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Activated Monocytes and Granulocytes, Capillary Nonperfusion, and Neovascularization in Diabetic Retinopathy

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Capillary occlusions are characteristic features of the early diabetic retinopathy and are presumed to initiate neovascularization. Activated leukocytes can cause microvascular occlusions and cell damage by release of cytotoxic products. To explore the role of leukocytes in capillary occlusion, nonperfusion, and neovascularization of diabetic retinopathy, a rat model was used, in which a diabetic state was induced by alloxan. Retina flat preparations were differentially stained for monocytes and granulocytes. Capillary occlusion, nonperfusion, and neovascularization were assessed microscopically in the center, midperiphery, and periphery of the retina. In contrast to control retinas, 2- to 9-month diabetic rats showed many capillary occlusions by leukocytes, especially monocytes, endothelial cell damage, extravascular macrophage accumulation, and tissue damage. The percentage of activated monocytes and granulocytes in the circulating blood of diabetic rats was greatly increased, and areas of capillary 'loss' and neovascularization in the retina coincided with sites of extravascular leukocytes. The authors' results suggest a potential role of monocytes and macrophages in the pathogenesis of diabetic retinopathy. (Am J Pathol 1991, 139:81–100)

Capillary occlusions constitute a characteristic pathologic feature in early diabetic retinopathy, and are presumed to initiate neovascularization.[1–3] Microaneurysms, intraretinal microvascular abnormalities, vasodilation, selective loss of intramural pericytes, hypertrophy of the basement membrane, and swelling of endothelial cells are commonly found in the early stages of diabetic retinopathy and have been correlated to capillary occlusions, although the sequence of events and the cause of the capillary occlusion are as yet unknown.[4–6]

Blood flow in the microcirculation is determined by various factors such as blood pressure, the structure of the vascular network, and by the rheologic properties of the circulating cells. Multiple hemorheologic abnormalities have been described in diabetic patients, but their respective pathogenetic roles have not been demonstrated.[7,8]

In the last decade, strong evidence has been gathered suggesting that leukocytes can cause capillary obstruction. This is due to their large cell volume and high cytoplasmic rigidity.[9] Significantly higher forces are required to deform leukocytes in a capillary than are necessary for erythrocyte deformation and passage. Single leukocytes often completely fill the capillary lumen, which is rarely observed with erythrocytes, except in extremely narrow capillaries. Furthermore leukocytes have a natural tendency to adhere to the vascular endothelium,[10–12] whereas red cells do not usually adhere to endothelial cells.

Leukocytes can become trapped in capillaries under conditions of reduced perfusion pressure (eg, caused by vasoconstriction, as seen in early stages of diabetes[13]) or in the presence of an elevated adhesive stress between leukocytes and the endothelium, endothelial swelling, or narrowing of the capillary lumen by perivascular edema. Elevated adhesive stress can result from release of chemotactic factors or expression of adhesion molecules on leukocytes or endothelial cells. Once this occurs, even a normal perfusion pressure may fail to dislodge the

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leukocyte.[14,15] Irreversible microvascular occlusion of capillaries by leukocytes in ischemia and shock has been observed in a number of organs.[16–19]

Among leukocytes, the monocytes and granulocytes are particularly likely to cause microvascular injury, because their activation can be accompanied by release of proteases and oxygen-derived free radicals.[20] The activation of neutrophils or monocytes can be induced by a number of mechanisms.[20] Although there is indeed evidence for neutrophil activation in patients with insulin-dependent diabetes mellitus,[21] the pathophysiologic significance of neutrophil activation in the development of diabetic complications remains largely unexplored.

The possible role of leukocytes and leukocyte-induced capillary occlusion in diabetic retinopathy cannot be studied, at least in the short term, by noninvasive methods in humans. Although no animal model truly reflects human diabetic pathology, a rat model was selected in which diabetes was induced by alloxan. Alloxan causes islet cell damage and subsequent insulin deficiency. Despite past controversy about some aspects of the end-stage retinopathy in alloxan-diabetic rats, and despite the obvious anatomic differences (lack of a macula in the rat retina), this model of diabetic retinopathy often shows elements characteristic of human retinopathy, such as capillary occlusion, microvascular degradation, and neovascularization. It therefore seemed worthwhile to explore the role of leukocytes in the animal model first. The potential contribution of mechanisms discovered in the animal model to human diabetic retinopathy will have to be explored in humans at a future stage.

Techniques were developed that allow the identification of occluded microvascular pathways in retina flat mounts, and the distinction between granulocytes and monocytes in microvessels of the retina, as well as the assessment of capillary destruction and neovascularization. The degree of neutrophil and monocyte activation in the circulating blood was determined with the nitroblue tetrazolium (NBT) test, a measure of superoxide formation by these cells.

