Neutrophils Are Associated With Capillary Closure in Spontaneously Diabetic Monkey Retinas

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Type 2 diabetes develops spontaneously in obese aging rhesus monkeys (*Macaca mulatta*). This study investigates the association between polymorphonuclear leukocytes and development of retinopathy. Blood pressure and plasma glucose levels were determined in 15 diabetic and 6 nondiabetic monkeys. The plasma levels of total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides were determined just before the start of the animal's final decline and elective necropsy. Retinas were incubated for ADPase (labels viable retinal blood vessels) and nonspecific esterase (labels neutrophils) activities. Polymorphonuclear leukocytes were counted per millimeter squared of retina. After the retina was flat-embedded in glycol methacrylate, tissue sections were taken through areas of interest and observed microscopically. Elevated numbers of intravascular polymorphonuclear leukocytes were present adjacent to areas with retinal capillary nonperfusion. There were significantly more polymorphonuclear leukocytes per millimeter squared in diabetic retinas (6.91 ± 5.01) compared with normal retinas (1.45 ± 1.62, $P = 0.018$). Severity of hypertension in diabetes was also significantly associated with greater numbers of polymorphonuclear leukocytes ($P = 0.02$). There was a significant positive exponential correlation between the number of polymorphonuclear leukocytes per millimeter squared and the level of total cholesterol ($R = 0.907$), LDL cholesterol ($R = 0.875$), the total cholesterol–to–HDL cholesterol ratio ($R = 0.86$), and total triglycerides ($R = 0.888$). This study demonstrates that severity of diabetes and the development of retinopathy are associated with increased numbers of polymorphonuclear leukocytes in the retina of diabetic monkeys. Hypertension, high plasma levels of LDL cholesterol and triglycerides, and low plasma levels of HDL cholesterol also are associated with increased polymorphonuclear leukocytes in retina. *Diabetes* 54:1534–1542, 2005

An early event in diabetic retinopathy is retinal capillary nonperfusion. Recent evidence suggests that leukocytes may initiate vaso-occlusion in diabetic retinal microvasculature (1). Miyamoto et al. (2) and Ogura (3) used acridine orange labeling and a scanning laser ophthalmoscope to observe leukocytes in diabetic rats. The number of leukocytes trapped in the retinal microcirculation was significantly elevated in streptozotocin-induced diabetic rats (2.5-fold) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats (2-fold) in the early stages of diabetes compared with nondiabetic rats. They hypothesized that accumulation of leukocytes in diabetic retinas during the preretinopathy stage could cause microvascular occlusions and dysfunction, in turn causing retinopathy. Barouch et al. (4) later demonstrated that the increase in leukocyte accumulation or leukostasis was dependent on the adhesion molecule responsible for modulating firm adherence of leukocytes to endothelial cells, intracellular adhesion molecule-1 (ICAM-1), which is elevated in human diabetic retina (5).

Leukocyte entrapment in the retinal microcirculation was enhanced in the early stage of hypercholesterolemia even in nondiabetic rats (6). Dyslipidemia has been associated with endothelial dysfunction and impaired vasodilatory response, and postprandial hypertriglyceridemia is exaggerated in type 2 diabetes (7). Hypertriglyceridemia was associated with a higher level of soluble vascular adhesion molecule-1, soluble ICAM-1, a smaller LDL cholesterol particle size, and reduction in flow-mediated vasodilation (8). In diabetic patients, LDL oxidation is promoted, and oxidized LDL increases production of reactive oxygen species (ROS) and adhesion molecules (ICAM-1) (9). Neutrophils (polymorphonuclear leukocytes) generate reactive oxygen radicals (ROS) when they undergo an oxidative burst; this may be an additional source of oxidative stress on endothelial cells when they are activated (10).

The pathogenesis of type 2 diabetes is associated with atherosclerosis, and morbidity is further increased in the presence of hypertension. The prevalence of vascular complications is greater in diabetic patients with hypertension, and the prevalence of hypertension is reported as higher in patients with diabetes (7). We hypothesize that dyslipidemia (hypertriglyceridemia, low HDL cholesterol) as well as chronic hyperglycemia and insulin resistance are risk factors for leukocyte accumulation and subsequent retinal vascular dysfunction in diabetes.

TABLE 1
Relation between polymorphonuclear leukocytes counts and medical and histological data of nondiabetic monkeys

Identifica-tion no.	Age (years)	Hyper-tension	PMNs/field	Capillary dropout	Large vessel pruning	Cotton wool spots	Micro-aneurysms	IRMAs	New vessels
1	14.1	−	0	None	None	None	None	None	None
2	16.1	++	1.1	None	None	None	None	None	None
3	18.9	++	1.4	None	None	None	None	None	None
4	20.2	−	4.6	None	None	None	None	None	None
5	27	++	1.1	None	None	None	None	None	None
6	27.8	++	0.5	None	None	None	None	None	None

−, none; ++, moderate. IRMA, intraretinal microvascular abnormality; PMN, polymorphonuclear leukocyte.

This study was conducted in a colony of spontaneously diabetic rhesus monkeys (*Macaca mulatta*). Their diabetes has been well characterized and has been shown to have all of the features in common with adult-onset diabetes found in humans (11). These features include predominantly middle-age onset and obesity association (12) and extended prodrome that includes insulin resistance (13–15), dyslipidemia (16), and β-cell hyperfunction followed by declining function (17). These animals develop all of the complications associated with diabetes in humans, including neuropathy (18) and nephropathy (19). These monkeys also have visual function loss (20) and develop preproliferative retinopathy similar to human diabetic retinopathy, which includes capillary nonperfusion, cotton wool spots, intraretinal hemorrhages, microaneurysms, and intraretinal microvascular abnormalities (21). In this study, the number of polymorphonuclear leukocytes in retina was correlated with total cholesterol, LDL, the total cholesterol–to–HDL cholesterol ratio, and triglycerides in a colony of monkeys that spontaneously developed type 2 diabetes.

