appeared very mild, eg, on light microscopic observation, there was only a questionable mild cytoplasmic densification as compared to adjacent normal cells. Electron microscopic observation of these cases failed to confirm diffuse cytoplasmic staining with HRP. Therefore, such areas were not included into Tables 2 and 3.

We found 13 tissue blocks with dense diffuse RPE staining in nine animals (four STZ-treated, five controls). In seven blocks the retina was attached; in two minimally detached by artifact, but present and evaluated; in four blocks the retina was artifactitiously detached and not available for evaluation. Tracer escape around retinal blood vessels occurred in four tissue blocks of three animals.

Discussion

The horseradish peroxidase (HRP) method of examining the blood-retinal barrier in STZ-hypergly-
Diffuse Cytoplasmic Staining of Cells by HRP

In the RPE this has been noted in both STZ-treated hyperglycemic and in untreated normoglycemic control rats. In the two studies cited experimental conditions and results varied. One group of authors examined both eyes of young rats, six diabetic and six control animals, initially weighing 145–185 g, 3 to 5 weeks after the beginning of the experiment allowing ten minutes tracer circulation time. The authors found occasional RPE staining in both diabetic and control eyes and attributed this not to the experimental condition. Instead, they directed attention to the fact that staining of the cytoplasm by HRP has been considered by some an artifact of the histochemical tracer method.

The second group of authors examined old rats, four STZ diabetic and four control animals, initially weighing 400–500 g, 8 days after the beginning of the experiment, allowing 30 min tracer circulation time. They found variable numbers of tracer stained RPE lesions. In tabulating their data they showed RPE lesions in six of seven diabetic eyes quantitatively studied, but in only one of four normal control eyes. RPE lesions in their STZ-hyperglycemic animals differed also in quality from RPE lesions in normoglycemic rats. In hyperglycemic animals three types of lesions were distinguished: (1) Diffuse dense tracer...
staining, (2) RPE necrosis with tracer extension within the subretinal space, and (3) RPE regeneration over degenerating RPE cells. In their control rats only type 1 lesions were seen.

Our results are consistent with some of their findings, but inconsistent with others. In control rats we also saw type 1 lesions only. Equivalents to their type 3 lesions were found only in STZ-treated animals and consisted of areas of hypopigmented RPE arranged in a single line associated with other pigmented cells underneath this line or on top of this line. On the other hand, we cannot confirm a significantly increased frequency of type 1 lesions in STZ animals. Also, type 2 lesions were not found in our material.

Type 1 and type 2 lesions may be seen as stages of the same process. Both indicate cell degeneration. Type 1 lesions may occur initially. They are described now in three reports in both groups of experimental animals, hyperglycemic and normoglycemic. Thus, the lesion seems nonspecific. Only frequency is con-
troversial making further study an affair of sample size and quantitative morphology.

Type 2 lesions were seen by only one group. RPE cells altered enough to permit cytoplasmic flooding by the foreign protein tracer HRP may at some time break down further. Tracer could then escape into the subretinal space, but be demonstrable only if tracer injection, death of the animal, and tissue sampling would have occurred at precisely the right time and location. Further clarification again would depend on more quantitative data.

Type 3 lesions were not found in one study; in the other, they were present only in STZ-treated hyperglycemic rats. We found type 3 lesions also in STZ-treated animals only but see difficulties in accepting this as a significant finding. Pathologically, type 3 lesions represent areas of RPE regeneration. These must have originated from acute changes comparable to those seen in type 1 and type 2 lesions. Why should acute changes be found in both control and STZ rats, but evidence of their repair only in the latter?

Diffuse cytoplasmic staining was also seen in the ciliary body which is particularly sensitive to changes of its environment. In the normal stage HRP enters the lateral and apical intercellular spaces of the pigmented epithelium, but passage between the cells of the nonpigmented layer is prevented by zonulae occludentes. Microvesicular transport across the cytoplasm bypassing the zonulae occludentes is considered insignificant, and does not amount to tracer deposition in posterior or anterior chambers. Similarly, in our animals, no intercellular passage of HRP was seen. In all animals some cells of the nonpigmented ciliary epithelium showed diffuse dense cytoplasmic staining. Statistically, no significant difference was recognized between hyperglycemic and control animals. A particular situation is present at the ora serrata where a physiologic leak of the blood-retinal barrier has been described by others. We confirmed, in the rat, the presence of an ora leak present in all STZ-treated and control animals with satisfactory tissue sections.

**HRP Leakage from Retinal Blood Vessels**

In experimentally diabetic dogs following five years of intentionally poor metabolic control, HRP seemingly permeated junctions between degenerate endothelial cells of retinal blood vessels. The junctions in normoglycemic control dogs remained tight. In STZ “diabetic” rats, maintained 3 to 6 months, a similar junctional insufficiency was demonstrated. It was also reported that after 2 months, the number of tracer filled pinocytotic vesicles in the cytoplasm of endothelial cells of retinal capillaries was significantly higher than those in the control group and in the diabetic group maintained for only 1 month. Both studies included extensive quantitative evaluation or morphometric analysis by electron microscopy.

In two other studies discussed before, quantitative data based on electron microscopy were not given. No leakage from the retinal vasculature was shown. Occasional dense diffuse staining of endothelial cells was noted in both diabetic and control eyes despite premedication of the animals with antihistamines.

Our findings in this present study were based on light microscopic screening. If a blood vessel was not suspected on light microscopic examination to leak tracer, a specific electron microscopic evaluation of the neurosensory retina was not performed. Blood vessel leakage occurred in two vessels of two control animals and in two vessels of one STZ-hyperglycemic animal. The appearance of leakage was comparable to the description given by others. Tracer impregnated the vascular basement membranes and filled intercellular tissue spaces around the vessels. We did not determine the morphologic pathway of leakage. The three animals showing vascular leakage belonged to groups not pretreated with antihistamines (groups A, B). In the third group (C) where such treatment was applied, no retinal vascular leakage was observed. We suspect, therefore, that leakage in our animals was influenced by HRP-toxicity known to be significant in rats. Additional information is needed in order to more conclusively assess the possible role of STZ-hyperglycemia on the permeability of retinal vessels.

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals</th>
<th>RPE</th>
<th>Retina</th>
<th>Ora</th>
<th>Ciliary body</th>
</tr>
</thead>
<tbody>
<tr>
<td>STZ</td>
<td>6</td>
<td>1 (1)</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Control*</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>STZ</td>
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<td>2 (1)</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Control</td>
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<td>0</td>
<td>0</td>
<td>1§</td>
<td>4</td>
</tr>
<tr>
<td>STZ</td>
<td>8</td>
<td>1 (2)</td>
<td>0</td>
<td>7§</td>
<td>8</td>
</tr>
<tr>
<td>Control†</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>4§</td>
<td>5</td>
</tr>
</tbody>
</table>

*† See footnotes †, and §, respectively, of Table 2.
‡ First figures give rats with diffuse cytoplasmic staining of the RPE. Listed in brackets are the animals which had “group 3 lesions” only. In addition, group 3 lesions were present in some STZ-rats that also had diffuse cytoplasmic staining, ie, in one rat each at 6 and 10 months.
§ In these groups only one, seven, or four animals, respectively, had sections passing through the ora.
Key words: blood-ocular barrier, retinal pigment epithelium, retinal blood vessels, horseradish peroxidase, streptozocin-induced hyperglycemia, rats.

Acknowledgments

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References