The experimental protocol we selected permits direct investigation of the pathogenesis of diabetic retinopathy in the rat model in a way not feasible in humans, although retinal network reconstructions have recently been initiated in man.[22,23] It provides a data set about the frequency and localization of capillary occlusions, necessary to model the hemodynamics of the diabetic rat retina and to compare the diabetic microcirculation with that of a normal retinal network.

Methods

Animal Model

A diabetic condition was induced in 180- to 200-g male Wistar rats (n = 17) by a single injection of 50 mg/kg alloxan (Sigma Chemical Co., St. Louis, MO) in physiologic saline into the tail vein, after a 12-hour fasting period. Control rats (n = 11) were injected with the same volume of saline. After administration of alloxan, the animals were allowed free access to food and water. The onset of diabetes, defined as a glucose concentration exceeding 200 mg/dl in blood samples obtained from the tail vein, was verified 1 week later with a Glucometer II Model 5550 (Miles, Elkhart, IN). Diabetic animals and matching controls were killed 2, 5, and 9 months after injection of alloxan or saline. All experimental procedures were approved by the Animal Subject Committee of the University of California, San Diego.

Tissue Preparation

To determine the microvascular pathology and the retinal leukocyte concentrations over time (at 2, 5, and 9 months of diabetes), a study was carried out in which the retinas of diabetic rats and age-matched controls were prepared without *in situ* perfusion and fixation. Rats were anesthetized by intramuscular injection of pentobarbital (40 mg/kg for diabetic rats; 60 mg/kg for control rats). After the animals had reached surgical anesthesia, a polyethylene catheter (inner diameter [ID] = 0.58 mm; outer diameter [OD] = 0.95 mm) was placed into the femoral vein and heparin was administered (100 units/ml blood; blood volume estimated as 6% of body weight). A 0.5 ml blood sample was taken by cardiac puncture from the left ventricle. The eyes then were enucleated and the optic nerve tied off quickly to prevent blood loss.

A second group of 2- and 5-month diabetic and control rats was used to investigate the occlusions of retinal capillaries by leukocytes. To eliminate errors due to the presence of nonadhering leukocytes, the blood was removed by perfusion with buffer, followed by *in situ* fixation under physiologic pressure, as described later. After general anesthesia with pentobarbital (40 to 60 mg/kg intramuscularly), the abdominal cavity was opened and a polyethylene catheter (ID = 1.2 mm, OD = 1.7 mm) was inserted into the vena cava. Heparin (100 units/ml blood) was administered through this catheter. A second catheter was inserted into the aorta, distal to the renal artery, pushed forward to a position just distal to the aortic arch, and a blood sample (0.5 ml) was taken. Pressure in the aortic inflow catheter was kept at a physiologic level of 120 mm Hg, ie, variations of systemic vascular tonus were compensated by variations in flow.

To remove the blood from the circulation, rats were perfused for 7 minutes with a modified Ringer's solution containing Plasmalyte A (294 mOsm electrolyte solution, pH 7.4, Travenol Laboratories, Deerfield, IL), bovine serum albumin (2.5 g/100 ml, Sigma), heparin (10 U/ml), and sodium nitroprusside (100 μg/100 ml) as a vasodi-

lator. Potassium chloride was added to the solution to a final potassium concentration of 30 mEq. This concentration induces cardiac arrest within 3 to 5 minutes, reducing the time required to exchange the blood with Ringer's solution and minimizing perfusion-induced tissue swelling. The final osmolarity was slightly adjusted to 300 mOsm by addition of distilled water.

Perfusion then was continued with formal-sucrose-solution (4% paraformaldehyde, 5% sucrose in Plasmalyte A, pH 7.4) for 10 minutes, maintaining the systemic pressure constant within 10 mm Hg during the turnover in perfusion medium. This was determined to be sufficient for an initial fixation of the retinal microvessels. The eyes were then enucleated and the optic nerve tied off. After an additional fixation in formal sucrose for 48 hours, the eyes were washed in cacodylate buffer (pH 7.4, 300 mOsm), and coronary sections were made at the posterior portion of the ciliary body. The retinas were then teased from the underlying chorioid and stained as described below. In the following, the term 'perfusion-fixed' will be used in reference to these cleared retinas, whereas the term 'non–perfusion-fixed' will be used for the retinas of the preceeding group, in which the microvasculature still contains blood. This definition will be adhered to, to avoid confusion with the term 'nonperfused' commonly used for vessels that are not perfused by blood *in vivo*, eg, as a result of an occlusion.

