Increased permeability of retinal blood vessels in human diabetic retinopathy is well known clinically. Its morphologic equivalent is unknown. In dogs with 5 years of poorly controlled alloxan diabetes and nonproliferative diabetic retinopathy comparable to that of man, permeability and patency of retinal blood vessels were tested with the protein tracer horseradish peroxidase and evaluated by electron microscopy. A breakdown of the blood-retinal barrier was found associated with extensive tracer leakage around retinal blood vessels. Tracer had seemingly permeated endothelial junctions, and was not transported through the endothelial cytoplasm. Blood vessels which had lost their endothelial cells and were partially occluded by glial cells retained some patency to tracer. These findings suggest the following. (1) Endothelial tight junctions are not a static cell specialization but one that can open due to chronic metabolic or osmotic factors prevailing in diabetes. Opened tight junctions may account for plasma leakage seen clinically in human diabetic retinopathy. (2) In the absence of endothelial cells perfusion does not necessarily end abruptly. The tracer method and electron microscopy may show details of vascular obstruction that are not readily demonstrated clinically.

Key words: retinal blood vessels, experimental alloxan diabetes, intercellular junctions, permeability, patency, horseradish peroxidase.

Clinical evidence indicates that alterations of vascular permeability are among the very early changes of human diabetic microangiopathy. Even the normally tight blood-retinal barrier appears to be affected early: extravascular leakage of fluorescein is well known clinically, has been observed reportedly without recognizable anatomic changes, and may even precede any clinically visible lesions such as capillary closure and microaneurysms. The morphologic basis of these permeability changes...
of retinal blood vessels in diabetes is unknown and cannot be systematically investigated in man.

Disturbance of vascular patency is another early sign of diabetic retinopathy. Clinically in fluorescein angiograms areas of capillary nonperfusion have been observed early in the disease. Nonperfused capillaries seemingly correspond to blood vessels that have become acellular. By electron microscopy an equivalent of such an acellular blood vessel has been shown in diabetic dogs. It consisted of a basement membrane tube filled with cytoplasm of glial cells. Other electron microscopic observations on diabetic retinopathy have been confined mostly to the description of microaneurysms and of basement membrane thickening both in man and in dogs. Fine structural information on vascular patency during stages that precede occlusion is lacking.

Table I. Summary of animal material

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<th>Nondiabetic control dogs</th>
<th>Alloxan-diabetic dogs</th>
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<td>Dog 1</td>
<td>Dog 2*</td>
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<td>Age (yr.)</td>
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<td>Duration of diabetes (yr.)</td>
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<td>Minutes between HRP injection and enucleation</td>
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*Given alloxan, at age 1.5 years; transient mild hyperglycemia and glucosuria for 2 days; nondiabetic.
Fig. 2. Retinal capillaries from outer plexiform layer of nondiabetic control dog 1. (A, Right eye enucleated 10 minutes after tracer injection. B, Left eye enucleated 20 minutes after tracer injection.) Electron micrographs show reaction product confined to lumen. Pinocytotic vesicles (arrows) of endothelial cell cytoplasm do not contain reaction product. rbc, Red blood cell; tj, tight junction. The vessel basement membranes (bm) and perivascular tissue are free of reaction product. Note absence of basement membrane between endothelial cell and pericyte (p) near heavy arrows and "Swiss-cheese" vacuolization of basement membrane (double arrow) in B. (A, x11,700; B, x15,000.)

Dogs made diabetic experimentally have been shown to develop capillary aneurysms and other abnormalities common to diabetic retinopathy in man. The availability of this animal model presents an opportunity to examine permeability and patency of vessels during the development of retinopathy. In the present study, the vascular system of diabetic dogs has been injected with a tracer, horseradish peroxidase (HRP) at a time subsequent to the appearance of capillary aneurysms.