RESEARCH DESIGN AND METHODS

The monkeys studied included 21 diabetic and nondiabetic aging rhesus monkeys (*M. mulatta*) maintained in the Obesity and Diabetes Research Center of the University of Maryland in accordance with the National Academy of Sciences/National Research Council Guide for the Care and Use of Laboratory Animals and the tenets for humane care and use of animals in ophthalmic and vision research. We studied 15 obese diabetic monkeys and 6 nondiabetic monkeys that were killed electively because of failing health (Tables 1 and 2). Eyes were enucleated and dissected immediately after euthanasia with intravenous sodium pentobarbital, and tissues were immersion-fixed within 1 h of enucleation. The animals were not perfused before euthanasia and enucleation of the eyes.

Age, diabetic history (duration and degree), the presence of hypertension, and other abnormalities were carefully evaluated using the historical medical records and the Obesity and Diabetes Research Center database for each monkey. Blood pressure and plasma glucose levels were measured periodically through life under ketamine anesthesia, and the median for each was determined. Plasma total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides were sampled just before the start of the animal's final decline in health and were determined by enzymatic colorimetric method.

Hypertension was graded on the following scale: systolic pressure 120–129 mmHg was considered mild hypertension, 130–149 mmHg moderate hypertension, and ≥150 mmHg severe hypertension. Normal monkeys have blood pressure <110 systolic and <70 diastolic. Severity of diabetes was based on fasting plasma glucose levels: 126–149 mg/dl was mild diabetes, 150–250 mg/dl moderate, and >250 mg/dl severe. Duration of diabetes, when it could be determined, was established with onset defined as meeting the American Diabetes Association criterion for humans of two fasting glucose levels ≥126 mg/dl.

ADPase and nonspecific esterase staining. After enucleation, a deep incision was made 1.0 cm posterior to the limbus with a scalpel blade, and the anterior segment was removed. After removal of the vitreous, the retina was separated carefully from the retinal pigment epithelium and choroid and fixed in 2% paraformaldehyde in 0.1 mol/l cacodylate buffer overnight at 4°C. The retina was washed in 0.1 mol/l cacodylate buffer with 5% sucrose and then incubated for the enzyme histochemical demonstration of ADPase activity, as previously published (22).

TABLE 2
Relation between counts of polymorphonuclear leukocytes and medical and histological data of diabetic monkeys

Identifica-tion no.	Age (years)	Duration of diabetes (years)	Severity of diabetes	Hyper-tension	PMNs/field	Capillary dropout	Large vessel pruning	Cotton wool spots	Micro-aneurysms	IRMA	New vessels
1	16.3	1.25	++	++	2.3	None	None	None	None	None	None
2	18.7	5.5	++	+	9.0	None	None	None	None	None	None
3	21.1	4	+++	++	11.1	+†	±*	+†	None	None	None
4	22.1	4.3	+++	+++	7.5	+†	None	+†	None	None	None
5	24	>4	+++	?	1.4	None	None	None	None	None	None
6	26.1	5	+++	+	4.3	None	None	None	None	None	None
7	28	15	+++	++	1.1	None	None	None	None	None	None
8	28.3	4.4	+++	++	0.4	None	None	None	None	None	None
9	29.8	>7	+++	−	8.4	+†	None	None	None	None	None
10	30.8	9.5	+++	−	5.5	None	None	None	None	None	None
11	30.9	7	+++	+	3.1	+++*	+++*	None	++	+++	None
12	31.2	8.1	+++	+++	13.9	+++*	+++*	+	None	+	None
13	31.5	3	+++	+++	6.0	+++*	+++*	+	++	++	None
14	31.6	13	+++	+++	13.8	++†	None	+†	None	None	None
15	33.9	8.3	+++	+++	15.8	None	None	None	None	None	None

−, none; ±, very mild; +, mild; ++, moderate; +++, severe. * Throughout posterior pole; †scattered or focal. IRMA, intraretinal microvascular abnormality; PMN, polymorphonuclear leukocyte.
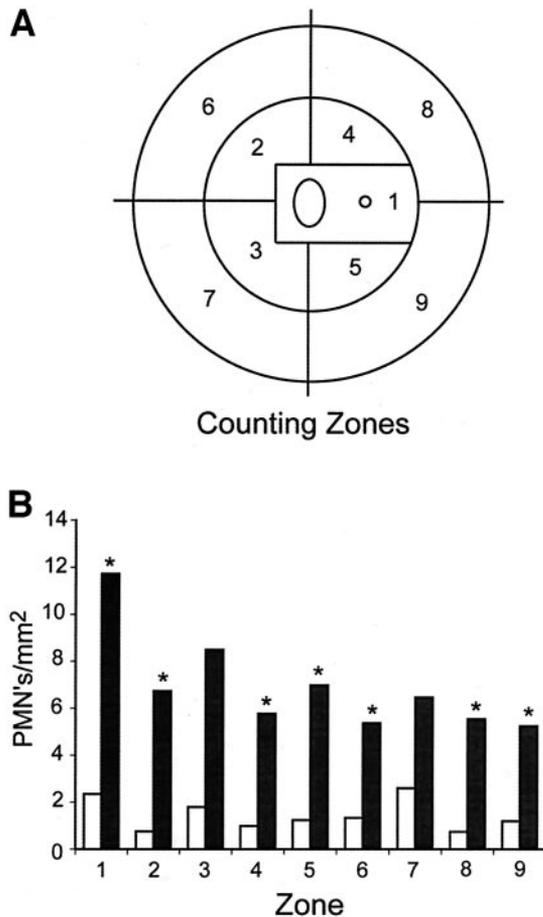
FIG. 1. *A*: Nine zones in which polymorphonuclear leukocyte number per millimeter squared were counted in retina from a left eye. The small circle represents the macula, and the small oval represents the optic disc. *B*: Mean number of polymorphonuclear leukocytes (PMNs) per millimeter squared in diabetic (■) and nondiabetic (□) retinas. The polymorphonuclear leukocyte number was elevated in all zones of the diabetic retina, but the differences were only significant in zones 1, 2, 4, 5, 6, 8, and 9 (zone 1, $P = 0.0083$; zone 2, $P = 0.0123$; zone 4, $P = 0.0085$; zone 5, $P = 0.0237$; zone 6, $P = 0.0098$; zone 8, $P = 0.01$; and zone 9, $P = 0.0261$). *Significant difference.