Differentiation of Leukocytes in the Retina

Monocytes and granulocytes contain different esterases, and the use of specific substrates to differentiate granulocytes and monocytes in blood smears, bone marrow films, and tissue touch preparations is well established.[24] Leukocyte-containing specimens are incubated with either α-naphthyl acetate (to detect monocytes) or naphthol AS-D chloroacetate (to detect granulocytes) in the presence of freshly formed diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds, which couple with the diazonium salt, forming highly colored deposits at sites of enzyme activity. We consecutively applied both staining methods for histochemistry of the retina, resulting in granulocytes with bright red granulation and monocytes with brown to black granulation that can be distinguished easily under the microscope.

Retinal monocytes were stained as follows: 0.25 ml sodium nitrite solution was added to an equal volume of Fast Blue BB Base Solution, mixed for 2 minutes, and added to 10 ml deionized water at 37°C, followed by addition of 1.25 ml Trizmal TM 7.6 buffer concentrate and 0.25 ml α-naphthyl acetate solution. The retinas were incubated in this solution for 1 hour at 37°C, protected from light. After thorough rinsing in deionized water, the retinas were stained analogously for granulocytes by 45-minute incubation in a solution with 0.25 ml sodium nitrite solution, 0.25 ml Fast Red Violet LB Base solution, 10 ml deionized water, 1.25 ml Trizmal TM 6.3 buffer concentrate, and 0.25 ml Naphthol AS-D chloroacetate solution (all reagents from Sigma).

Histology of the Retina

After completion of the staining procedure, the retinas were mounted on glass slides for light microscopy. A Nikon Microphot FX microscope, a Hitachi color video camera VK-C350, and a color video monitor were used for measurements. The capillary network of the rat retina can be subdivided in an upper layer, closely connected to the precapillary arterioles, and a deeper layer, in the vicinity to the postcapillary venules. These two layers were assessed together, and microphotographs taken in both planes.

For each retina, three concentric regions were separately analyzed: the retinal center, defined as a circular area around the optic nerve with a radius of half the retinal radius; the midperiphery, extending to three quarters of the retina radius; and the periphery. Within each of these regions, three areas were randomly selected. Within each such area, 10 visual fields were selected at random with a total area of about 0.66 mm^2. Thus, in each of the concentric regions, an area of about 2 mm^2 was analyzed, or 6 mm^2 per retina. The following parameters were determined:

1) The length of capillaries per unit area (L/A), measured with a stereologic method,[18] and the number of capillary segments per unit area (N_s/A). The capillary segments, defined as the part of a capillary between two branchpoints, were determined by direct counts on tissue regions of preselected surface area. These parameters serve as quantitative measures to detect vessel 'loss' or neovascularization.[18] In this context, we use the term 'capillary loss' to indicate the lack of perfused capillaries in a circumscribed retinal area. Typically we find loss of a clear endothelial definition at the light microscopical level, and often endothelial vacuolation in these areas. In some, but not all, cases, segments of nonperfused capillaries still can be traced from branch point to branch point.

2) The number of monocytes per vessel length (M/L) and the number of granulocytes per vessel length (G/L), determined in analogy with the methods in reference 18. In non–perfusion-fixed, ie, blood-filled retinas, these parameters indicate difference in leukocytes concentrations. In the perfused and *in situ* fixed retinas, the same parameters provide a measure of capillary occlusion by leukocytes. To correlate the total number of monocytes and granulocytes per capillary length in the entire retina

with the number of these cell types in the systemic circulation, and with their extracapillary accumulation in the retina, we assumed an average area of 23.8 mm^2 for the center, 29.8 mm^2 for the midperiphery, and 41.4 mm^2 for the periphery, based on an average retinal diameter of 11 mm in the rat.

3) The inner and outer diameters, D_i and D_o, of the capillary segments. This was carried out under 50× and 100× oil immersion objective, numerical aperture 1.4. The images were recorded by color video camera, and analyzed at a final magnification of 2250 to 4500× on a color monitor. Under these conditions, approximately 0.25 μ could be resolved. The inner diameter, Di, is of particular interest with regard to leukocyte capillary passage time and plugging. Even slight changes of diameter are of critical importance for leukocyte kinetics, as the typical capillary resting diameter in the rat is only about 3.5 μ. The average wall thickness h of the capillaries was calculated as:

$$h = \frac{D_o - D_i}{2}$$

The individual cross-sectional area A_n for each capillary wall in the examined retinal area and the average cross-sectional area $\bar{A}$ were calculated as a measure of the wall volume per unit vessel length, with D_o^n, D_i^n being inner and outer diameters at randomly selected sites along the capillaries, with n = 1,..,N measurements. The capillary wall cross-sectional area at location n is then

