HISTAMINE, ZO-1 AND INCREASED BLOOD-RETINAL BARRIER PERMEABILITY IN DIABETIC RETINOPATHY

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ABSTRACT

Purposes: First, to develop an improved retinal capillary endothelial cell culture system which exhibits some of the physiologic features of the blood-retinal barrier; second, to use this model to determine how histamine and chemical conditions of diabetes effect expression of the tight junction protein, ZO-1; and third, to discuss application of the Henle-Koch postulates to the problem of diabetic retinopathy.

Methods: Bovine retinal capillary endothelial cells were exposed to varying serum and growth factor concentrations, as well as astrocyte-conditioned medium, in order to establish a model of the blood-retinal barrier. Cells were also exposed to varying concentrations of histamine, and of insulin and glucose. The expression of ZO-1 tight junction protein was determined by immunocytochemistry and immunoblotting.

Results: Modified concentrations of growth factors reduced endothelial cell proliferation, without reducing viability. Astrocyte conditioned medium increased ZO-1 protein content. Histamine reduced ZO-1 protein content. Both high glucose (20mM) and low insulin (10^{-12}M) reduced ZO-1 protein content compared to control conditions (5mM glucose and 10^{-9} M insulin).

Conclusions: Control of culture conditions results in a more physiologic in vitro model of the blood-retinal barrier. Soluble factors from astrocytes promote tight junction formation. Both histamine and chemical conditions of diabetes diminish tight junction formation. These factors may mediate blood-retinal barrier breakdown in diabetic retinopathy. Henle-Koch postulates for diabetic retinopathy are presented.

INTRODUCTION

Diabetic retinopathy remains a leading cause of blindness, in spite of laser and vitreous surgery.[1] Long-term prospective clinical trials have proved recently that strict metabolic control retards the development of retinopathy in patients with diabetes.[2,3] However, many patients are unable to maintain the degree of tight long-term glucose control attained in the rigorous environment of clinical trials. The cellular, biochemical, and molecular events

that lead to retinopathy remain uncertain, and at this point a definitive cure for diabetes remains elusive.[4] Diabetic retinopathy is likely to remain a major cause of visual impairment as the frequency of diabetes increases.[5] For these reasons, the mechanisms that lead to visual loss must be understood so that retinopathy may be prevented or delayed despite imperfect metabolic control.

Increased blood-retinal barrier (BRB) permeability is one of the cardinal pathophysiologic features of diabetic retinopathy, which ultimately results in retinal damage and may cause macular edema, the leading cause of visual loss in diabetes.[6] Histamine, a recognized mediator of microvascular permeability, has been recently proposed as an important pathogenic agent of BRB breakdown in a rat model of diabetes.[7-10] This study further explores the effects of histamine and antihistamines in vitro. The overall purpose of this study is twofold: first, to develop an improved retinal capillary endothelial cell culture system that exhibits some of the physiologic features of the BRB; and second, to use this model to determine how histamine and chemical conditions of diabetes effect expression of the interendothelial cell tight junction protein, ZO-1. These data regarding the basic mechanisms of histamine and antihistamines on the BRB may help to lay the foundation for a novel pharmacologic treatment for diabetic retinopathy.

The final aspect of this thesis deals with cause-and-effect relationships in the pathogenesis of disease, and the application of the Henle-Koch postulates to the problem of diabetic retinopathy. These postulates should help to clarify the role of histamine, as well as other potential mechanisms, in the initiation and progression of diabetic retinopathy. An understanding of the mechanisms of retinopathy will allow safe and effective means to prevent visual loss from diabetes.

HISTORICAL REVIEW

Retinopathy was first recognized as a complication of diabetes by von Jaeger in 1855.[11] He described the ophthalmoscopic findings of a 22-year-old man with "blood-red flecks...in the plane of the retinal vessels. Between the flecks, at some distance from the optic disc, there also appear numerous irregular, rounded, light-yellow spots whose brightness makes them very obvious."[12] This description suggests that the patient probably had macular hemorrhages and lipid exudates, the consequence of chronic BRB breakdown.

Diabetic retinopathy was not described commonly until the 20th century, because many patients with diabetes, particularly children, did not live long enough to develop chronic complications. The isolation of insulin by Banting and Best in 1922 initially offered a means to prevent short-term death. However, insulin and other improvements in the treatment of diabe-

tes have extended the life span of diabetic persons and paradoxically increased the problem of chronic microvascular and macrovascular complications. Since diabetic retinopathy was recognized, physicians have taken numerous indirect and direct medical and surgical approaches to achieve metabolic homeostasis and minimize microvascular complications.

TREATMENTS FOR DIABETIC RETINOPATHY

Great strides have been made in the classification and treatment of retinopathy since the days of von Jaeger, as shown in Table I.

TABLE I:

TIME LINE OF PROGRESS IN CLASSIFICATION AND TREATMENT OF DIABETIC RETINOPATHY, 1855 TO 1995

1855	1955-1959	1960-1969	1970-1979	1980-1989	1990's
Description of retinopathy	diet therapy; xenon photocoagulation	Natural history; Airlie House classification; pituitary ablation	Ruby, argon lasers; Pars plana vitrectomy; DRS	ETDRS; DRVS; standards of care by AAO, ADA	DCCT; Diabetes 2000

DRS: Diabetic Retinopathy Study
ETDRS: Early Treatment Diabetic Retinopathy Study
DRVS: Diabetic Retinopathy Vitrectomy Study
DCCT: Diabetes Control and Complications Trial
AAO: American Academy of Ophthalmology
ADA: American Diabetes Association

The first attempts to control diabetic retinopathy addressed the metabolism of the diabetic patient, specifically through diet. Diabetic patients often have elevated serum cholesterol and triglyceride levels, and these lipids and lipoproteins leak through diseased vessels to cause hard exudate deposition. Restriction of fat intake was shown to cause regression of hard exudates in patients with macular edema.[13,14] Oral hypolipidemic drug therapy of hyperlipidemic patients for 12 to 24 months also caused significant reductions in macular lipid exudates.[15-17] Neither diet nor drug control improved impaired visual acuity despite reducing lipid exudates, probably because of photoreceptor and pigment epithelium damage from chronic macular edema. However, the fact that control of hyperlipidemia could substantially improve retinopathy suggested that circulating lipids may impair vascular function. Recent laboratory findings that low-density lipoproteins are toxic to cultured retinal vascular endothelial cells and pericytes

support this early clinical observation.[18]

Investigators recognized early in the study of diabetes that hormones other than insulin may also influence the course of retinopathy. For example, testosterone reduces pituitary corticotropin (ACTH) production and insulin requirements.[19] However, testosterone administration did not produce any consistent beneficial effect on retinopathy in clinical trials.[20,21] Nevertheless, these observations, and the finding that hypophysectomy ameliorated diabetes in pancreatectomized dogs, suggested the importance of growth hormone; thus arose the first indirect surgical treatments for retinopathy, pituitary ablation.[22] This was accomplished through transsphenoidal hypophysectomy, external beam radiation, or insertion of Yttrim-90 radioactive implants.[23-26] While effective at reducing retinal neovascularization, these nonocular surgical approaches also carried a high systemic morbidity and mortality.

The results of pituitary ablation demonstrated that neovascularization could be controlled, but more direct ocular surgical approaches to diabetic retinopathy were needed in order to avoid neurosurgical complications and the systemic effects of hormonal manipulation. Therefore, direct treatment of retinopathy was developed. Xenon arc light photocoagulation was developed in Europe in the late 1950s; several years later the ruby and then the argon laser photocoagulators were introduced in the United States.[27-30] Photocoagulation treatment was directed initially onto visible neovascular fronds, and later to the ischemic peripheral retina to treat proliferative retinopathy. The xenon and laser photocoagulators were subsequently used to treat diabetic macular edema and rubeosis irides.[6,31] The availability of efficient photocoagulators, clarification of the natural history of untreated retinopathy, and the standardized Airlie House classification gave rise to the multicentered randomized clinical trials that proved that photocoagulation surgery is highly effective for the treatment of diabetic retinopathy.[32-35] In particular, the Early Treatment Diabetic Retinopathy Study established the importance of treating clinically significant macular edema to reduce the risk of moderate visual loss; previous pharmacologic studies of lipid-lowering agents had not resolved this point.

To treat more advanced complications of diabetic retinopathy that were not amenable to photocoagulation, Machemer and Blankenship applied pars plana vitrectomy to diabetes.[36] This method allowed surgeons to clear hemorrhagic vitreous cavities, remove preretinal membranes, treat traction retinal detachments, and thus rescue previously unsalvageable eyes. The effectiveness of vitrectomy for severe diabetic retinopathy has also been established in large randomized clinical trials.[37,38]

Because of the success of the laser treatment trials, the risk of bilateral severe visual loss (visual acuity < 5/200) from diabetic retinopathy has been reduced to about 1% when laser surgery is applied in a timely manner.[39] However, nearly 20 years after the first Diabetic Retinopathy Study report,

at least half of the persons at risk for visual loss from retinopathy fail to receive an eye examination.[40] Therefore, guidelines for ophthalmologic assessment of persons with diabetes have been established, and the Diabetes 2000 program has been initiated to educate physicians and patients about diabetic retinopathy.[41,42] Translation of the research findings of the 1970s and 1980s remains a major public health challenge in the 1990s and beyond.