After ADPase incubation, nonspecific esterase enzyme histochemical staining was performed using a naphthol AS-D chloroacetate esterase kit (Sigma Diagnostics, St. Louis, MO) (23). This method preferentially stains red the granulocyte population of leukocytes. The incubation solution was made as recommended by the manufacturer. The retina was incubated for 45 min at 37°C while protected from light. The ADPase- and nonspecific esterase–stained retina was washed in 0.1 mol/l cacodylate buffer and kept in 2% paraformaldehyde in 0.1 mol/l cacodylate buffer until further examination and analysis.

Counting neutrophils. A total of 15 diabetic and 6 nondiabetic monkey retinas were analyzed for the number and location of polymorphonuclear leukocytes in the retina. After flat-mounting the retinas in cacodylate buffer, the whole retina was divided into nine zones (zones 1–9) (Fig. 1*A*). On an image of each retina, a horizontal line was drawn through the centers of the optic disc and the macula, and a vertical line was drawn through the optic disc at 90° to the horizontal line (Fig. 1). A circle was drawn 6 disc diameters (DD) from the optic disc center to separate central or posterior zones from peripheral zones. Additional horizontal lines were drawn 2 DD inferior and superior to the horizontal midline, and a vertical line 1 DD nasal to disc was also drawn. Zone 1 was then defined as the rectangular area including the macula from 1 DD nasal to 6 DD temporal to the disc and 2 DD inferior and superior to the disc. The rest of the retina was divided with posterior regions (zones 2, 3, 4, and 5) and periphery regions (zones 6, 7, 8, and 9) separated by the circle 6 DD from the optic disc. Finally, the zones in the periphery and posterior represented each quadrant formed by the central vertical and horizontal lines.

Using a Zeiss microscope at 160× magnification (field area = 1 mm²), neutrophils were counted in eight random microscopic fields with viable capillaries in each zone. Areas of viable capillaries were determined by the

presence of ADPase⁺ microvasculature. Neutrophils were stained intensely red after nonspecific esterase incubation, and they appeared elongated in capillaries and rounded in larger vessels under brightfield illumination. Darkfield illumination, which allows the lead ADPase reaction product to be clearly seen, was used in conjunction with brightfield illumination to determine whether neutrophils were intralumenal and to determine their location within the vascular hierarchy. The data presented represents only polymorphonuclear leukocytes in the microvasculature, not arteries and veins. The mean density of neutrophils in each monkey retina was calculated using the zonal mean counts from eight fields in each zone. The number of neutrophils in each group of monkeys was determined as the means ± SE per millimeter squared of retina. Comparisons between groups were performed using two-tailed Student's *t* test. $P \leq 0.05$ was considered significant.

Flat-embedding of retinas. The regions described above from each retina were excised and placed between two pieces of Nytex screen in a holder, as previously published (22). The tissue was fixed in 25% Karnovsky's fixative, pH 7.4, at 4°C until processing. The tissue was then washed, dehydrated, and infiltrated with freshly catalyzed glycol methacrylate (JB-4; Polysciences, Warrington, PA), as previously published (22). After infiltration, the retina was embedded in JB-4, and the blocks were stored in a desiccated environment. The cured blocks were observed using both brightfield and darkfield illumination. Sections 2.5 μ thick were cut on a dry glass knife with a Sorvall NT2 microtome. Some sections were stained with periodic acid Schiff and hematoxylin, whereas others were developed with ammonium sulfide to stain ADPase activity in viable blood vessels and also with 0.1% thionin to counterstain the cell nuclei blue.