Materials and methods

Animals. Three eyes of three diabetic dogs and three eyes of two nondiabetic dogs were used for this report (Table I). Healthy young adult female beagle dogs with normal ocular fundi were made diabetic by alloxan. Diabetes was intentionally poorly controlled for 5 years by daily doses of insulin insufficient to prevent constant severe hyperglycemia and glucosuria. Nondiabetic control dogs were housed and fed for 5 years together with the alloxan-diabetic animals.

Tracer technique and tissue processing. Each of the five animals was placed under surgical anesthesia with pentobarbital (Nembutal) and injected intravenously with HRP (Type II; Sigma Chemical Co., St. Louis, Mo.), 180 mg/kg, of body weight dissolved in normal saline. The eyes were enucleated at various intervals after injection as specified in Table I, opened coronally, and promptly fixed in a mixture of 1 percent glutaraldehyde and 1 percent formaldehyde in cacodylate buffer at pH 7.4. In all animals a nasal sector of nontapetal retina adjacent to the tapetum and comprising approximately ⅓ to ⅔ of the retina was dissected after 1 hour of fixation and the remainder of the tissue was processed for conventional light microscopic evaluation. The nasal sector was returned into fixative for a total fixation time of 3 hours. This tissue was then thoroughly rinsed in cacodylate buffer, left overnight at 4°C, and arbitrarily divided into posterior, midperipheral, and anterior thirds. Each of these thirds was sectioned at 40 μ slices and incubated for peroxidase activity. Subsequently the tissue was postfixed for 1 hour in Caulfield's solution, dehydrated in graded alcohols, and embedded in Epon. Sections 1.5 μ thick were stained with toluidine blue and studied by light microscopy. Thin sections were stained with uranyl acetate and lead citrate and studied in an AEI Corinth 275 electron microscope. Some tissue portions were stained in the block with 0.5 percent uranyl acetate for 1 hour prior to dehydration and embedding. The sections evaluated were from the posterior and midperipheral retina. The terms “tracer,” “peroxidase,” and “reaction product” will be used...
Fig. 3. A. In diabetic dog 5, reaction product (RP) impregnates walls of two blood vessels shown between double arrows of Fig. 1, C. rbc, Red blood cell. B and C. Two capillaries from diabetic dogs 4 (B) and 5 (C) show pinocytotic vesicles (arrows) containing tracer which does not stain basement membrane (bm). n, Nucleus of endothelial cell. Note degenerate pericyte (p) in B and thickened basement membrane in C. (A, ×2,000; B, ×10,600; C, ×9,300.)

Results
The fundi of the control dogs were unremarkable on clinical observation and on inspection at autopsy. Histologic examination of digest preparations by criteria detailed elsewhere12 showed the retinal vasculature to be normal. On light microscopic examination of retinal cross sections from tissue processed for the HRP reaction and embedded in Epon, the dark tracer was found only in vascular lumina.

Clinical evaluation of the fundus of diabetic dogs was impractical since they developed severe cataracts within 2 years of poorly controlled diabetes. At autopsy their fundi contained retinal hemorrhages (Fig. 1, A). In trypsin digest preparations, 22 to 76 capillary aneurysms, many pericyte ghosts, and numerous acellular capillaries were found (Fig. 1, B). In Epon sections from dog 5 (enucleated 20 minutes following injection of HRP) and dog 3 (enucleated 6 minutes after injection), but not from dog 4 (enucleated 10 minutes after injection), brown reaction product

interchangeably to designate presence or absence of HRP in the tissue.
Fig. 4. In diabetic capillary near triple arrow of Fig. 1, C, reaction product (RP) extends into interendothelial cleft (heavy arrow), impregnates basement membrane, and fills intercellular spaces of perivascular tissue (arrows). Note abundant cytoplasm and numerous RP-containing cytoplasmic vesicles of endothelial cells (end). n, Nucleus; rbc, red blood cell; p, pericyte. Inset A, RP is diffusely dispersed (arrow) in endothelial cytoplasm. Inset B, RP is localized to abluminal vesicles (arrow). In neither A nor B does RP stain basement membrane or fill intercellular space of perivascular tissue. (xl0,000; inset A, x9,500; inset B, x10,000.)

was seen to have escaped the lumen and to have impregnated vessel walls (Fig. 1, C).