$$A_n = \frac{\pi}{4}\left[(D_o^n)^2 - (D_i^n)^2\right]$$

and the average cross-sectional area is

$$\bar{A} = \frac{1}{N}\sum_{n=1}^{N} A_n$$

Determination of Blood Parameters

The arterial blood sample obtained before perfusion was used to determine the blood glucose level, the hematocrit, and the central leukocyte concentration. The percentage of monocytes and granulocytes was established by differential count. The percentage of activated monocytes and granulocytes generating oxygen free radicals was determined with the NBT test, using the fact that oxygen free radicals cause a spontaneous reduction of the NBT substrate (pale yellow) to formazan crystals (blue-black).[25,26] The NBT solution (0.1%) was prepared by reconstituting a NBT vial (Sigma) with 1.0 ml distilled water. An aliquot of 0.1 ml NBT solution then was mixed with 0.1 ml heparinized blood in a siliconized vial (Sigma). Because most forms of blood manipulation may induce cell activation, and may overshadow the activation induced by the diabetic condition, no cell separation procedure was carried out before the NBT test. The fresh sample was incubated for 10 minutes at 37°C and allowed to stand at room temperature for an additional 10 minutes. The blood–NBT mixture was gently mixed by rolling the vial before and after the incubation. Smears were prepared, stained with Wright's stain (Sigma), and examined microscopically to determine the percentage of activated monocytes and neutrophils, which show either a diffuse intracytoplasmic distribution or a single dense deposit of formazan crystals.

To investigate whether alloxan or hyperglycemia could by themselves induce leukocyte activation in whole blood, samples from normal rats were incubated *in vitro* with alloxan and glucose. The incubation with alloxan was carried out at a concentration equivalent to the estimated blood concentration (assumed blood volume: 6% of body weight) used to induce diabetes in rats. An alloxan stock solution (8.33 mg/ml in phosphate-buffered saline [PBS], pH 7.4, 300 mOsm), 0.1 ml, was added to 0.9 ml blood and incubated at 37°C for 15 minutes. The effect of hyperglycemia was studied by adjusting the glucose concentration of a blood sample to 400 mg/dl by addition of glucose stock solution (40 mg/ml in PBS, pH 7.4) and 15 minutes incubation at 37°C. Using the NBT test, the percentage of activated monocytes and neutrophils was determined and compared with that in control blood, incubated with PBS.

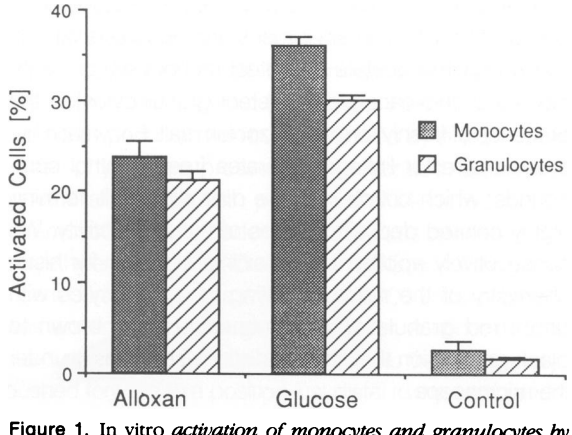


Figure 1. In vitro *activation of monocytes and granulocytes by alloxan and hyperglycemia, determined with the NBT test. Normal rat blood was incubated for 15 minutes at 37°C with alloxan at the same final concentration (0.833 mg/ml) used to induce diabetes in the rat, and with glucose at a final concentration of 400 mg/dl. Control blood was incubated with PBS. Both alloxan and glucose groups are higher than controls (P ≤ 0.001). Data represent mean ± SEM (n = 5).*

Statistical Analysis

Student's *t*-test for paired or unpaired data sets was used for statistical interpretation of the results.

Results

Normal rats had a blood glucose level of 129.3 ± 7.7 mg/dl (mean ± standard error of the mean [SEM]). All alloxan-injected animals showed clinical symptoms of diabetes mellitus, eg, muscle atrophy, polydipsia, and polyuria. Their body weight after 2 months of diabetes was 295 ± 25 g *versus* 409 ± 89 g in controls; after 5 months of diabetes, 322 ± 38 g *versus* 565 ± 70 g; and after 9 months, 415 ± 46 g *versus* 734 ± 46 g. Their blood glucose levels exceeded 300 mg/dl in all cases. After 5 and 9 months of diabetes, all rats presented with cataracts in both eyes. The blood hematocrit was 43.3% ± 1.37% (mean ± SEM) in diabetic rats and 45.1% ± 1.47% in controls ($P > 0.05$).