RESULTS

Neutrophils in the retinal vasculature. In ADPase/nonspecific esterase–incubated retinas, the ADPase reaction product clearly labeled the vasculature. It was visualized as a lead phosphate precipitate by darkfield illumination. Blood vessels that expressed little ADPase activity were assumed to be less viable and were suspected to have degenerative or dysfunctional endothelial cells, as demonstrated previously (22). The nonspecific esterase incubation resulted in bright red–stained neutrophils, whereas monocytes, if stained, had only a light pink color. Only neutrophils in the microvasculature were counted in this study. In the diabetic retinal vasculature, neutrophils were numerous, especially around the optic disc and macula (Fig. 1*B*), and their relation to compromised vasculature (low or no ADPase activity) was apparent (Fig. 2). Neutrophils were often observed in blood vessels that lacked ADPase activity and were seen in large numbers in blood vessels adjacent to areas with capillary dropout, presumed nonperfused areas (Figs. 2 and 3). When the blood vessels documented en bloc were sectioned, the loss of ADPase adjacent to the polymorphonuclear leukocytes was apparent in some capillaries (Fig. 3). At the borders of regions with capillary dropout, vascular remodeling was apparent and exemplified by venous loops, which were also often associated with neutrophils. Neutrophils were also frequently observed in microaneurysms (Fig. 2*D*). In four diabetic monkeys, a large area of capillary loss was apparent between the optic disc and fovea, with the area temporal to the fovea having viable blood vessels and the area nasal to the fovea having no viable blood vessels (Fig. 2*A*).
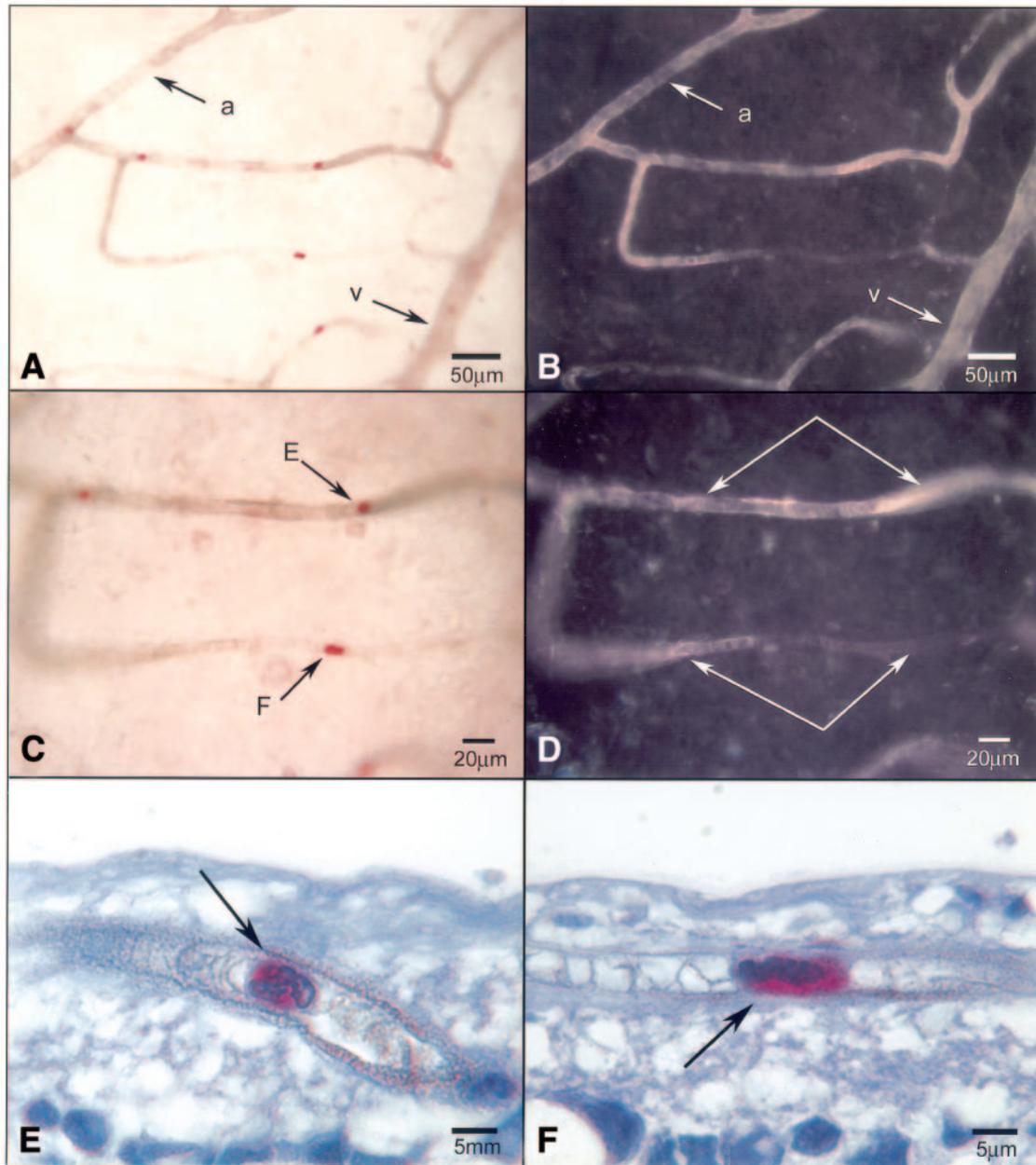
The number of polymorphonuclear leukocytes was elevated in diabetic monkeys compared with nondiabetic monkeys in every zone of the retina (Fig. 1*B*). This difference was significant ($P \leq 0.01$) in zones 1, 2, 4, 5, 6, 8, and 9, but the greatest difference was in zone 1 (11.73 polymorphonuclear leukocytes/mm² in diabetic monkeys compared with 2.35 in nondiabetic monkeys), which was also the most significant difference ($P = 0.0083$). It seems
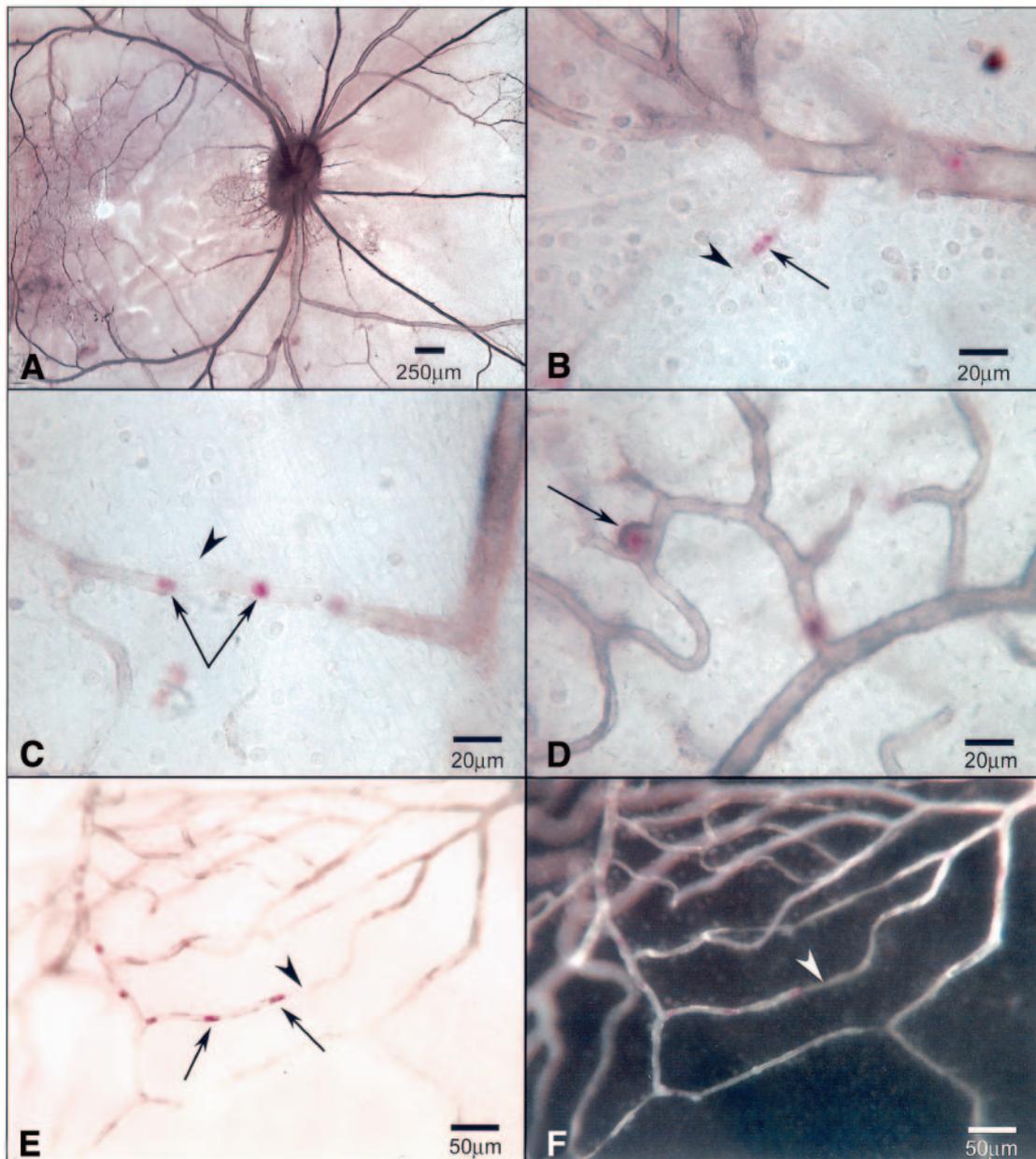
FIG. 2. Nonspecific esterase and ADPase labeling in a 31.5-year-old diabetic monkey (no. 14) (*A–D*) and a 31.2-year-old diabetic monkey (no. 13) (*E* and *F*). *A*: A large area of capillary loss extends from fovea to optic disk within and beyond the vascular arcades as well as a large area nasal to optic disk. An apparent cilio-retinal vascular system temporal to the disc remains viable (i.e., has ADPase activity). *B*: Red nonspecific esterase–positive neutrophils (arrow) are present in a vascular segment that lacks ADPase activity (arrowhead). *C*: Two neutrophils (arrows) are present on either end of a vascular segment that lacks ADPase activity. There is a branch of the capillary (arrowhead) that also lacks ADPase activity. *D*: A neutrophil is present in an aneurysm that has formed at a branch point in a capillary. *E*: Brightfield illumination shows neutrophils (arrows) in a capillary segment adjacent to a nonperfused area (arrowhead). *F*: Darkfield illumination of the same area in *E* shows reduction in ADPase activity in some of the capillaries in this area of capillary dropout. *A–E*: Bright field illumination of flat mount retina. *F*: Darkfield illumination of flat mount retina.