Electron microscopic observations clarified these findings. In nondiabetic control animals reaction product remained confined to vessel lumina. It was rarely found within pinocytic vesicles on the luminal side of the endothelial cytoplasm (Fig. 2) and was absent from abluminal vesicles. The tight junctions (zonulae occludentes) between adjacent endothelial cells had a normal appearance consisting of a series of fusion points between the outer leaflets of the plasma membrane (inset, Fig. 7). Generally, tracer was noted within the luminal portion of the intercellular cleft, but was absent beyond the first fusion point. No peroxidase was seen in the subendothelial space, within the basement membrane or within the extracellular space of the perivascular tissue.

In the diabetic dogs, reaction product had escaped from the lumina of some small and large blood vessels and had infiltrated the basement membrane (Fig. 3, A). In other blood vessels, no evidence of such leakage was seen. In both leaky and nonleaky ones, tracer was invariably present in pinocytotic vesicles on the luminal side of and within the cytoplasm (Fig. 3, B and C) and also was occasionally present in abluminal vesicles (Fig. 4, inset B). In some instances, tracer appeared to be diffusely dispersed within large regions of
Fig. 5. Figs. 5, 6, and 7 show retinal blood vessels from diabetic dog 5. Reaction product fills lacuna of interendothelial cleft between segments where endothelial cell membranes appear fused (arrows). Lumen (L) and basement membrane (bm) also contain reaction product. This print and others (Figs. 7 and 8) have been intentionally underdeveloped so that dark tracer does not overshadow cellular detail. (x33,000.)

Leaky blood vessels, small and large, showed alteration of the interendothelial tight junctions. Tracer was observed within long portions of the interendothelial clefts (Figs. 4 to 6). Throughout junctions there was gaping between the outer leaflets of the plasma membrane without points of fusion, and tracer was present within the interendothelial space and within the basement membrane (Fig. 7). At times, tracer seemed to continuously extend from the lumen through the interendothelial cleft into the basement membrane and into the extracellular space of the perivascular tissue (Fig. 8). Leaky blood vessels were lined by two types of endothelial cells. In one type, nuclei were encountered frequently (Fig. 4) and the cytoplasm was abundant and contained numerous organelles (Figs. 4, 5, 7, and 8). These cells were resting upon a thick (and therefore most likely preexisting) basement membrane. They were interpreted as newly formed dedifferentiated cells which were replacing others. In the second type of leaky blood vessel (Fig. 6), the basement membrane was also thick, but nuclei were seen less frequently and the cytoplasm was scant and contained few organelles. These cells were interpreted as resting endothelial cells. Leakage was found more often between the newly formed cells than between the resting ones.

In several instances, small retinal blood vessels were noted in which endothelial cells lined a perfused lumen, but lacked a basement membrane (Fig. 9). These vessels were interpreted as examples of intraretinal neovascularization examined at a stage prior to the production of basement membrane material. The few examples of such blood vessels which we have studied so far were not associated with tracer leakage.

A spectrum of occlusive changes was
seen in small and large vessels. Endothelial cells were often degenerate and reaction product sometimes was noted within the remaining lumen despite disintegration of the endothelium into globular debris (Figs. 10 and 11). An occluded vessel was observed in which the endothelium was absent (Fig. 12). The vascular basement membrane was discontinuous, and the space inside the basement membrane was partially filled by cytoplasmic extensions of active-appearing unidentified paravascular cells. Peroxidase was absent from this vessel. In other blood vessels which lacked endothelial cells the vessel walls were collapsed with apposition of the basement membrane leaflets to each other (Fig. 13, A), or the basement membrane tube was filled by cells interpreted as astrocytes (Fig. 13, B and C). In several instances, peroxidase was seen within and around such vessel remnants (Fig. 13, C).