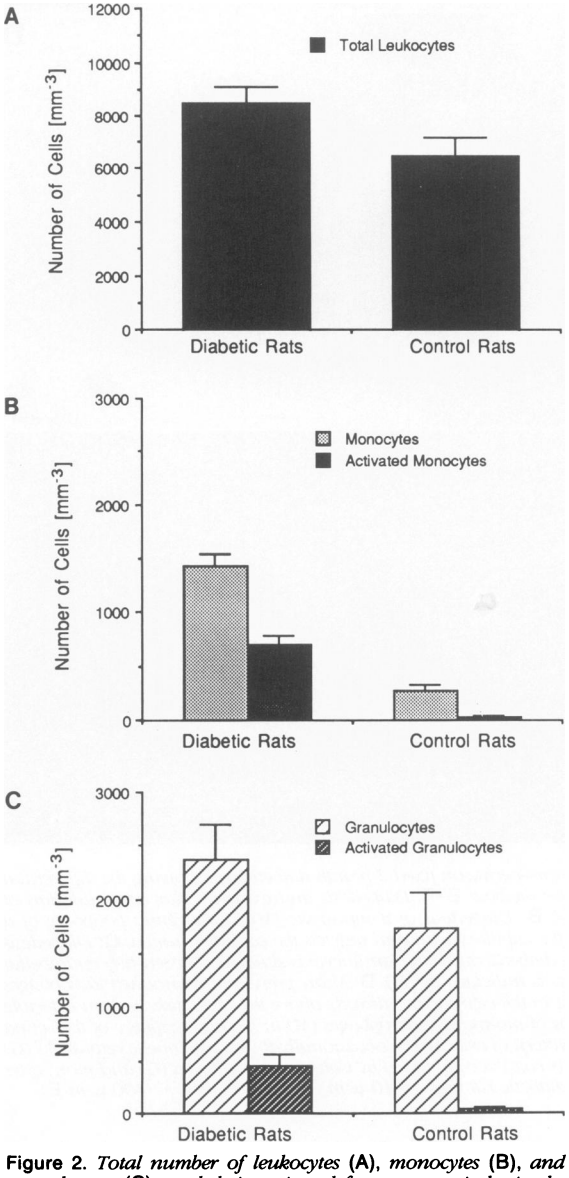


Figure 2. *Total number of leukocytes* **(A)**, *monocytes* **(B)**, *and granulocytes* **(C)**, *and their activated forms, respectively, in the systemic blood of diabetic and control rats. The data for 2-, 5-, and 9-month diabetic animals and control animals, respectively, were pooled (mean ± SEM, n = 17 for diabetic rats, and n = 11 for controls).*

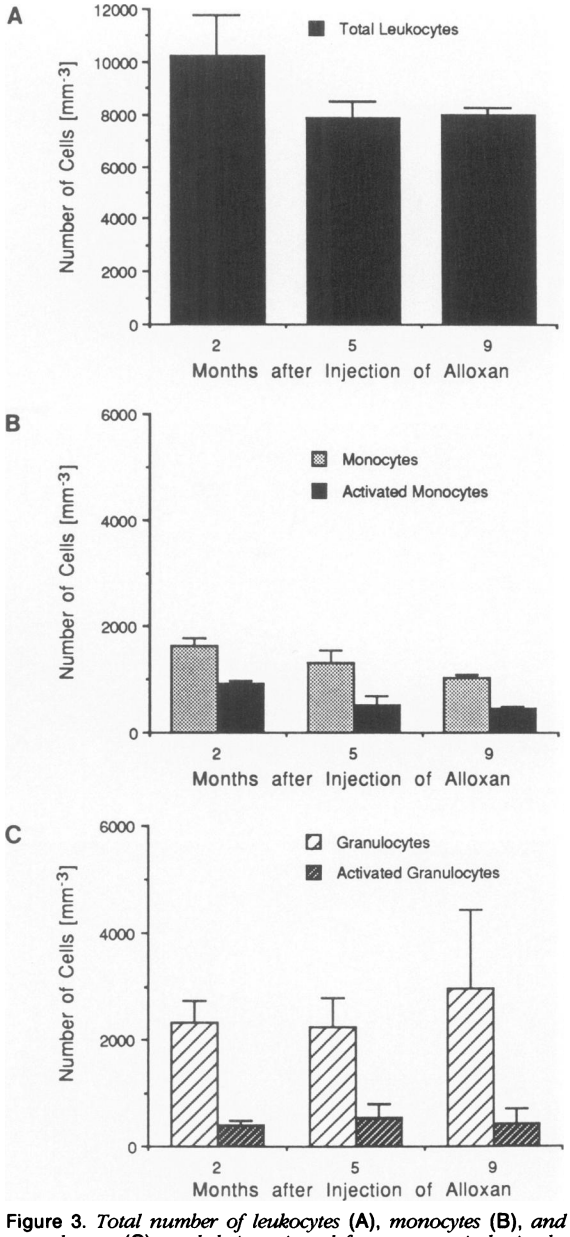


Figure 3. *Total number of leukocytes* **(A)**, *monocytes* **(B)**, *and granulocytes* **(C)**, *and their activated forms, respectively, in the systemic blood of 2-, 5-, and 9-month diabetic rats (mean ± SEM).*