Despite the benefits of both direct and indirect medical and surgical approaches, none of these treatments address the systemic metabolic perturbations or underlying pathophysiology of diabetic retinopathy. Patients who undergo successful laser or vitrectomy surgery may still lose vision. For example, in the Diabetic Retinopathy Study, loss of ≥ 5 lines of visual acuity developed in 13.7% of the treated eyes (versus 27.1% of untreated eyes).[34] In the Early Treatment Diabetic Retinopathy Study, eyes that underwent focal or grid treatment for clinical significant macular edema still had a 24% rate of persistent macular thickening at 36 months posttreatment.[35] Ten-year follow-up of eyes treated with vitrectomy and 15-year follow-up of eyes treated with photocoagulation in the Diabetic Retinopathy Study also showed continued visual loss.[43,44] In addition, there are substantial side effects, such as permanent visual field loss from photocoagulation. With these points in mind, much effort has been directed toward understanding the systemic and ocular factors that contribute to diabetic retinopathy.

GLYCEMIC CONTROL AND SYSTEMIC FACTORS IN DIABETIC RETINOPATHY

The relationship of diabetic retinopathy to glycemic control has been studied in animal models of diabetes, with epidemiologic studies, and through prospective clinical trials.

Retinopathy that most closely resembles that of humans is found in the alloxan-diabetic dog. Engerman and associates[5] found that dogs randomly assigned to good glycemic control developed significantly fewer microaneurysms, acellular capillaries, and pericyte ghosts than those animals randomized to poor glycemic control; dogs that received poor control further developed retinal hemorrhages and intraretinal neovascularization.[45] Engerman and Kern[46] found that the timing of institution of good glycemic control was important. Dogs assigned to good glycemic control from the outset developed very little retinopathy over 5 years, but those animals that were under poor control for the first 2 1/2 years and then under good control for the second 2 1/2 years, developed retinopathy at a rate essentially the same as those animals that were under poor control from the outset.

Epidemiologic studies have also found that the incidence and severity of retinopathy is strongly correlated with glycemic control, as reflected by glycosylated hemoglobin levels.[47-51] Specifically, Klein and associates[47] found that the risk of developing any retinopathy in patients in the highest quartile

of glycohemoglobin values was 1.9 times that of patients in the lowest quartile, and that the risk of developing proliferative retinopathy was 21.8 and 4.0 times greater, for younger- and older-onset patients, respectively.

Numerous short- and long-term prospective clinical trials have shown a clear benefit of tight glycemic control.[2,3,52-57] The Diabetes Control and Complications Trial demonstrated that tight glycemic control not only delays the onset of retinopathy in patients with no baseline lesions, but also slows the progression of mild nonproliferative retinopathy over a period of 6 years.[3,58] The benefit of achieving tight control in patients with established retinopathy contrasts to the findings in diabetic dogs, in which good glycemic control did not stop progression of incipient retinopathy.[46] The positive findings in humans may be due to the longer duration of the clinical trial (mean, 6 years versus 2 1/2 years for the dogs).

In addition to the association between metabolic control and retinopathy, genetic factors have also been associated with diabetic retinopathy. Specifically, the HLA haplotypes DR-3 and DR-4 have been reported to increase the risk of proliferative retinopathy.[51] However, a recent study refutes this finding and suggests an association with immunoglobulin IgG2.[59] The possibility remains that genetic factors unrelated to HLA antigens determine susceptibility to diabetic complications.

From a clinical standpoint, these trials of metabolic control are of great value in the treatment of patients with diabetes. Moreover, they establish the priniciple of specific pharmacologic treatment for retinopathy. From an etiologic viewpoint, animal, epidemiologic, and clinical studies also illustrate how hyperglycemia and other factors interact over the course of time to result in retinopathy. That is, the longer any metabolic disturbance persists, the more likely it is to damage microvascular function and structure. However, these studies do not address the issue of the specific factors that initiate or accelerate diabetic retinopathy, because insulin treatment affects many aspects of carbohydrate, lipid, and protein metabolism. For this purpose experimental models of diabetes have been instructive.

THEORIES ON THE PATHOGENESIS OF DIABETIC RETINOPATHY

Molecular, biochemical, and cellular alterations have been described in animals and cell culture models of diabetic retinopathy. Deficient insulin action (from insulin deficiency or insulin resistance) and hyperglycemia are the major systemic metabolic alterations in diabetes mellitus. Alone or in combination, these abnormalities likely initiate biochemical events that lead to diabetic retinopathy. Glucose-mediated alterations in cellular metabolism have been explored extensively, and the two most popular hypotheses have been activation of the polyol pathway and nonenzymatic glycation.

In the polyol schema, aldose reductase converts glucose to sorbitol, and sorbitol dehydrogenase converts sorbitol to fructose. These reactions may damage cells by osmotic stress, reduced intracellular *myo*-inositol levels, or

alterated redox states.[60] Polyol pathway abnormalities have been found in peripheral nerves, kidneys, and retinas of diabetic animals, and in retinal vascular cells cultured in excess glucose.[61,62] Greene and associates[61] have proposed that inadequate discrete intracellular *myo*-inositol pools lower inositol availability for phosphoinositol synthesis. The means by which reduced inositol would lead to retinopathy is, however, not well explained. Sodium-potassium ATPase activity, which is thought to change secondary to polyol pathway activation, is not universally decreased in the retinas of diabetic animals.[63] In addition, *myo*-inositol levels are increased, not decreased, in most tissues of diabetic rabbits.[64] From a practical standpoint, potent aldose reductase inhibitors do not inhibit development of retinopathy in diabetic dogs, or short-term progression of very mild retinopathy in humans, although they may do so in rats.[65-67] A long-term (7-year) clinical trial of the aldose reductase inhibitor Tolrestat is near completion, and could show some benefit.

The polyol pathway hypothesis of retinopathy was constructed largely on two observations. The first is that pericytes are "selectively" lost in human retinal vessels from subjects with diabetes.[68] However, this widely quoted light microscopic study lacked important details regarding the anatomic regions from which the images were taken, gave no statistical analysis of pericyte dropout or other morphologic lesions, and did not determine if pericyte loss occurred in areas without microaneurysms. Hence, it cannot be interpreted to determine that pericytes are lost first or preferentially in diabetic retinopathy. Another study found no definite pericyte loss.[69] The second observation is that aldose reductase is found primarily in pericytes, rather than endothelial cells.[70] However, these features do not alone adequately explain the features of diabetic retinopathy that account for decreased vision—increased retinal vascular permeability and capillary closure. Thus, although polyol pathway abnormalities occur in diabetes, a definite causal relationship with retinopathy remains to be established.

A different approach suggests that nonenzymatic glycation of proteins and nucleic acids occurs when glucose, fructose, and triose phosphates react with free amino groups. Subsequent rearrangements, dehydration, and cleavage reactions result in advanced glycation end-product formation. Advanced glycation end products stimulate endothelial cell interleukin-1 and tumor necrosis factor production, increase cross-linking of Type IV collagen in vascular wall basement membranes, and impair synthesis of extracellular matrix components of basement membrane, such as fibronectin and laminin.[71,72] These abnormalities might increase vascular wall permeability. Increased levels of circulating advanced glycation end products have been found in humans with diabetic retinopathy.[73] In a rat model, aminoguanidine treatment to inhibit advanced glycation end-product formation reduced experimental diabetic retinopathy.[74] However, aminoguanidine, a semicarbazide derivative, also inhibits other reactions, including aldose re-

ductase, catalase, and S-adenosylmethionine decarboxylase, nitric oxide synthase, and histidine decarboxylase, so aminoguanidine cannot be used as a specific probe to provide evidence for the role of nonenzymatic glycation.[75-78] Although nonenzymatic glycation is certainly increased in diabetes, "direct proof that these glycosylation processes can cause tissue injury in diabetes in humans is currently lacking...."[79] Hopefully, more specific nonenzymatic glycation inhibitors will be developed to further explore this fascinating mechanism.

Alternative explanations for extracellular matrix changes in diabetes have also been considered in light of the potential impact of matrix on endothelial cell viability, migration, and replication. Roth and associates[80] have recently found overexpression of fibronectin and the integrin receptor for fibronectin, laminin, and collagen in human umbilical vein endothelial cells exposed to 30 mM glucose. The cells exposed to high glucose attached more tightly to fibronectin and laminin. They also found increased immunostaining for β_1 integrin in the retinal vessels of humans with diabetes versus those without diabetes.[80] However, these findings of increased endothelial cell adhesion to extracellular matrix in response to high glucose appear to be in contrast to those of Mandarino,[81] who found decreased adhesion in vitro.

In contrast to the mechanisms already discussed, which are based on factors within the vascular wall, other hypotheses deal with intraluminal events in diabetic retinopathy. Increased platelet aggregation has been reported in patients with diabetic retinopathy, along with decreased fibrinolysis; together, these abnormalities could produce capillary closure.[82] However, treatment trials of aspirin, dipyramidole, or ticlopidine in patients with mild to moderate nonproliferative retinopathy did not produce clear evidence of benefit.[83-85] Therefore, either the laboratory alterations in blood elements lack a cause-and-effect relationship to the progression of retinopathy, or the medications do not adequately inhibit the abnormalities.

Although the sequence and relative importance of intracellular and intercellular biochemical disturbances remain highly controversial, there is general agreement that the hemodynamic changes of increased BRB permeability, vasodilation, and increased retinal blood flow accompany the onset of clinically evident diabetic retinopathy.[86-90] Parving and associates[88] have suggested that increased microcirculation hydrostatic pressure and vasodilation lead to enhanced leakage of plasma proteins through the vascular wall. Disturbances in the renin-angiotensin system, prostaglandins, and nitric oxide release have been offered as signal transduction mechanisms for loss of retinal vascular autoregulation.