Comment

In this study on dogs with 5 years of experimental alloxan diabetes the permeability of retinal blood vessels to HRP, a protein tracer of 40,000 m.w. and approximately 50 Å particle size, was pathologically increased. The evidence for increased permeability consisted of the demonstration that in two diabetic dogs tracer reaction product had accumulated within the vascular basement membrane and the extravascular retinal parenchyma. This distribution pattern suggested retinal
blood vessels as the source of leakage and prompted search for the morphologic pathway of leakage.

The normal retinal vasculature is impervious to protein, a property shown to rest in the blood-retinal barrier.3-4 This barrier, in blood vessels, is a result of two components: (1) focal obliterations of the intercellular space between adjacent endothelial cells (such areas of obliteration have been termed tight junctions, or zonulae occludentes) and (2) absence of transport of protein by vesicular transfer through the endothelial cytoplasm. Insufficiency of either or both of the components could cause a breakdown of the barrier resulting in a pathologic increase of permeability.

Evidence highly suggestive of a junctional insufficiency was found in our diabetic dogs. Normally “tight” junctions had apparently split and allowed tracer passage out of vascular lumina into the surrounding tissue. This interpretation is based on three observations: (1) absence of fusion areas from the membranes of adjacent endothelial cells, leaving a gap between the outer leaflets (Fig. 7); (2) apparent continuity of reaction product from a vessel lumen through an interendothelial cleft into the basement membrane (Fig. 8); and (3) aggregation of tracer within interendothelial pools between successive tight junctions (Fig. 5). Alternate explanations for our observations seem less satisfactory.

With regard to observation 1, the objection could be raised that the luminal portion of the interendothelial cleft shown in Fig. 7 is not sectioned at right angles to both cell membranes and does, therefore, not allow an interpretation of func-
Fig. 8. Retinal blood vessel of diabetic dog 3 (enucleated 6 minutes after tracer injection). Reaction product fills lumen (L), interendothelial cleft (heavy arrow), basement membrane (bm), and intercellular spaces of perivascular tissue (arrows). end, Endothelial cells. (x21,000.)

Differential openness or tightness. On the other hand, for the greatest part of the cleft, the cell membranes can be followed and can be observed to lack a pentalaminar configuration in two areas of closer approximation, i.e., to lack fusion areas.

With regard to observation 2, one could point out that the terminal portion of the abluminal end of a gaping junction in Fig. 8 is sectioned slightly obliquely, permitting an assumption that either (a) the junctional cleft might not have been depicted at the very end of its tortuous course onto the basement membrane, actually terminating elsewhere, or (b) an area of membrane fusion might be hidden within the blurry obliquely sectioned zone.

With regard to observation 3, one might argue that tracer might escape the circulation at some distance from a junction and penetrate the abluminal end of a junction in a retrograde fashion. If such a distant source of leakage were present but not observed, retrograde tracer flux from this source would not explain the presence of peroxidase in the middle of an interendothelial cleft unless fusion areas had been opened. If this is accepted, however, then it would be more reasonable to assume that orthograde flux supported by local blood pressure has led to opening of successive fusion areas.

Two other factors support our interpretation that opened endothelial junctions were responsible for the observed perivascular leakage of tracer protein: (1) splitting of tight junctions in other experimental conditions and (2) lack of appreciable cytoplasmic transport of HRP in this study.

Conditions under which junctional cleavage has been previously observed include experimental hypertension, osmotic stress, tumor growth, and regeneration after mechanical injury. In experimental hypertension, tight junctions of endothelial cells of retinal blood vessels were penetrated by HRP. Rapid protein escape
Fig. 9. Figs. 9 to 12 are from same retina of diabetic dog 5. Newly formed retinal blood vessel of diabetic dog 5 near inner limiting membrane (lim). Endothelial cells (end) lacking a continuous basement membrane are joined by tight junctions (arrows) and form a lumen (L) filled with reaction product. Perivascular cells (p) may represent prospective pericytes. One of them is in close apposition (heavy arrow) to endothelial cell. (×4,300.) Inset A, Interendothelial cleft at left of main picture, by higher magnification. The terminal end of interendothelial cleft is sectioned obliquely and appears blurry. Reaction product stops before terminal end. (×11,000.) Inset B, Another example of new vessel formation near inner limiting membrane. (×3,800.)