unlikely that the large number of polymorphonuclear leukocytes in the posterior pole of diabetic monkeys was caused by increased numbers of blood vessels since this was not the area that had the greatest number of polymorphonuclear leukocytes in nondiabetic monkeys (Fig. 1*B*). The number of neutrophils per millimeter squared of retina (mean $\pm$ SE) was 1.45 ± 1.62 (range $0–4.6$) in nondiabetic monkeys and 6.91 ± 5.01 ($0.4–15.8$) in diabetic retina ($P = 0.018$) (Fig. 4). In the diabetic group, the neutrophil number increased with the presence of retinopathy (4.98 ± 5.21 in retinas without retinopathy vs. $9.11 \pm$ 4.04 in retinas with retinopathy), but the difference was not significant ($P = 0.113$) (Fig. 4*B*). Retinopathy was defined as any of the following characteristics: areas of capillary dropout, aneurysms, and intraretinal microvascular abnormalities. In 13 severe diabetic monkeys (excluding the two youngest diabetic monkeys), the number of neutrophils increased as severity of hypertension increased. The number of neutrophils per millimeter squared of retina was $4.84 \pm$ 3.86 in diabetic monkeys with hypertension that was mild to moderate and 11.4 ± 4.35 in diabetic monkeys with severe hypertension ($P = 0.02$) (Fig. 4*C*).

FIG. 3. Neutrophils in capillaries from the ADPase/nonspecific esterase–incubated retina of a 31.6-year-old diabetic monkey (no. 15) viewed in brightfield (*A* and *C*) and in darkfield (*B* and *D*). Some polymorphonuclear leukocytes (arrow E in panel *C*) are in capillaries with normal ADPase activity in the flat perspective and when sectioned (*E*). Other polymorphonuclear leukocytes (arrow F in panel *C*) were associated with loss in ADPase activity at the site of polymorphonuclear leukocyte (*D*). The polymorphonuclear leukocyte at this site in cross section (*F*) appears flat and fusiform. *A–D*: ADPase and nonspecific esterase activity en bloc. *E–F*: Cross sections stained with toluidine blue and treated with ammonium sulfide to develop the ADPase activity.