Another potent vasoactive agent, histamine, produces microvascular vasodilation and increased permeability. This study now examines evidence that histamine could account for at least one aspect of impaired autoregulation in experimental and human diabetic retinopathy—BRB breakdown.

HISTAMINE, BLOOD-RETINAL BARRIER BREAKDOWN, AND DIABETIC RETINOPATHY

To understand the BRB, it is instructive to consider the blood-brain barrier. These two barriers share common neuroectodermal embryologic origins and structural and functional characteristics.[91] The blood-brain barrier has very impermeable endothelial junctions and associated high electrical resistance, up to 1,800 $\Omega \cdot cm^2$. The barrier feature is induced by astroglial cells whose processes wrap around brain blood vessels.[92,93] Müller cells, the primary glial cells of the retina, provide the same function in the eye.[94] Studies with in vitro models of the blood-brain barrier have shown that exposure of cultured brain capillary endothelial cells to astrocytes or astrocyte-conditioned medium and cyclic AMP analogs induces high levels of electrical resistance and low levels of permeability, indicative of tight junction formation.[95,96] Such studies have not been performed previously on the BRB, but similar changes would be predicted.

The mechanism by which vasoactive compounds and diabetes induce breakdown of the BRB is fundamental to the pathogenesis of diabetic retinopathy. Tracer studies have shown that horseradish peroxidase migrates through opened interendothelial cell tight junctions (zonula occludens) in retinal vessels of rats and dogs with diabetes.[97,98] Histamine, one of the most potent vasoactive agents, induces endothelial cell contraction and opens interendothelial cell tight junctions in rat cremaster muscle of nondiabetic rats.[99] This effect of histamine is most pronounced in postcapillary venules, where histamine H_1 and H_2 receptors are most densely concentrated.[100] Intravascular histamine infusion causes leakage and reduced electrical resistance of cerebral vessels, which is reversed by histamine H_2 receptor antagonists.[100-105] Furthermore, diabetic rats developed increased permeability of the blood-brain barrier within 2 to 4 weeks of the onset of hyperglycemia, which is reversed by histamine H_1 receptor antagonists.[106-109]

Histamine is present in three distinct pools within the body.[110] Two of these pools exhibit slow turnover and are found in mast cells and abdominal viscera. The third pool has a short half-life (3 to 6 hours) and undergoes rapid depletion following histamine synthesis inhibition. This rapid turnover, inducible histamine pool has long been known to play a role in microcirculation control.[110] Non–mast cell histamine is formed from histidine precursor in both large and small vessel walls via histidine decarboxylase.[111-113] Histamine increases aortic endothelial cell permeability.[113] Intrinsic vascular wall histamine causes vasodilation and vascular hyperpermeability in various microcirculatory beds, including the kidney.[114-118] Antihistamines induce contractile responses of microvascular smooth muscle mediated by both histamine H_1 and H_2 receptors and, in part, by norepinephrine-induced vasoconstriction.[119,120] Histamine infusion in nondiabetic rats mediates leakage of fluorescein isothiocyanate-bovine serum albumin (FITC-BSA) into the vitreous cavity. This leakage is reversed by histamine H_1 receptor antagonists.[121]

The evidence for a pathogenic role of induced histamine in vascular manifestations of diabetes follows. First, aortic endothelial and smooth muscle cell histamine content and histidine decarboxylase activity are increased 138% and 250%, respectively, over control values in streptozotocin diabetic rats. Insulin therapy returns these values to normal.[122] Aortic histamine synthesis inhibition and FITC-BSA accumulation in the aortic wall are significantly correlated.[123] This indicates that macromolecules gain entry to the vessel secondary to increased permeability and contribute to vascular wall injury. Second, blockade of aortic histamine synthesis by the specific histidine decarboxylase inhibitor, *a*-hydrazino histidine (*a*-HH), prevents increased aortic intima-media^{125}I albumin mass transfer rates, despite persistent hyperglycemia.[124] Aortic albumin accumulation is also inhibited.[125] Third, renal histamine is significantly increased by 45% in streptozotocin diabetic rats. *a*-HH and insulin each reduce renal histamine concentration and total histamine content to normal.[126] Fourth, in addition to vascular tissue, plasma histamine levels in untreated diabetic rat are increased 67% above nondiabetic control values. Insulin and *a*-HH each return histamine levels to control levels.[127] The finding of elevated plasma histamine in diabetic rats has recently been confirmed.[128] Gill and associates[129,130] have also found significantly increased histamine content in aortas and kidneys of streptozotocin-diabetic rats and in plasma of diabetic patients. Together, these data show that histamine synthesis is specifically stimulated by the diabetic state, is reversible by insulin and histidine decarboxylase inhibitors, contributes to vascular wall injury, and is systemic.

In addition to its location in large vessels and the kidney, non-mast cell histamine has also been localized to the P-1 centrifugation fraction of retina containing microvessels, and in human retina.[131-133] Mast cells are not present in the retina.[132] Histamine H_1 receptors are found in the retina and on rat retinal vessels, with a dissociation constant (K_d) of 2.2 to 3.8 nM.[134-137] Histamine has an inhibition constant (K_i) for [^{3}H]-mepyramine binding of 7.3 x 10^{-5} M in bovine retina.[137] Histamine induces endothelium-dependent relaxation of bovine retinal arteries.[138] Histamine infusion into nondiabetic rats for 1 week, in a concentration found in diabetic rat plasma (45 ng/mL) causes a statistically significant increase in vitreous FITC-BSA accumulation compared with controls. Cotreatment with the H_1 antagonist diphenhydramine (Benadryl normalized leakage to control values, but the H_2 receptor antagonist cimetidine (Tagamet) effected no significant reduction of vitreous FITC-BSA accumulation.[121]

Hollis and associates[10] have developed the following evidence for a role of histamine in BRB breakdown in streptozotocin diabetic rats. First, vitreous FITC-BSA accumulation increases 98% in diabetic animals after 4 weeks of hyperglycemia. When administered via subcutaneous osmotic minipump for the last week of a 4-week study period, diphenhydramine and ranitidine (an H_2 antagonist, Zantac) lowered vitreous FITC-BSA content 35% and

51%, respectively, compared with untreated diabetic animals ($P<.05$); the combination of diphenhydramine and ranitidine lowered the same parameter by 71%.[7] In a parallel study, insulin treatment significantly lowered vitreous FITC-BSA content 37% below untreated diabetic values, while insulin and diphenhydramine, when combined, lowered FITC-BSA content 63%. However, a different H_2 receptor antagonist, cimetidine (Tagamet), in contrast to ranitidine, had no significant effect.[9] Astemizole (Hismanal), an H_1 receptor antagonist that lacks the sedative side effects of diphenhydramine, also significantly reduces BRB permeability in diabetic rats.[10]

Second, retinal histidine decarboxylase activity, a highly sensitive indicator of non-mast cell induced histamine synthesis, is elevated 197% over control values after 21 days of hyperglycemia. Histidine decarboxylase activity in insulin-treated diabetic animals was not significantly different from control values, despite persistent hyperglycemia and other manifestations of experimental diabetes. Also, there were no significant differences in retinal histidine decarboxylase activity between insulin and *a*-HH–treated diabetic animals.[8]

Third, histamine H_1 and H_2 receptor antagonists significantly reduce rat retinal capillary basement membrane thickening after 12 to 16 weeks of diabetes.[139,140] Fourth, horseradish peroxidase appeared within retinal vascular endothelial tight junctions of diabetic rats; however, rats that were pretreated with intravenous diphenhydramine did not develop leaking junctions, and the authors of the latter reports concluded that the permeability defect in diabetes might lie at the retinal pigment epithelium instead of at the vascular endothelium.[97,98,141-143] In contrast to horseradish peroxidase, FITC-BSA does not induce mast cell histamine release.[144] Therefore, the data cited above suggest that these studies with diphenhydramine pretreatment should be reinterpreted to support the hypothesis that histamine does, in fact, mediate BRB leakage, probably at the level of the retinal vascular endothelium.[141-143]

In sum, histamine is present in vascular tissues of all species, has known vascular actions in humans, and exists in human retina. Furthermore, histamine has a role in the BRB in experimental diabetes. Hence, it is reasonable to predict that histamine may mediate BRB dysfunction in human diabetes, as postulated by Bresnick.[145]

Nevertheless, two older studies found no effect of histamine on the BRB.[146,147] In these experiments, histamine was applied on the external side of the vessel, and no permeability change occurred. Such findings are consistent with the more recently defined nil effect of topically applied histamine on brain capillaries and do not mitigate the current findings of histamine's role on the retinal circulation.[148]

Histamine acts on vascular cells by means of two receptor subtypes with distinct signal transduction pathways.[149,150] Histamine H_1 receptors

induce vasodilation by inhibition of norepinephrine release, release of prostaglandin I_2, and release of nitric oxide.[150,138] In contrast, histamine H_2 receptors stimulate a G_S-protein and mediate their intracellular actions by means of cyclic AMP. The mode of action of histamine on microvascular permeability has been examined in cultures of endothelial cells and in vivo. H_1 receptor stimulation increases free cytosolic [Ca^{++}], and alters F-actin content and distribution, protein kinase C activation, vimentin reorganization, and accumulation of inositol phosphate.[152-159] However, no direct connection between histamine receptor stimulation and tight junctions has been reported previously.