through apparently open tight junctions was seen in another experiment following a pulse of heightened hydrostatic pressure and suggests that the mechanism of junctional opening in hypertension is due to mechanical distention.19 In osmotic junctional cleavage of endothelial cells of brain vessels studied by HRP13 or in osmotic cleavage of tight junctions of hepatocytes studied by the freeze-fracture technique,16 a similar mechanical distention can be assumed. In fact, osmotic shrinkage of endothelial cells has been postulated to produce physical separation of the external membrane leaflets.20 This separation can be reversible, implying that also graduated openings occur.21

Interendothelial clefts are also open and lack the expected pentalaminar fusions in blood vessels of malignant brain tumors17 and in capillaries of the spinal cord proliferating after mechanical injury.18 Regenerating or proliferating capillaries are morphologically and functionally dissimilar from mature capillaries. Leakage in immature capillaries may be related to incomplete junctional differentiation of the endothelial cells, although this cannot be demonstrated in cross sections. Once junctions have split, the junctional membranes are indistinguishable from adjacent nonjunctional membranes.16 Thus, in immature blood vessels with dedifferentiated endothelial cells, study of thin-sectioned ma-
Fig. 10. Three capillaries showing occlusive changes. Endothelial cells of capillary at lower right (1) and at upper left (2) are degenerated into globular debris. Capillary 1 contains reaction product (RP). Capillary 2 has a discontinuous basement membrane (arrows) and is invaded by cells containing numerous filaments in their cytoplasm (heavy arrow). Capillary at upper right (3) has been invaded by similar cells filling the previous lumen. (×10,500.)

Material does not reveal whether a junction is open because of a lack of full differentiation or whether the junction had achieved full competence and was then cleaved.

Distentional damage and dedifferentiation should not be perceived as the only possible factors causing junctional splitting. In this investigation we observed a number of leaky retinal blood vessels in which newly formed endothelial cells were present. In these examples junctional immaturity may have been the mechanism of opening and leakage. Yet there were other examples of leaky junctions in seemingly mature cells. There is no reason to suspect distentional damage from high blood pressure or from acute severe osmotic stress. The condition under which they had become leaky was chronic in contrast to the acute nature of some of the conditions that induced junctional splitting in other studies. It seems likely, then, that other, presumably metabolic, factors may have rendered vascular endothelial cells and their junctional specializations more vulnerable. Junctions having reduced resistance conceivably might be cleaved, for instance, by osmotic stresses resulting from repeated daily excursions of blood sugar levels.

The extent to which the opening of the junctions in the diabetic dog may be relevant to diabetic retinopathy in man is difficult to assess. Protein leakage is a well-known clinical finding in the human diabetic patient presenting with hard exudates or macular edema. Also, subtle chronic plasma insudation might occur long before this and play a part in diabetic capillary basement membrane thickening.

Opening of supposedly tight junctions was discussed first in this comment because the pathologically increased permeability of retinal blood vessels after 5 years of experimental diabetes seemed to result from opening of tight junctions be-
tween vascular endothelial cells. Other routes of leakage were not apparent. Evidence for vesicular transport sufficient to account for protein leakage through endothelial cells reportedly consists of numerous peroxidase-filled cytoplasmic pits and vesicles on the abluminal side associated with extracellular tracer deposits onto and within the vascular basement membrane. Such evidence was not seen in the diabetic dogs. Two cytoplasmic routes other than vesicular transfer can also be ruled out as ways in which peroxidase crossed the endothelium. (1) Tracer can become freely dispersed in the cytoplasm to reach the basement membrane diffusely and passively rather than being actively carried in quanta bound to vesicles. Diffuse tracer dispersion was observed occasionally in the present study within retinal endothelial cells of diabetic dogs, but also in endothelial cells of nondiabetic control dogs and was not associated with leakage beyond the abluminal cell membrane. Identical diffuse cytoplasmic staining was seen in endothelial cells of cerebral vessels of rabbits after infusion of hyperosmolar urea. In that investigation diffuse cytoplasmic staining was regarded as an artifact in which the endothelial cell membrane had been injured by high concentrations of peroxidase or fixative, or by plasma hyperosmolarity, allowing subsequent passive flooding of the cytoplasm with tracer reaction product. (2) Tracer might cross the cytoplasm through transverse channels possibly originating from a confluent row of vesicles. While this has not been ob-