The plasma level of total cholesterol was 240.9 mg/dl in diabetic monkeys and 123.4 mg/dl in nondiabetic monkeys ($t = -3.725$, $P = 0.0018$). The plasma LDL cholesterol was 130.1 mg/dl in diabetic monkeys and 59.8 mg/dl in nondiabetic monkeys ($t = -3.15$, $P = 0.0060$), and the plasma total cholesterol–to–HDL cholesterol ratio was 9.28 in diabetic monkeys and 2.68 in nondiabetic monkeys ($t = -2.55$, $P = 0.0217$). The plasma triglyceride level was 636.2 mg/dl in diabetic monkeys and 119.2 mg/dl in nondiabetic monkeys ($t = -3.08$, $P = 0.0071$). There was a significant exponential correlation between the density of neutrophils in retina and the level of total cholesterol ($R = 0.907$), LDL cholesterol ($R = 0.875$), the total cholesterol–to–HDL cholesterol ratio ($R = 0.86$), and triglycerides ($R = 0.888$) (Fig. 5).

Finally, although the animals were not perfused before enucleation, there was no significant correlation between the number of circulating white blood cells immediately before death and the number of polymorphonuclear leukocytes counted in retina (Fig. 6). This analysis included control and diabetic monkeys as well.

DISCUSSION

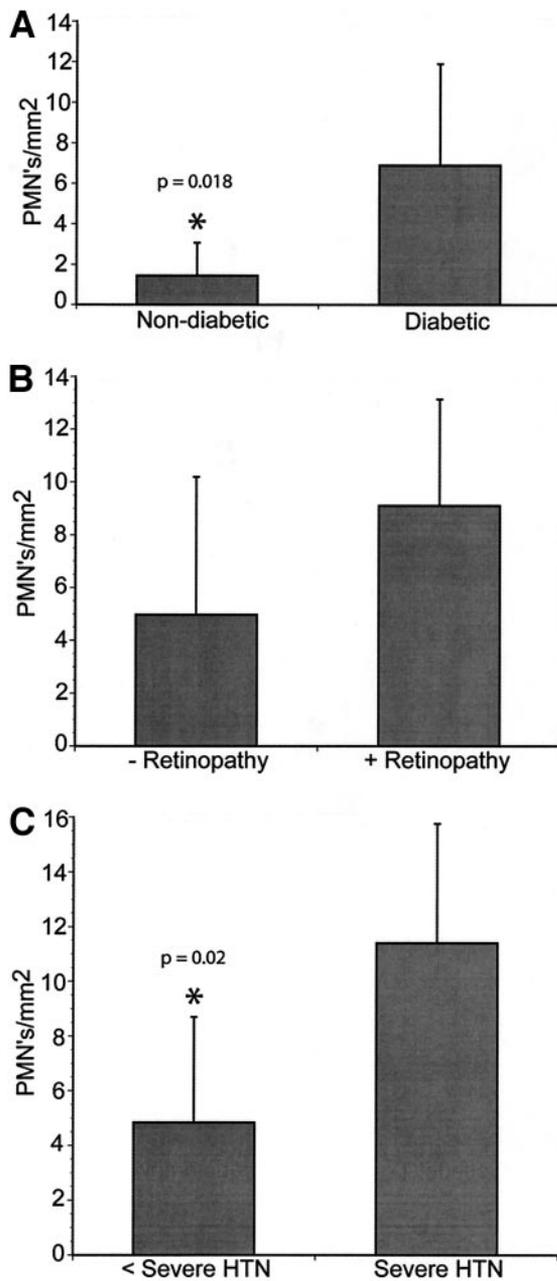Spontaneously type 2 diabetic monkeys develop retinopathy that has characteristics in common with human dia-