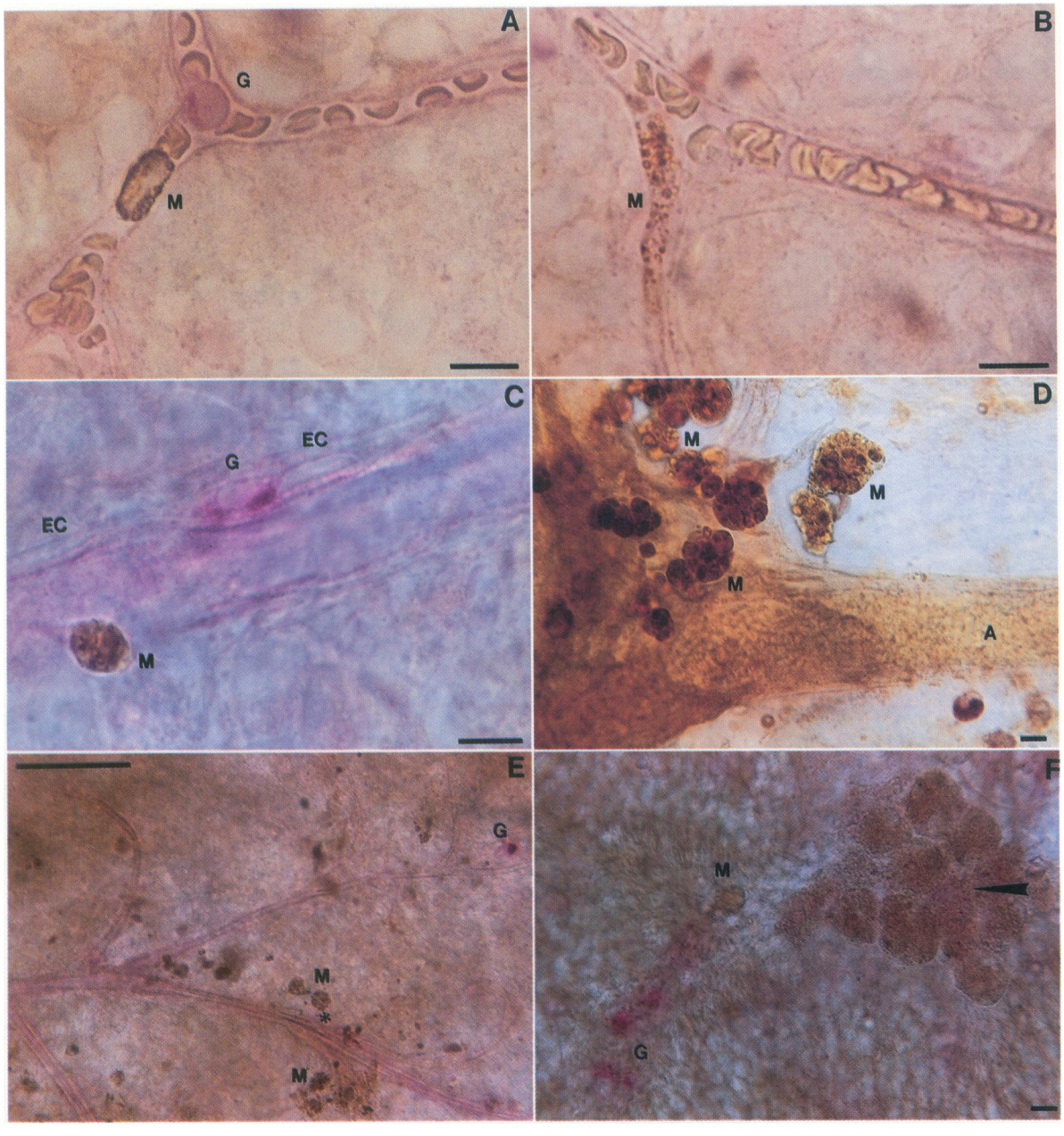


Figure 4. **A**: *Intracapillary leukocytes in the periphery of the retina of a non–perfusion-fixed 2-month diabetic rat, showing the differential staining of a monocyte (M) and a granulocyte (G) by the specific esterase method.* **B–F**: *Diapedesis and extravascular accumulation of leukocytes in diabetic rat retinas, stained by the specific esterase method.* **B**: *Diapedesis of a monocyte (M) in the retina periphery of a non–perfusion-fixed 2-month diabetic rat. The monocyte has penetrated the capillary wall and restricts the capillary lumen.* **C**: *Diapedesis of a granulocyte (G) in the retina periphery of a perfusion-fixed 2-month diabetic rat. The granulocyte is situated between two endothelial cells (EC) in the wall of a postcapillary venule. An extravascular monocyte is indicated by (M).* **D**: *Many preretinal monocyte/macrophages (M) are found in the center of a non–perfusion-fixed 2-month diabetic rat in the vicinity and directly above the optic disk. A main arteriole (A) is filled with erythrocytes.* **E**: *Preretinal and intraretinal accumulation of monocyte/macrophages (M) in the midperiphery of the retina of a non–perfusion-fixed 9-month diabetic rat. Trichotomous branching pattern of arterioles is occasionally found in diabetic retinas (*). (G) indicates a granulocyte.* **F**: *Interstitial cluster of monocyte/macrophages (arrow) accompanied by isolated granulocytes (G) and monocytes (M) in the midperiphery of the retina of a non–perfusion-fixed 5-month diabetic rat (bar = 10 μ in* **A–D** *and* **F**; *bar = 100 μ in* **E**).

Activation of Monocytes and Granulocytes by Alloxan and Hyperglycemia In Vitro

The incubation of normal rat blood with alloxan, at a dosage equivalent to that used to induce diabetes, led to a significant increase of monocyte and granulocyte activation ($P = 0.001$ and $P \leq 0.001$), measured by the NBT test (Figure 1). The incubation of normal blood with glucose (400 mg/dl) also resulted in a significantly elevated number of activated monocytes ($P \leq 0.001$) and granu-

Table 1A. *Number of Monocytes (M/L) and Granulocytes (G/L) per Capillary Length in Non–perfusion-fixed Rat Retinas*