ZO-1 AND OTHER TIGHT JUNCTION PROTEINS

The presence of an intercellular cement was proposed in 1947.[160] Since 1988, six proteins that compose the interendothelial junction have been identified.[161-167] ZO-1, the first component to be identified, is a 225-kD serine phosphorylated protein molecule found originally in liver cell membranes.[161,162] More recently, it has been found in endothelial cell tight junctions, and it has two isoforms, ZO-1 a^+ and a^-.[168] The a^- isoform is identified in endothelium from both arteries and veins. ZO-1 protein expression is related to the degree of confluence and parallels the development of electrical resistance in cultures of rat brain capillary endothelial cells.[169,170] It has been identified in both rat and human blood-brain barriers.[171]

Although the influences of astrocytes on blood-brain barrier induction are well known, and ZO-1 is associated with tight junctions, the relationship between astrocytic influences and ZO-1 protein content has not been explored in the retina. Moreover, the relationships between histamine and ZO-1, and conditions of diabetes and ZO-1, have not been explored. This thesis has examined these questions.

SPECIFIC AIMS

A cell culture model of the BRB was developed in order to examine the cellular effects of histamine, and the conditions of diabetes, on ZO-1 protein expression. Cell culture studies were used so that specific variables could be controlled.

Experiment 1—to establish a cell culture model of bovine retinal capillary endothelial cells with properties of the BRB. This experiment was designed to determine how culture conditions affect endothelial cell growth and viability, and to test the hypothesis that retinal endothelial cells in culture express increased tight junction protein secondary to astroglial influences.

Experiment 2—to determine the effect of histamine, and of histamine receptor blockers, on ZO-1 protein expression in cultures of bovine retinal capillary endothelial cells by immunocytochemistry and immunoblotting.

This experiment was designed to test the hypothesis that histamine receptor stimulation decreases ZO-1 protein expression.

Experiment 3—to determine how the conditions of diabetes, and antihistamines affect ZO-1 protein expression in cultures of bovine retinal capillary endothelial cells by immunocytochemistry and immunoblotting. This experiment was designed to test the hypothesis that the conditions of diabetes decrease ZO-1 expression.

MATERIALS AND METHODS

Methods common to experiments 1, 2, and 3 are presented together, then details unique to each of these experiments are given separately.

Experiment 1—to establish a cell culture model of retinal capillary endothelial cells with properties of the BRB.
Bovine Retinal Capillary Endothelial Cell Cultures--Bovine retinal endothelial cells (BREC) were isolated and cultured by modifications of the methods of Wong and associates[172] and LaTerra and Goldstein[173] to enhance the yield and purity. Sterile conditions were used throughout the procedure. Thirty bovine whole eyes were transported on ice from a local abattoir, soaked in povidone-iodine solution, and opened circumferentially 5 mm posterior to the limbus with a knife. The retinas were removed and rinsed three times in ice-cold Modified Eagle's Medium with D-Valine (Gibco, Gaithersburg, MD) containing 30 mM HEPES buffer. In a laminar flow hood, the pooled retinas were washed with the same solution over a 183-μ nylon mesh stretched over a sterile porcelain funnel inserted into a sidearm flask connected to a vaccum to remove retinal pigment epithelial cell clumps. The retinas were then homogenized six times in a Teflon/glass Potter-Elvehjem type tissue grinder with 0.25-mm clearance at 250 rpm. The resulting retinal homogenate was spun at 400x g for 10 minutes at 4°C, and the pellet resuspended in Ca^{++},Mg^{++} free Dulbecco's phosphate-buffered saline. The microvessel fragments were then trapped on an 88-μ nylon mesh. The mesh was excised and transferred with the trapped microvessels to a glass petri dish and rinsed with Ca^{++}, Mg^{++} free Dulbecco's phosphate-buffered saline. The collected microvessel fragments were then collected and spun at 2,000x g, 4°C for 10 minutes, and resuspended in 10 mL of Ca^{++}, Mg^{++} free Hank's balanced salt solution containing 500 μg/mL Type I collagenase (Worthington Biochemicals, Freehold, NJ) and 200 mg/mL DNase (Sigma). The microvessels were incubated at 37°C for 50 minutes on a rotating platform. The microvessel digestion was halted when observation of vessel fragments with a Nikon phase contrast microscope showed release of pericytes. The preparation was then passed over a 53-μ mesh without suction and the mesh was cut out and transferred to a 50-mL centrifuge tube. The vessel fragments were washed off the mesh with ice-cold

Modified Eagle's Medium with D-Valine, spun at 400x g for 5 minutes at 4°C, resuspended in 10 mL of Modified Eagle's Medium with D-Valine, and respun. The resultant pellet containing microvessel fragments was resuspended in 5 mL of medium and seeded in a 25-cm^2 tissue culture flask (Falcon) precoated with fibronectin at 25 μg/cm^2. The standard growth medium consisted of Modified Eagle's Medium with D-valine, 20% fetal calf serum, 50 μg/mL of endothelial cell growth supplement (Collaborative Research), 16 U/mL heparin (Sigma), 10 mL antibiotic/antimycotic solution (Sigma), and 5 mL glutamine (Sigma). The cells were grown in a humidified incubator at 37° C with 95% CO_2,, 5% O_2.

After 7 to 9 days, discrete colonies of endothelial cells were removed with 0.1% trypsin and reseeded onto another fibronectin-coated 25-cm^2 tissue culture flask. Contaminating pericytes remained adherent to the original flask, and subsequent cultures were essentially free of pericytes as observed by phase contrast microscopy. The cells were repeatedly subcultured with 0.1% trypsin when approximately 80% confluent and expanded for experimental use. Verification of endothelial cell characteristics was based on typical morphology, uptake of acetylated low-density lipoprotein (Biomedical Technologies, Stoughton, Mass), and positive immunostaining for Factor VIII–related antigen (Endotech, Indianapolis, Ind).[174,175]

The number of cell doublings was monitored to be certain that the cells continued to proliferate at a steady rate. These data were obtained when the cells were split, counted in a hemocytometer, and calculated as:

$$\text{Number of doublings} = \frac{\ln.\dfrac{(\text{number of cells harvested})}{(\text{number of cells seeded})}}{.693}$$

Endothelial cells were used for experiments between 6 to 10 passes after the primary culture, and only cells that had undergone 10 or fewer doublings were employed in these studies.

Neonatal Rat Brain Astrocyte Cultures

Neonatal rat brain astrocytes were grown by a modification of the method of McCarthy and DeVellis.[176] Briefly, brains are removed from 12- to 18-hr-old postnatal Sprague-Dawley rat pups, the meninges removed, and the frontoparietal cortices isolated, minced, and trypsinized with 2x trypsin and 0.2 mg/mL DNase in an equal volume of buffer with 137 mM NaCl, 0.17 mM $Na_2HPO_4^-$, 0.2 mM $KH_2PO_4^-$, 5 mM glucose, 59 mM sucrose, 5.4 mM KCl, 1% penicillin-streptomycin, pH 7.2. An equal volume of Dulbecco's Modified Eagle's Medium with 10% fetal calf serum with 2% $NaHCO_3$, 1% penicillin-streptomycin was added, and the digested tissue was spun at 300x g for 15 minutes at 4°C. The cell pellet was passed through a 72-μ nylon mesh, recentrifuged, and resuspended in medium onto poly-l-lysine–coated 75-cm^2 tissue culture flasks. The cells were grown to confluency, then shaken on an orbital shaker at 260 rpm for 12 to 18 hours at 37°C to remove oligodendrocytes,

microglia, and other process-bearing cells. The cultures were trypsinized with 1x trypsin, seeded in flasks, and grown to confluency. Medium was collected every 3 days from confluent secondary astrocytes.

Effect of Astrocyte-conditioned Medium on ZO-1 Protein Expression
Confluent bovine retinal capillary endothelial cells were exposed for 7 days to either "defined" medium or "defined" medium mixed in a 1:1 ratio with astrocyte-conditioned medium. The cells were grown on fibronectin-coated glass coverslips for immunocytochemical staining and in 75-cm^2 plastic cell culture flasks (Corning) for immunoblotting studies.

Effects of Culture Conditions on Endothelial Cell Proliferation
To reduce the rate of endothelial cell proliferation, concentrations of fetal calf serum from 20% to 5% were tested over 14 days. The rate of cell proliferation was determined by measurement of [^{3}H]-thymidine incorporation into confluent endothelial cells grown in 24-well culture plates (Falcon), and by cell counting. Total cell protein content was determined with the Bio-Rad protein reagent on a Hitachi spectrophotometer, with bovine serum albumin as standard.

The effect of astrocyte-conditioned medium on bovine retinal endothelial cell proliferation was examined by seeding cells in six-well plates with the proliferative medium, and half the wells were switched to 50% astrocyte-conditioned medium/50% BREC working medium when the cells reached 50% confluence. When the monolayer covered the well, the cells were scraped and counted in a hemocytometer.

For experiments 1, 2, and 3, the endothelial cells were grown to 90% confluence in standard growth medium and then changed to appropriate medium according to the given protocol. Cells were harvested when confluent. For experiment 1, the "defined" medium consisted of Modified Eagle's Medium with D-Valine with 10% fetal calf serum and 5 μg/mL of endothelial cell growth supplement to reduce the rate of cellular proliferation. This medium contained 5 mM glucose.