FIG. 4. Relationship between retinal density of neutrophils and diabetes and hypertension. *A*: **The density of neutrophils was significantly different between diabetic and nondiabetic animals.** *B*: **There were more neutrophils in diabetic animals with than without retinopathy, but the difference was not significant.** *C*: **There was a significant difference in neutrophil numbers between diabetic animals that had severe hypertension versus those that did not have severe hypertension ($P = 0.02$). *Significant difference. HTN, hypertension; PMN, polymorphonuclear leukocyte.**
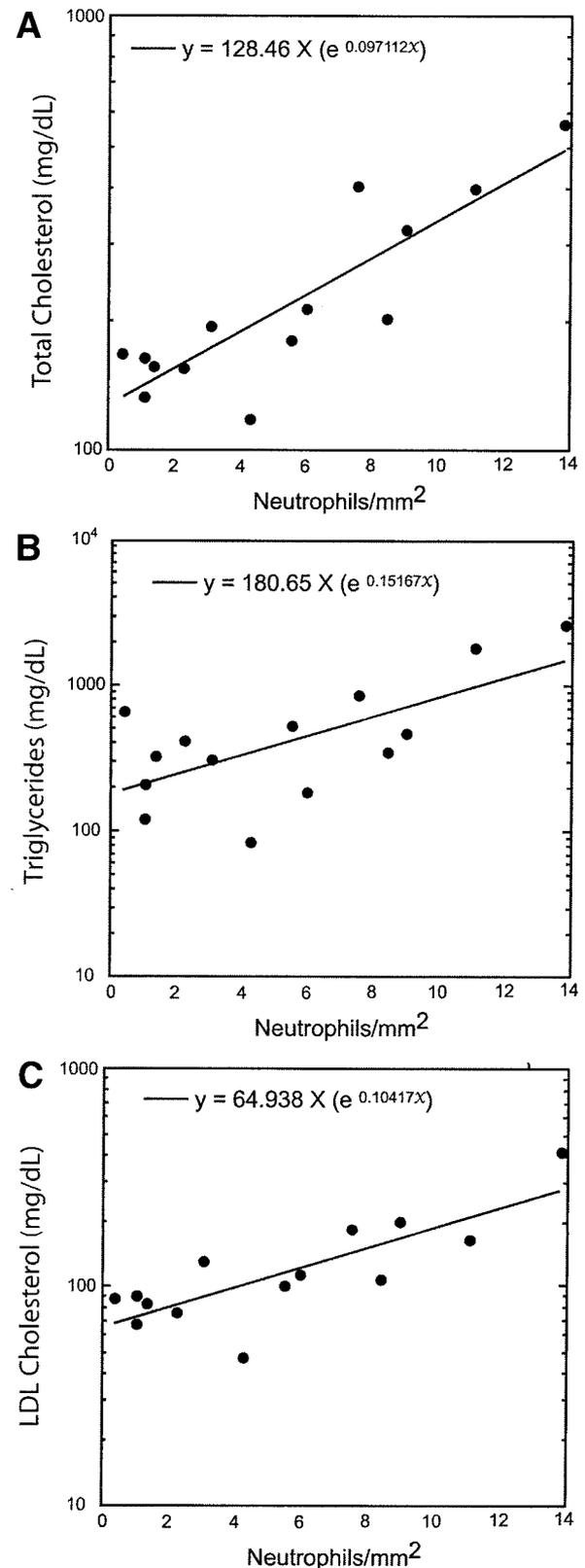

FIG. 5. Linear regression analysis of polymorphonuclear leukocyte number per millimeter squared of retina and log levels (mg/dl) of total cholesterol (*A*), triglycerides (*B*), and LDL cholesterol (*C*).

betic retinopathy; these include capillary nonperfusion, cotton wool spots, intraretinal hemorrhages, microaneurysms, and intraretinal microvascular abnormalities (21). In our study, the number of neutrophils was significantly higher in diabetic than in nondiabetic retinas. Although not statistically significant, the neutrophil number was higher in diabetic retinas with retinopathy than in diabetic retinas without retinopathy. This increase in neutrophils was associated with loss in capillary viability (loss in ADPase) and probable endothelial cell injury. Interest-

ingly, the greatest number of neutrophils was adjacent to regions with capillary dropout. We often observed neutrophil accumulation in microaneurysms. Stitt et al. (24)
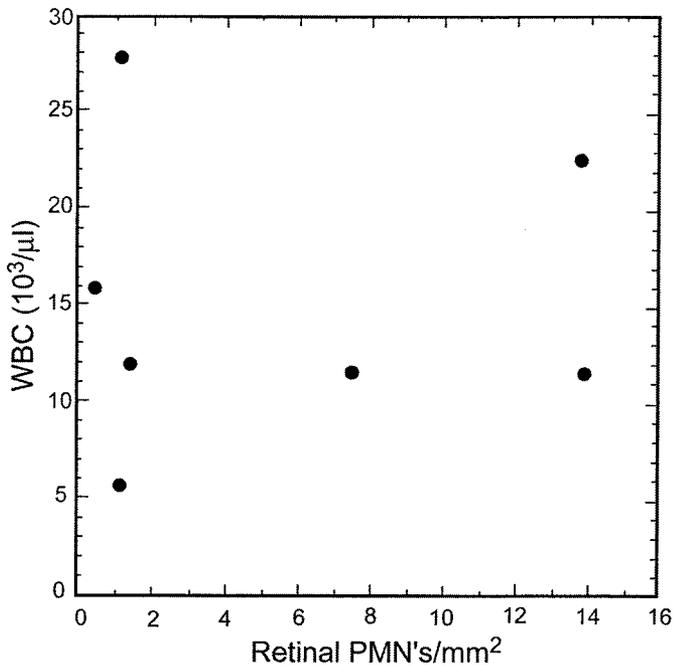
FIG. 6. Relationship between circulating white blood cells (per µl blood) and polymorphonuclear leukocytes (per mm² of retina) at the time of death. There was no significant relationship between polymorphonuclear leukocyte numbers and the number of circulating white blood cells immediately before death. PMN, polymorphonuclear leukocyte; WBC, white blood cell.

observed extensive accumulation of neutrophils in the lumen of microaneurysms of human type 1 diabetic subjects in which the endothelium remained intact but pericytes were variably absent.

Four of the diabetic monkeys had extensive areas of capillary nonperfusion in the posterior pole. One possible explanation for this phenomenon is that neutrophil numbers were greatest in the posterior pole, and neutrophils initiated occlusions and increased perfusion pressure in the area. A similar large central area of nonperfusion was observed in pancreatectomized cats (25). The pattern of the vascular loss in the monkeys was noteworthy because the capillary network on the nasal side of the fovea is lost, whereas capillaries on the temporal side of the fovea remained viable. Capillary nonperfusion within 1.5–2.5 DD nasal to the optic disc is known to proceed rapidly and involve the farther periphery in some human subjects with diabetes (26), whereas the peripapillary zone (<1 DD away from the disc margin) was consistently spared from vaso-occlusions (27). In the spontaneously diabetic monkeys, the temporal peripapillary zone was spared in three of the four monkeys with severe retinopathy but these spared vessels were cilioretinal in origin (21). The loss of viable capillaries is likely to result from neutrophil plugging or endothelial cell dysfunction while the large areas of vascular loss may have their origins in hemodynamics of the retinal vascular system, which changes in hypertension.