Non–perfusion-fixed retina	Duration of diabetes (months)	M/l [$10^{-5}/\mu$]			G/l [$10^{-5}/\mu$]		
		Center	Midperiphery	Periphery	Center	Midperiphery	Periphery
Diabetic 1	2	0	7.2	10.0	0	0	12.1
Diabetic 2	2	1.3	1.0	11.7	0	0	17.6
Diabetic 3	2	1.6	0.5	0.2	3.2	0	0.1
Diabetic 4	2	0.9	1.3	3.6	0	0	0
Diabetic 5	2	2.5	3.9	21.9	0	2.1	3.4
Diabetic 6	2	0	12.8	5.1	0	0	0
Diabetic 7	5	6.3	21.7	12.6	1.2	2.5	20.0
Diabetic 8	5	2.4	5.4	7.9	1.8	1.4	15.7
Diabetic 9	5	2.6	0.8	5.5	0	0	2.4
Diabetic 10	5	1.0	1.3	1.2	1.0	0	0
Diabetic 11	5	3.6	0	12.7	0	0	0
Diabetic 12	5	7.7	10.0	12.2	0	3.0	0
Diabetic 13	9	4.9	4.8	15.1	0	0	1.2
Diabetic 14	9	1.8	5.1	10.3	0	0.4	2.2
Diabetic 15	9	24.9	9.2	19.0	3.6	9.2	37.9
Diabetic 16	9	4.4	3.5	5.2	6.7	4.9	14.7
Diabetics*		4.1 (1.5)	5.5 (1.4)	9.6 (1.5)	1.1 (0.5)	1.5 (0.6)	8.0 (2.7)
Controls* (n = 12)		0.8 (0.3)	1.0 (0.6)	2.4 (0.8)	0	0.3 (0.2)	0.8 (0.8)

* Mean (SEM).

locytes ($P \leq 0.001$). Monocytes showed a slightly higher activation by glucose than granulocytes ($P = 0.059$).

Activation of Monocytes and Granulocytes in the Systemic Circulation

Diabetic rats showed slightly higher leukocyte counts than age-matched controls, and also higher average granulocyte counts (Figure 2A, C), but the differences were not significant. The number of monocytes in the systemic circulation, however, was significantly increased ($P \leq 0.001$) (Figure 2B). Furthermore the number of circulating activated monocytes in the diabetic rats was dramatically increased ($P \leq 0.001$), and so was the number of activated granulocytes ($P = 0.013$) (Figure 2C). The comparison of the three diabetic groups (2-, 5-, and 9-months diabetic) showed no significant changes in the total number of leukocytes in the systemic circulation (Figure 3A). The granulocyte or activated granulocyte counts remained essentially constant during the duration of diabetes (Figure 3C). By contrast, the number of circulating monocytes was significantly elevated in early diabetes, ie, after 2 months compared with the 9-months diabetic animals ($P = 0.036$) (Figure 3B). The number of activated monocytes, too, was significantly elevated in the 2-month diabetic rats, compared with both 5-month ($P = 0.044$) and 9-month diabetic rats ($P = 0.009$). Other hematologic parameters did not vary significantly.