Immunocytochemistry of ZO-1 Protein
Bovine retinal capillary endothelial cells grown to confluence on fibronectin-coated glass coverslips were washed three times with ice-cold phosphate buffered saline, soaked in -20°C methanol for 4 minutes, rinsed with -20°C acetone, and dried at room temperature for 10 minutes. Nonspecific binding was blocked with 3% bovine serum albumin in phosphate buffered saline for 30 minutes at room temperature. The cells were then incubated with 250 μL of rat anti-ZO-1 primary antibody (Chemicon, Temecula, Calif; 1:150) for 60 minutes at room temperature in a moist, covered petri dish. The cells were then rinsed three times with phosphate buffered saline and incubated with 250 μL of anti-rat IgG secondary antibody–labelled fluores-

cein isothiocyanate (Accurate, Westbury, NY; 1:300) for 60 minutes at room temperature. The cells were then washed three times with phosphate buffered saline, dried, and covered with Vecta-Shield and a coverslip. Coverslips that served as negative controls received only the secondary antibody. The ZO-1 immunofluorescence was imaged and digitized on a Zeiss LSM 10 confocal scanning laser microscope with software version 2.0.5. Images of representative fields were enhanced with Adobe Photoshop software (version 2.5) on a Macintosh Quadra 950 computer and printed on a Colortone color printer.

Endothelial Cell Membrane Preparation and Immunoblotting

Crude bovine retinal endothelial cell membranes were prepared by rinsing confluent 75-cm^2 flasks twice with ice-cold phosphate buffered saline, scraping the flask with a rubber policeman, and pelleting the collected cells at 300x g for 5 minutes at 4°C. The pellet was then resuspended in ice-cold buffer of 10 mM TRIS, pH 7.4, 4 mM EGTA, 4 mM EDTA, and the following protease inhibitors: leupeptin (2.5 μg/mL), pepstatin (0.5 μg/mL), aprotinin (0.5 μg/mL), soybean trypsin inhibitor (30 μg/mL), dithiothreitol (0.2 mM), and phenylmethylsulfonyl fluoride (0.2 mM). The cells were then homogenized with 5 up-and-down strokes in a Wheaton 15-mL mortar with pestle A. The homogenate was spun at 100,000x g, 4°C for 20 minutes in a Beckman ultracentrifuge. The pellet was resuspended in buffer and respun at 100,000x g for 20 minutes, the pellet resuspended, and the protein concentration determined with the Bio-Rad protein reagent on a Hitachi spectrophotometer, with bovine serum albumin as standard.

The retinal endothelial cell membranes were adjusted to equal protein concentrations, and run on 6% SDS-polyacrylamide gels. The gels were transferred to Nytran using a Hoefer transfer chamber. The Nytran membrane was stained with Ponceaus to confirm equivalent protein loading.[177] After blocking with low-detergent Blotto in 0.2% Tween, the blot was probed with a rat monoclonal anti-ZO-1 antibody (Chemicon; 1:1000) overnight at 4°C. Rabbit anti-rat IgG (Accurate; 1:1000) was used as a second antibody, and 0.2 μCi^{125} I-IgG was added as tertiary antibody. The membrane was then exposed to Kodak film at -80°C for 72 hours and developed.

Experiment 2—to determine the effect of histamine, and of histamine receptor blockers, on ZO-1 protein expression in cultures of bovine retinal capillary endothelial cells by immunocytochemistry and immunoblotting.

As preliminary experiments, the effect of histamine on endothelial cell proliferation was studied. Histamine (10^{-8} M, 10^{-6} M, or 10^{-4} M) was added daily for 5 days to BRECs, beginning at 50% confluence. After 5 days the cells were harvested by scraping. The cell number was counted in a hemocytometer, and total protein was determined.

For the studies of histamine on endothelial cell ZO-1 expression, the

following conditions were applied for 7 days to cells on fibronectin-coated coverslips or in T75 flasks:

a. controls: defined medium only, or

b. histamine-treated: 10^{-4} M (K_i histamine ≈ 10^{-6} M), or

c. histamine plus antihistamines—mepyramine (H_1 receptor blocker, K_d = 0.8 nM) or ranitidine (H_2 receptor blocker, K_d = 200 nM)—were applied at 4 x 10^{-9} M and 4 x 10^{-7} M concentrations, respectively. The histamine and drugs were applied daily, and the antihistamines were administered 15 minutes prior to histamine to allow for receptor binding.[137] The cells were prepared for immunocytochemistry or immunoblotting as in Experiment 1.

Experiment 3—to determine how the conditions of diabetes affect ZO-1 expression in cultures of bovine retinal capillary endothelial cells by immunocytochemistry and immunoblotting.

To maintain constant glucose concentrations in the medium during the course of the experiments, the rate of glucose consumption was determined by measuring glucose concentrations in aliquots of cell culture medium by the glucose oxidase technique (Sigma). Similarly, insulin degradation in the medium was monitored by measuring insulin concentrations with a solid phase radioimmunoassay (Diagnostic Products Corp).

Preliminary studies revealed that the glucose concentration in medium removed from confluent endothelial cells remained within 10% of the set value (5 mM or 20 mM) for 48 to 72 hours (data not shown). Therefore, we routinely changed the medium every 2 days in subsequent experiments. The insulin concentration in the stock medium was found to be 10^{-12} M.

For this experiment, the standard growth medium was replaced with glucose-free Dulbecco's Modified Eagle's Medium (Gibco) with added glucose and/or pork insulin (Eli Lilly), and the cells were treated as follows for 7 days:

Protocol	[Insulin]	[Glucose]
1	Basal	Basal
2	Low	Basal
3	Basal	High
4	Low	High

For insulin, basal = 10^{-9} M and low = 10^{-12} M; for glucose, basal = 5 mM and high = 20 mM. These conditions were designed to mimic diabetes and to compare the effects of hyperglycemia with normal and deficient insulin concentrations and of normoglycemia and insulin deficiency with basal conditions of normoglycemia and of normal insulin concentrations. The insulin levels are based on mean plasma insulin concentrations of 10^{-9} M in nondiabetic humans.[178]

To determine if the conditions of diabetes had an effect on bovine retinal endothelial cell proliferation, 50% confluent cells were exposed to the four protocols for 5 days, the cells were harvested, and cell counts and total protein were determined.

The cells were grown on fibronectin-coated coverslips or in T75 flasks and prepared for immunocytochemistry or immunoblotting, as described for experiment 1.

RESULTS

Experiment 1—to establish a cell culture model of bovine retinal capillary endothelial cells with properties of the BRB.
The endothelial cell nature of the cultures was confirmed by demonstration of typical cobblestone morphology under phase contrast microscopy, uptake of acetylated low-density lipoprotein (Fig 1A), and positive staining for factor VIII–related antigen (Fig 1B). Astrocyte cultures maintained typical morphologic appearance, and the cultures showed no evidence of neuronal or oligodendroglial cell contamination.

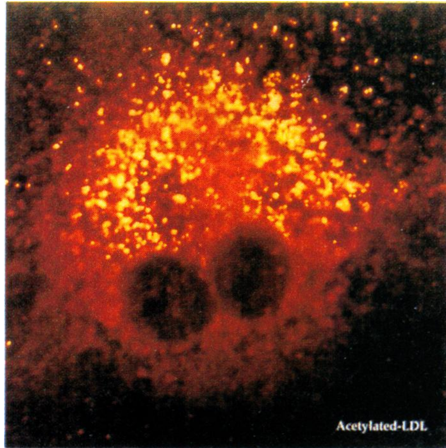

FIGURE 1A
Uptake of fluorescein-labelled acetylated low-density lipoprotein in cultures of bovine retinal capillary endothelial cells (X400). Fluorescence was imaged with Zeiss confocal microscope, and yellow color was applied with Adobe Photoshop software.

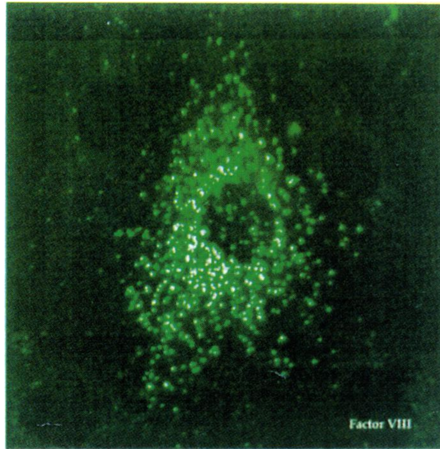

FIGURE 1B

Staining for Factor VIII–related antigen in cultures of bovine retinal capillary endothelial cells (X400).

As shown in Table II, reduction of the fetal calf serum concentration in the culture media from 20% to 10% or 5% dramatically reduced [^{3}H]-thymidine incorporation into bovine retinal capillary endothelial cells but had little effect on the total cell protein content. This effect on [^{3}H]-thymidine incorporation was in addition to the expected reduction when the cells became confluent. Although 5% serum produced the lowest rate of cell proliferation, the cells exhibited increased polymorphism and cytoplasmic vacuolation after extended exposure to 5% serum. As a result of these findings, the fetal calf serum concentration was reduced in subsequent experiments from 20% to 10% when the endothelial cells reached confluence.

TABLE II:

EFFECT OF MEDIA CONDITIONS ON ENDOTHELIAL CELL PROLIFERATION AND PROTEIN CONTENT

Days after seeding	[Fetal calf serum]	[^{3}H]-thymidine incorporation (% of standard*)	Protein content (% of standard*)
3	10%	65	84
	5%	53	79
6	10%	81	97
	5%	22	99
9	10%	67	74
	5%	19	128
14	10%	22	82
	5%	6.6	86

* Standard = 20% fetal calf serum.