In the early stage of diabetes, reduced retinal blood flow is thought to result from increased vasoconstriction at the capillary level, and this may increase neutrophil plugging in the microcirculation. Human diabetic leukocytes have been shown to be immobilized easily and to plug the retinal microvasculature (28). Leukocytes are not sufficiently deformable and more are activated under conditions of low perfusion (27,29,30). Leukocyte plugging may be involved in capillary nonperfusion, endothelial cell damage, and vascular leakage in the retinal microcirculation (1,30). We have observed significantly increased numbers of polymorphonuclear leukocytes (more than fourfold greater) in human diabetic choriocapillaris compared with nondiabetic choriocapillaris, and the polymorphonuclear leukocytes were associated areas of capillary loss (23,31). Decreased deformability can be caused by changes in membrane lipid composition and subsequently altered membrane fluidity of neutrophils (32). Increased glycation of the cell membrane or intracellular proteins may cause stiffening of cells. A further possibility is the presence of a state of chronic low-grade neutrophil activation that could increase basal cytoskeletal F-actin, leading to a reduction in neutrophil deformability (30). It is possible that the declining health of the monkeys at the time of necropsy in the current study contributed to the number of polymorphonuclear leukocytes, but all monkeys, including controls, were declining in health at the time of necropsy, and there were significantly more polymorphonuclear leukocytes in the diabetic monkeys than in normal controls.

Neutrophil–endothelial cell interactions are known to play a role during retinal vascular diseases, including diabetes. Neutrophil adhesion to endothelium in vitro can be prompted by hyperglycemia as low as 5.5 mmol/l (33). After hyperglycemic treatment, neutrophils exhibit increased adhesion to pulmonary arterial endothelial and retinal endothelial cells. A two- to threefold increase in retinal leukocyte adhesion was observed in streptozotocin-induced diabetic rats (34). One reason for increased adhesion could be upregulation of leukocyte–endothelial cell adhesion molecules (1). ICAM-1 immunoreactivity is elevated in human diabetic retinal blood vessels, and this is accompanied by an increased number of neutrophils in the retina (5). Diabetic monocytes and neutrophils also show higher surface expression of CD11b/CD18, a ligand for ICAM-1 (35,36). Miyamoto et al. (37) found enhanced expression of adhesion molecules in the capillary endothelium of the retina in diabetic rats. Neutralization of ICAM-1 or CD18 decreased diabetes-induced retinal leukostasis and prevented endothelial cell injury and death in diabetic rats (37).

Leukocyte–endothelial cell adhesion enhances the ability of leukocytes to generate ROS. Superoxide radical production from diabetic subjects' neutrophils exceeds that of nondiabetic subjects, and increases in superoxide production may damage vascular endothelial cells and result in subsequent vascular occlusion (10). The result of this injury could be the loss of critical ectoenzymes like ADPase on endothelial cells, which prevents ADP-initiated platelet aggregation. ADPase was reduced in many areas adjacent to polymorphonuclear leukocytes in the current study.

In our study, the number of neutrophils was significantly increased in diabetic monkeys with severe hypertension, compared with diabetic monkeys with mild to moderate hypertension ($P = 0.02$). This suggests that the increased number of neutrophils and their interaction with endothelial cells are related to the severity of hypertension in diabetic individuals. Hypertensive diabetic patients have

neutrophils that are less deformable than normotensive diabetic individuals, but the deformability of neutrophils from subjects with impaired glucose tolerance was similar to those of subjects with normal glucose tolerance (38). Insulin itself acutely promotes vasodilation and inhibits platelet aggregation; however, in insulin resistance, as is present in the spontaneously diabetic monkeys, these actions of insulin are reduced, resulting in endothelial dysfunction, platelet aggregation, hypertriglyceridemia, and eventual loss of glycemic control. Essential hypertension seen in insulin resistance is likely associated with elevated production of angiotensin II. Angiotensin II is either directly or indirectly responsible for additional vascular effects, including increased proinflammatory adhesion molecule expression, increased cytokine and growth factor expression, increased endothelin-1 formation, and an increase in endothelial scavenger receptors for oxidized LDL cholesterol (7).

There was a significant exponential correlation between the number of neutrophils in the retina and the plasma level of total cholesterol, LDL cholesterol, the HDL cholesterol–to–total cholesterol ratio, and triglycerides in these monkeys. Dyslipidemia, particularly elevated serum triglycerides; low HDL cholesterol; and elevated intermediate-density lipoprotein levels are among the hallmarks of the insulin resistance syndrome and particularly common among type 2 diabetic human subjects (7). Postprandial hypertriglyceridemia is more exaggerated in type 2 diabetes. In addition, LDL cholesterol is elevated in many diabetic patients. These lipid abnormalities are known to decrease blood flow and may be associated with endothelial dysfunction and impaired vasodilatory response. Hypertriglyceridemia is associated with a higher level of soluble vascular adhesion molecule-1 and soluble ICAM-1, a smaller LDL cholesterol particle size, and a reduction in human flow-mediated vasodilation (8). Chen et al. (39) reported that dyslipidemia, not hyperglycemia, induces inflammatory adhesion molecules in human retinal vascular endothelial cells. In fact, leukocyte entrapment in the retinal microcirculation is enhanced in the early stage of hypercholesterolemia even in nondiabetic rats and was increased as the period of hypercholesterolemia was prolonged (6).

In this study using spontaneous type 2 diabetic monkeys, severity of diabetic retinopathy was associated with increased numbers of neutrophils in the retinal vasculature. Hypertension and dyslipidemia were associated with the increased numbers of neutrophils. This study provides further evidence for diabetes being an inflammatory disease and for neutrophils being responsible for capillary loss in diabetic retina.

ACKNOWLEDGMENTS

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