Fig. 11. Small retinal arteriole with disintegrated remnants of endothelium (end) contains residual lumen filled with reaction product (RP). (×8,200.)
Fig. 12. Occluded blood vessel with discontinuous basement membrane (arrows) is surrounded and invaded by paravascular cells containing irregular nuclei (n) and abundant cytoplasm with numerous dense bodies (db) resembling lysosomes. (×4,700.)

Fig. 13. Occluded capillaries lacking endothelial cells. A and B are from diabetic dog 4; C from diabetic dog 5. A, Vessel wall is collapsed with apposition of basement membrane leaflets. B and C, Previous lumen is filled by cytoplasmic processes containing numerous oriented filaments (fil). Reaction product is absent from A and B. In C, Reaction product fills intercellular spaces within and outside previous lumen (arrows). (A, ×9,700; B, ×6,900; C, ×9,600.)

served in the present study, it does apparently occur as discussed elsewhere.\textsuperscript{24}

We observed absence of tracer leakage after 10 minutes of HRP circulation, yet presence of leakage after 6 and 20 minutes. The absence of leakage after 10 minutes seems best attributed to one of the following two possibilities. (1) No leakage indeed occurred and the blood-retinal barrier to protein remained competent for 10 min-
utes because this retina possibly was insufficiently affected by the diabetes at the time of the experiment. (2) Alternately, leakage might have been present in at least some retinal areas but may have been missed in our morphologic evaluation due to the inherent technical problems of the sampling procedure. Cataracts develop in dogs within a year or two of poorly controlled diabetes, preventing selection of specific areas of clinical pathology for microscopic evaluation.

Some findings on vascular patency seem suitable to compare vascular occlusion as seen in this study by electron microscopy in eyes of diabetic dogs, with vascular occlusion as seen in previous observations by fluorescein angiography and retinal digest preparations in human diabetic eyes. In the diabetic dogs, retinal capillaries and also large blood vessels showed severe endothelial degeneration and even replacement by glial cells which invaded the previous vessel lumen; yet often there were still remnants of perfusion as interpreted by presence of intravascular reaction product. In the diabetic human patient, retinal blood vessels that lacked an endothelium reportedly were nonperfused. Our observations suggest that in the absence of endothelial cells perfusion apparently need not end abruptly, but may taper off for some distance into small intercellular channels within and around the old blood vessel. Perhaps perfusion of vessels with partial obstruction or slow flow is less readily demonstrated by fluorescein angiography than by the tracer method used in this study.

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REFERENCES
Experimental myelin intrusion in the nerve head

David G. Cogan and Robert D. Yee

On the assumption that myelin intrusion into the papilla and peripapillary region might occur with traumatic lesions of the optic nerve, a study was made of the clinical and histopathologic changes that might be expected. Nine monkey eyes were subjected to hemostat compression of the nerve close to the globe and studied over various periods up to 28 days. Myelin was demonstrable ophthalmoscopically, followed by variable and increasing amounts of hemorrhage. The myelin was demonstrable histopathologically only during the first 2 weeks after the manipulation and was then masked by the associated hemorrhage and gliosis. The optic nerve showed expected myelinolytic reactions.

Key words: myelin intrusion, optic nerve papilla, histopathology, demyelination.

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Myelin intrusion into the nerve head, adjacent subretinal space, prepapillary vitreous and retinal veins is a frequent finding in routine histopathology (Figs. 1