In addition to the [^{3}H]-thymidine incorporation studies, cell counts were also performed. When 1.5 x 10^5 endothelial cells per well were seeded per well (n = 3) after 5 days, those grown in 20% fetal calf serum numbered 1.46 x 10^6 per well (± 1.6 x 10^5) versus 4.7 x 10^5 per well (± 9.3 x 10^4) when grown in 10% fetal calf serum; this is a 68% decrease, P = .002, Student's t test).

Exposure of cultured endothelial cells to 50% astrocyte-conditioned medium combined with 50% "defined" medium for 7 days resulted in three prominent changes. First, the cells rapidly (within 24 to 48 hours) adopted a more uniform cobblestone morphology compared with the spindle morphology more typical of unexposed confluent endothelial cells (Fig 2 A and B). Second, 50% astrocyte-conditioned medium increased endothelial cell proliferation. That is, 3 days after seeding at equal densities (2 x 10^5 per well), there were 17.5 x 10^5 (± 1.9 x 10^5) cells in each of 6 wells of conditioned medium versus 13.3 x 10^5 (± 2.2 x 10^5) cells per well of defined medium (P = .01, Student's t test). Third, endothelial cells exposed to astrocyte-conditioned medium showed more uniform and more intense immunofluorescence at the interendothelial cell junctions when stained with anti-ZO-1 antibody (Fig 3 A and B). Correspondingly, membranes from astrocyte-conditioned medium-treated cells contained approximately twofold more ZO-1 protein immunoreactivity compared with cells grown in "defined" medium without astrocyte-conditioned medium (Fig 4).

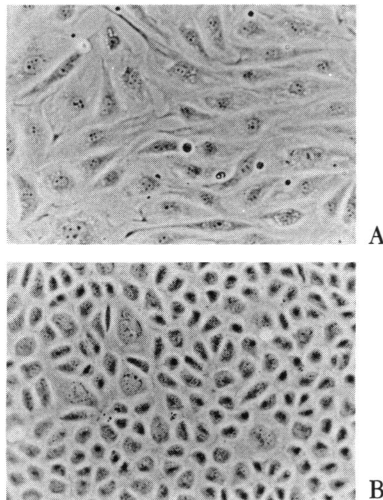

FIGURE 2

Phase contrast micrograph of bovine retinal capillary endothelial cells (X200) grown in "defined" medium (A) and in defined medium combined 1:1 with 50% astrocyte-conditioned medium (B). Cells exposed to conditioned medium exhibit more uniform cobblestone appearance.

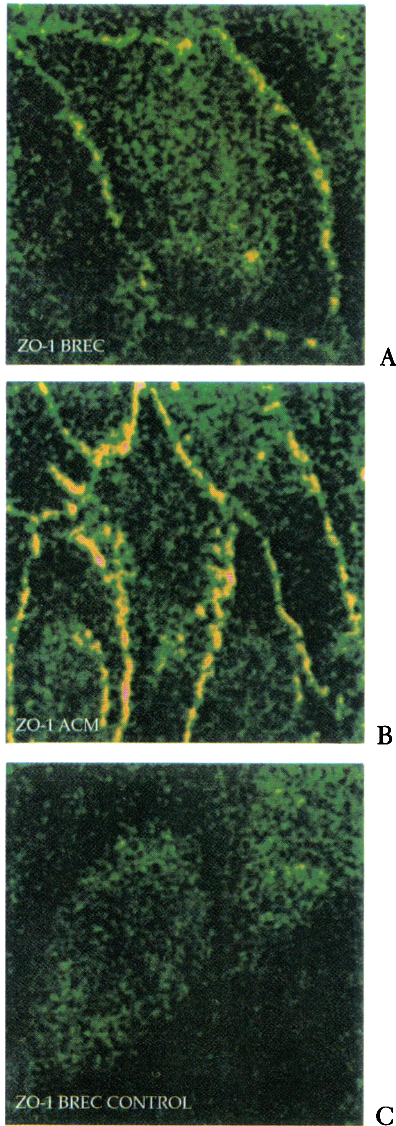

FIGURE 3
Immunofluorescent micrographs of cultured bovine retinal capillary endothelial cells exposed to anti-ZO-1 antibody and then fluorescein-labelled IgG (X400). Cells grown in "defined" medium (A) had less uniform and less intense immunofluorescence at interendothelial cell junctions than cells grown in 50% astrocyte-conditioned medium (B). Cells that received only secondary antibody to serve as negative controls are shown in C and exhibit minimal background fluorescence.

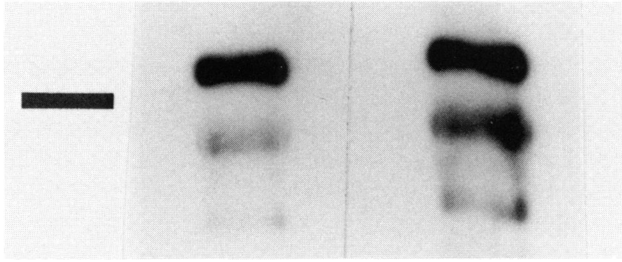

FIGURE 4
Representative autoradiogram of bovine retinal capillary endothelial cell membranes (100 μg protein per lane) probed with anti-ZO-1 antibody. From left: Lane 1, molecular weight standard myosin, 205 kD; lane 2, "defined" culture medium; lane 3, "defined" medium with 50% astrocyte-conditioned medium.

Experiment 2—to determine the effect of histamine, and of histamine receptor blockers on ZO-1 protein expression in cultures of bovine retinal capillary endothelial cells by immunocytochemistry and immunoblotting. Table III shows that histamine, in a range of concentrations known to increase permeability, had no effect on proliferation. There was also no change in cell morphology.

TABLE III

EFFECT OF HISTAMINE ON BREC PROLIFERATION

[Histamine]	Total Protein ± SE (mg) (n=3)	Cell Number (x 10^5) (n=3)
0	61.7 ± 5.8	1.2 ± 0.6
10^{-8} M	65.0 ± 0.0	1.2 ± 0.1
10^{-6} M	61.7 ± 2.9	1.2 ± 0.1
10^{-4} M	63.3 ± 2.9	1.2 ± 0.2

BREC, bovine retinal endothelial cell.

Fig 5 shows that 10^{-4} M histamine dramatically decreases ZO-1 immunofluorescence at interendothelial cell junctions in cultured bovine retinal endothelial cells compared with control conditions over 7 days. Moreover, there is reduced intracellular ZO-1 immunofluorescence throughout the histamine-exposed cells. In confirmation of the immunocytochemical study, endothelial cell membranes blotting with anti-ZO-1 monoclonal antibodies exhibited a dramatic reduction of ZO-1 protein. Proteolytic fragments are

noted as described previously.[161] Ranitidine, an H_2 receptor antagonist, prevented the decrease in ZO-1 expression, but mepyramine, an H_1 receptor antagonist, did not (Fig 6).

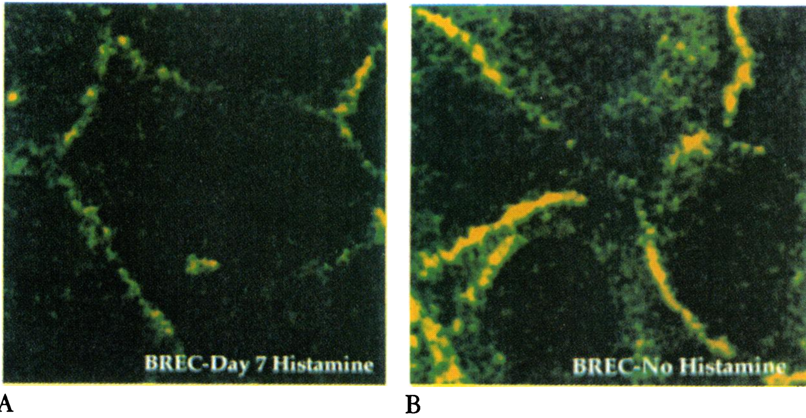

FIGURE 5
Immunofluorescent micrographs of cultured bovine retinal capillary endothelial cells exposed to anti-ZO-1 antibody and then fluorescein-labelled IgG (X400). Cells exposed to 10^{-4} M histamine for 7 days (A) showed reduced ZO-1 immunofluorescence at plasma membranes, compared with controls (B).

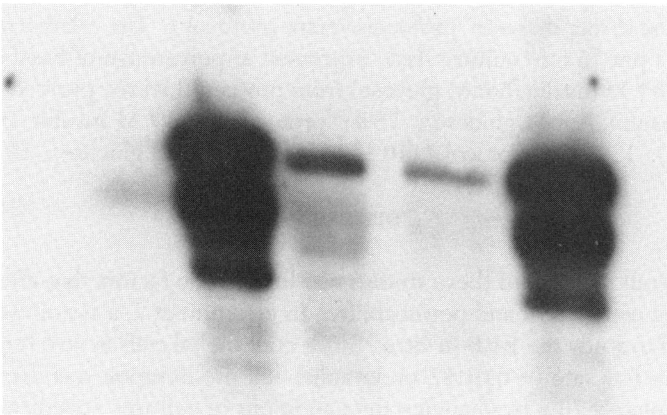

FIGURE 6
Representative autoradiogram of bovine retinal capillary endothelial cell membranes (100 μg protein per lane) probed with anti-ZO-1 antibody. From left: Lane 1, molecular weight standard, myosin, 205 kD; lane 2, control; lane 3, 10^{-4} M histamine exposure daily for 7 days; lane 4, treatment with 4×10^{-9} M mepyramine; lane 5, treatment with 4×10^{-7} M ranitidine.

Experiment 3—to determine how the conditions of diabetes, and an H_1 receptor blocker, affect ZO-1 expression in cultures of bovine retinal capillary endothelial cells by immunocytochemistry and immunoblotting.
Table IV shows that high glucose, but not low insulin, modestly reduced bovine retinal endothelial cell proliferation compared with basal glucose and insulin levels. These conditions had no effect on bovine retinal endothelial cell morphology as viewed by phase contrast microscopy.

TABLE IV:
EFFECT OF CHEMICAL CONDITIONS DIABETES ON BREC PROLIFERATION

Protocol	Cell Number (x 10^3) ± SE (n=3)
1	480 ± 26
2	653 ± 136
3	367 ± 163
4	300 ± 108

BREC, bovine retinal endothelial cell.

As shown in Fig 7, ZO-1 immunofluorescence is reduced in all three protocols that mimic the diabetic state, compared with protocol 1 with physiologic concentrations of glucose and insulin. While it is difficult to differentiate between the relative ZO-1 fluorescence of the diabetic conditions, all three show less uniform and less intense fluoresence than the basal control. Figure 8 is a representative autoradiogram showing reduced total ZO-1 protein in the three "diabetic" protocols versus protocol 1. The relative protein contents per 75 cm^2 culture flask, expressed as percentage of basal conditions (10^{-9} M insulin, 5 mM glucose) from protocol 1 were: protocol 2 (10^{-12} M insulin, 5 mM glucose): 78%; protocol 3 (10^{-9} M insulin, 20 mM glucose): 139.0%; protocol 4 (10^{-12} M insulin, 20 mM glucose): 127%.

DISCUSSION

The overall purpose of these studies was to examine factors that affect endothelial cell growth and permeability. In experiment 1, a system was developed to study the BRB in vitro. Since endothelial cells in vivo turn over at a very low rate ($\approx$ 0.01%), endothelial cell proliferation was decreased approximately 70% by changing the conditions of culture; specifically, the concentration of fetal calf serum was reduced in half, and the concentration of the mitogenic endothelial cell growth supplement was reduced by 90%. Nevertheless, the cells maintained normal morphologic features and remained viable, as shown by cell viability assays and stable total cell protein content.

In addition to reducing the rate of cell proliferation, this model also

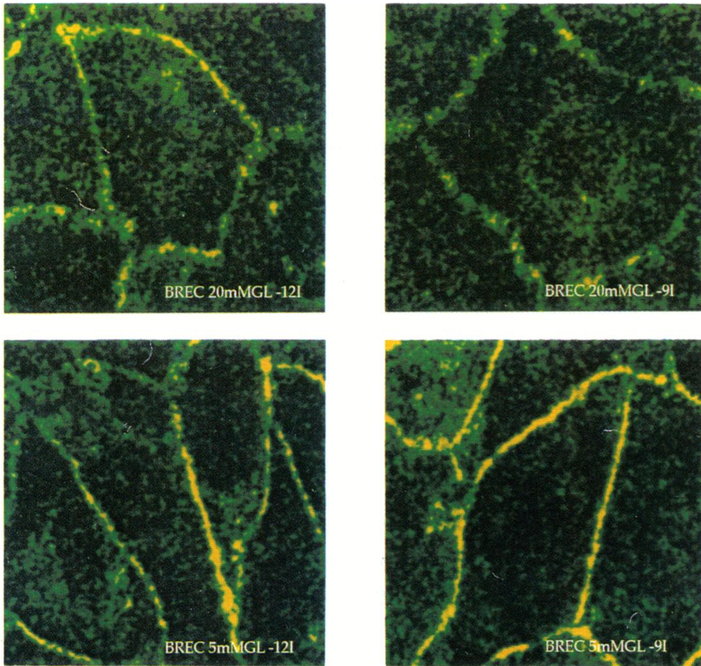

FIGURE 7
Immunofluorescent micrographs of cultured bovine retinal capillary endothelial cells exposed to anti-ZO-1 antibody and then fluorescein-labelled IgG (X400). Cells were exposed to either 5 mM or 20 mM glucose (GL), and 10^{-9} M or 10^{-12} M insulin (I) for seven days. All three diabetic protocols show less intense and less uniform fluorescence than basal control.

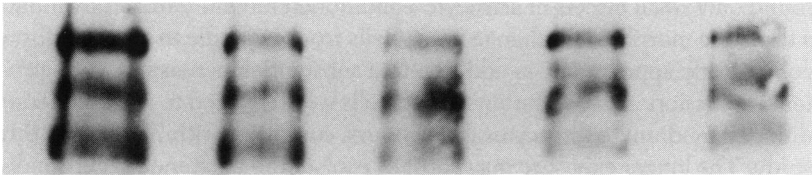

FIGURE 8
Autoradiogram of bovine retinal capillary endothelial cell membranes (100 μg protein per lane) probed with anti-ZO-1 antibody. From left: Lane 1, 5 mM glucose, 10^{-9} M insulin; lane 2, 5 mM glucose, 10^{-12} M insulin; lane 3, 20 mM glucose, 10^{-9} M insulin; lane 4, 20 mM glucose, 10^{-12} M insulin; lane 5, 20 mM glucose, 10^{-9} M insulin plus mepyramine. Immunoreactivity in each of diabetic protocols is reduced compared with nondiabetic control.

differs from standard retinal endothelial cell cultures in that endothelial cells were exposed to influences from astrocytes. Astrocytes induce tight endothelial barrier properties in the brain and retina, and this high degree of tight junction (zonula occludens) formation distinguishes brain and retinal blood vessels from those in the peripheral vascular system. In these experiments astrocyte-conditioned medium induced an increase in ZO-1 tight junction protein expression compared with control conditions. Although astrocyte-conditioned medium increased endothelial cell proliferation and altered cell morphology, equal amounts of protein were loaded per lane on the immunoblotting studies, so the effect on ZO-1 is likely specific. Likewise, the immunocytochemical studies clearly show more intense fluorescence in the cells exposed to conditioned medium, even considering the different morphology. This is the first known demonstration of this phenomenon in the retina. The increase in ZO-1 expression by bovine endothelial cells by factor(s) derived from rat brain astrocytes is also evidence for the evolutionary conservation of this effect.[179] While the permeability and electrical resistance of this model remain to be investigated, it appears to more accurately reflect the in vivo state, with a lower endothelial cell turnover rate and a higher level of tight junction molecules than other retinal endothelial cell culture systems, which do not consider these factors.[81] Although these results demonstrate the importance of culture conditions on endothelial cell growth, further work will be required to refine the system to possess all the features of the BRB in vivo.

These findings support the results of Rubin and associates,[96] who found increased ZO-1 expression in a cell culture model of the BRB. However, they did not report any data regarding the proliferation rates or viability of their cells. In this study, the investigators were careful to maintain equivalent degrees of confluency between sets of experimental conditions, since ZO-1 protein expression varies with confluency.[169] While the Rubin study found only small effects of astrocyte-conditioned medium, this study found a dramatic morphologic change in the cells from a spindle to more uniform cobblestone appearance, in addition to a substantial increase in ZO-1 protein expression. Also, in Rubin's study, cells were exposed to astrocyte-conditioned medium to astrocytes for 48 hours, compared with 72 hours in this study. The longer exposure may partially explain the greater effect of conditioned medium.

ZO-1 protein content correlates directly with transendothelial electrical resistance in cultures of rat cerebral endothelial cells, even in the absence of astroglial influences.[169] Therefore, this model system with enhanced ZO-1 protein expression likely exhibits the tight junctional characteristics of the BRB. Furthermore, these data suggest that the factor(s) from astrocytes that induces ZO-1 protein expression and, presumably, the BRB, is soluble, and direct astrocyte-endothelial cell contact is not necessary. Astrocytes are separated from endothelial cells by a basement membrane in vivo.[93]

The influence of astrocytes on endothelial cell properties demonstrated by this study raises the possibility that a primary defect in retinal Müller cell metabolism could, in turn, impair retinal vascular barrier function. Bresnick and Palta[180] found that reductions of oscillatory potentials on the a wave of the electroretinogram, an indicator of Müller cell function, correlate with capillary leakage on fluorescein angiograms. Müller cells of diabetic rats exhibit glycogen and acid phosphatase accumulation, and partial necrosis, within 12 months of onset of diabetes.[181,182] A recent preliminary study increased immunohistochemical staining of retinal Muller cells for glial fibrillary acidic protein after 3 months of streptozotocin-induced diabetes in rats.[183] This finding suggests that glial cell injury may occur early in the course of diabetes. Further work is required to determine whether Müller cell dysfunction precedes or follows endothelial cell damage.

While the first phase of this study examined means to represent physiologic conditions and increase ZO-1 content, the second and third specific aims explored the effects of pathologic conditions. Using this cell culture model, results from experiment 2 clearly demonstrate that histamine reduces the interendothelial tight junction protein, ZO-1. This effect occurs at histamine concentrations found in pathologic states in vivo (10^{-5} to 10^{-4} M), which increases permeability of endothelial cell monolayers and edema in the rat microcirculation.[159] To my knowledge, this observation is the first direct explanation for histamine-induced microvascular permeability at the level of the interendothelial cell plasma membrane. Previous explanations of the effect of histamine on endothelial cell permeability have been limited to acute intracellular events, such as increased free cytosolic Ca^{++}, reduced F-actin fiber content, redistribution of actin filaments, and activation of protein kinase C.[152-157] Moreover, treatment with the H_2 receptor antagonist ranitidine prevented the reduction in ZO-1 protein expression, as shown by immunocytochemistry and immunoblotting. It was anticipated that the histamine H_1 receptor would have a greater effect than the H_2 receptor on ZO-1 expression, on the basis of prior studies of BRB permeability in rats.[7,9] However, the histamine H_2 mediates blood-brain barrier permeability and may also be active in the retina. It is also possible that our cell culture system selects for endothelial cells that express the H_2 receptor. This study did not find any effect of histamine on retinal microvascular endothelial cell proliferation, at least under the conditions examined, as has been reported for other cell types.[158]

The decrease in total cellular in ZO-1 immunofluorescence suggests that the effect of histamine, at least after 7 days' exposure, is on total ZO-1 protein content, rather than an internal redistribution of plasma membrane tight junction protein. Further studies are necessary to determine if histamine exerts its effect on ZO-1 directly at the plasma membrane, indirectly via the cytoskeleton, or by decreasing expression of the ZO-1 mRNA. While it might be anticipated that continued histamine exposure would down-regu-

late the endothelial cell receptors and mitigate the response, the dramatic changes at 7 days suggest that the effect is cumulative and may have bearing on conditions such as diabetes, with prolonged histamine production.

The endothelium serves important homeostatic functions for its surrounding tissues and participates in inflammatory reactions.[184] Histamine release is a prominent aspect of the response of endothelium to injury. Hollis[126] has proposed that histamine production serves as a response to the injury of diabetes and that histamine receptor stimulation may trigger a cascade of intracellular events that lead to vascular cell damage through chronic inflammation. The known effects of histamine receptor stimulation, such as increased free cytosolic Ca^{++}, breakdown of inositol phospholipids, activation of protein kinase C, and nitric oxide release, are changes that have been described by other investigators as potential pathways of vascular damage in diabetes and chronic inflammation.[4] Histamine release, like von Willebrand's factor and angiotensin-converting enzyme, may reflect endothelial cell dysfunction in diabetes.[185]

Further evidence for a chronic inflammatory component to diabetic retinopathy, at least in eyes with macular edema, is found in the response of eyes with severe diabetic macular edema to systemic corticosteroid therapy. Deutman and coworkers reported their results in treating patients with diabetic macular edema with oral prednisone at the meeting of the Club Jules Gonin, Vienna, September 1992. Of the 15 eyes with macular edema in eight patients who received oral prednisone, there was a reduction of macular edema in all 9 eyes of the patients who received prednisone for 6 to 48 months (mean, 21.1). The visual acuity improved more than 2 lines on a Snellen chart in 8 eyes. The mechanism by which corticosteroids might improve macular edema is unknown. Prednisone and antihistamines both have anti-inflammatory properties, but antihistamines do not disrupt glycemic control or aggravate peptic ulcers, hypertension, or osteoporosis, and if they are effective for retinopathy, they may be better suited for long-term treatment of patients with diabetes. Antihistamines such as astemizole, which do not cross the blood-brain barriers, do not cause sedation.

At this point it remains unclear if the effect of histamine or antihistamines is limited to the endothelium. Histamine also activates platelet aggregation, possibly by enhancing the effect of platelet activating factor.[186] Thus, it is possible that a component of the antihistaminic effect is also on the events of the vascular lumen that contribute to microvascular closure. Additional studies are needed to clarify this issue.

In keeping with the vasodilation and excessive BRB permeability associated with loss of autoregulation, the potential contribution of histamine, which is known to induce these changes in microcirculation, must be considered in the pathogenesis of diabetic retinopathy. Likewise, antihistamines may improve the autoregulatory function of the retina by mediating vasoconstriction and reducing abnormal permeability.

In contrast to experiment 2, which studied a direct mediator of vascular permeability, experiment 3 was designed to examine the effect of chemical conditions of diabetes on bovine retinal endothelial cell proliferation and ZO-1 protein expression. The conditions of high glucose decreased bovine retinal endothelial cell number, without affecting morphology. After 7 days of exposure to these conditions, ZO-1 protein content was decreased in all of the three "diabetic" protocols compared with the basal, physiologic conditions of insulin and glucose concentrations. The largest decreases occurred in protocols 3 and 4, in which the cells were exposed to 20 mM glucose. The lack of an inibitory effect of mepyramine on decreased ZO-1 protein expression may be due to the relatively low dose of mepyramine, or the apparent predominance of histamine H_2 receptors on these cultured endothelial cells, as shown in experiment 2. Alternatively, the reduction in ZO-1 protein expression may not be histamine receptor–mediated. Additional work to clarify this point is in progress.

Although exposure to high glucose appears to influence ZO-1 protein expression, the mechanism for this change remains uncertain. The time course is too rapid to be accounted for by advanced glycation end-product formation. These data suggest that changes in tight junction proteins could occur early in the course of diabetes, and may help explain increased BRB permeability as an early feature of diabetic retinopathy. It is also possible that this rapid change in ZO-1 protein is unique to the cell culture model and would not occur in vivo where additional homeostatic mechanisms may protect the retina from injury. Additional cell culture studies should permit determination of the specific factors that regulate ZO-1 protein and messenger RNA expression. Studies are currently under way to determine the effect of diabetes on vascular ZO-1 protein expression in vivo.

THE HENLE-KOCH POSTULATES AND THE PATHOGENESIS OF DIABETIC RETINOPATHY

Mutiple factors and mechanisms have been proposed to account for the pathogenesis of diabetic retinopathy and associated visual loss. Establishing a causal relationship between multiple variables and the initiation or progression of chronic conditions such as diabetic retinopathy has been extremely difficult. To address similar challenges of cause and effect in chronic infectious diseases such as tuberculosis, Henle and Koch postulated a framework by which various hypotheses could be tested. They put forth three criteria:

First, the parasite occurs in every case of the disease in question and under circumstances that can account for the pathologic changes and clinical course of the disease.

Second, it occurs in no other disease as a fortuitous and nonpathogenic parasite.

Third, after being fully isolated from the body and repeatedly grown in

pure culture, it can induce the disease anew.[187] These postulates continue to be applied to modern problems of pathogenicity.[188]

If these points are adapted to the problem of diabetic retinopathy, they could be stated as follows:

First, the key abnormalities must be found in vascular tissue of animals and humans with diabetes and should be temporally associated with the stage of the disease for which they are proposed.

Second, the abnormalities must account for the cellular, functional (BRB leakage), and structural (basement membrane thickening, capillary closure) alterations, directly or indirectly.

Third, induction of the abnormalities in an appropriate animal model should simulate functional and structural characteristics of diabetic retinopathy.

A fourth criterion, which was not available to Henle and Koch, is that specific inhibition of the abnormality should prevent or retard the development of cellular, functional, and structural alterations.

The hypothesis of histamine-mediated BRB leakage may be evaluated by the guidelines listed above. The first condition is satisfied by the findings of increased vascular histamine synthesis in diabetic rats and humans.[122,123,126-130] The second point is fulfilled by studies of histamine receptor mediation of BRB breakdown in rats and humans with diabetes, and by the findings that histamine and conditions of diabetes independently reduce ZO-1 protein expression in cultures of endothelial cells.[7,9,10,189] The third point is partially satisfied by the finding that histamine infusion for 1 week increases BRB permeability in nondiabetic rats.[121] The effects of longer exposure to histamine are unknown. The fourth and most important issue is whether specific histamine receptor blockers can improve retinal vascular function in patients with diabetic retinopathy. This point is being addressed by a randomized clinical trial now in progress that examines the effect of astemizole on nonclinically significant diabetic macular edema.

Since diabetes is a complex systemic disease, there are undoubtedly multiple factors involved in the pathogenesis of diabetic retinopathy at various stages. Conceivably, histamine may contribute to increased BRB permeability and macular edema, and other factor(s) lead to capillary occlusion, microaneurysms, or neovascularization. As pointed out by Greene and associates,[61] "Prevention or treatment of a diabetic complication may not demand correction of all contributing factors—only those required for its clinical expression." The data included in this study provide evidence for a factor that may contribute to one of the most common complications of diabetes, and for which there appears to be a safe, readily available therapy.

Finally, it is premature to predict what events will later be recorded in the time line of diabetic retinopathy for the 1990s and beyond. These future achievements will certainly be built on the successes and failures of the past. With better transfer of existing knowledge into clinical practice, and

of knowledge from research laboratories into clinical trials, Dr Thomas Duane's question, "Is diabetic retinopathy uncontrollable?" may eventually be put to rest.[190]

SUMMARY

This thesis utilizes an improved in vitro model of the blood-retinal barrier to provide a novel explanation for the actions of histamine and diabetes on tight junction protein expression in retinal microvascular endothelial cells. The Henle-Koch postulates have been adapted to the problem of diabetic retinopathy and may serve as guidelines with which to evaluate causal relationships between histamine and other pathophysiologic mechanisms in the long time line of diabetes and its retinopathy. Hopefully, the experimental data and principles in this thesis will contribute a small step in the effort to eliminate blindness from diabetes.

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Penn State University holds patent rights for use of antihistamines in diabetes.

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