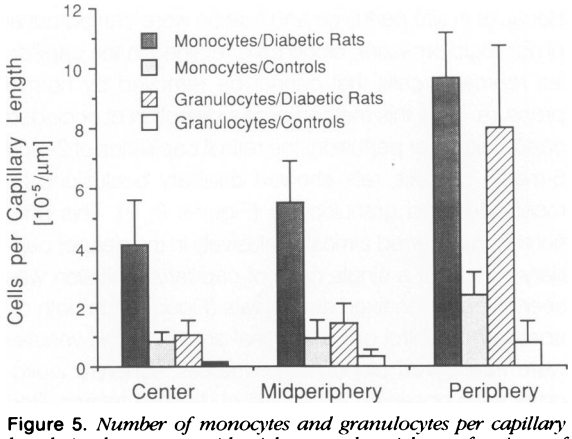


Figure 5. *Number of monocytes and granulocytes per capillary length in the center, midperiphery, and periphery of retinas of non–perfusion-fixed rats. The data for 2-, 5-, and 9-month diabetic rats and control rats, respectively, were pooled. Note that there were no granulocytes in the center of control retinas. (mean ± SEM, n = 16 for diabetic, and n = 12 for control retinas)*

Monocytes and Granulocytes in Non–Perfusion-fixed Retinal Capillaries

The studies of non–perfusion-fixed capillaries served to document the freely circulating as well as the noncirculating leukocytes. An example of the specific staining of monocytes and granulocytes in the retinal capillaries is given in Figure 4A. The individual numbers of intracapillary leukocytes for each diabetic retina showed marked differences (Table 1A). In diabetic rats, the numbers of both intracapillary monocytes and granulocytes were elevated compared to control animals (Figure 5). The difference in the number of monocytes between diabetic animals and controls was significant in the midperiphery ($P = 0.05$) and the periphery of the retina ($P = 0.001$). The intracapillary leukocyte concentration varied among the regions of the retina. In the retina periphery of diabetic

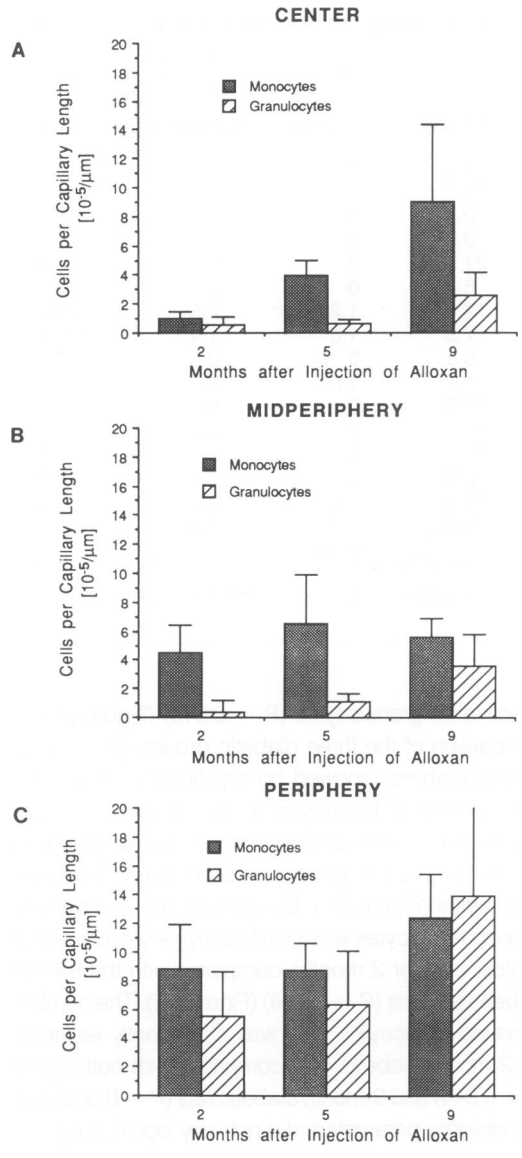


Figure 6. *Number of monocytes and granulocytes per capillary length in the center, midperiphery, and periphery of retinas of non–perfusion-fixed 2-, 5-, and 9-month diabetic rats. (mean ± SEM).*

rats, the number of monocytes as well as that of granulocytes was significantly higher than in the center and in the midperiphery ($P \leq 0.05$). In control rats, the increase of the leukocytes from the center toward the periphery did not reach statistical significance.

In diabetic rats, significantly more monocytes than granulocytes were observed in the retina center ($P = 0.045$) and the midperiphery ($P = 0.009$) of the retina (Figure 5). A comparison of the intracapillary concentrations of monocytes and granulocytes of 2-, 5-, and 9-month diabetic rats is given in Figure 6. The numbers of both cell types increased with duration of diabetes. After 5 months of diabetes, the number of monocytes in the retina center was significantly elevated compared with 2 months ($P = 0.028$). Figure 7 shows that in non–perfusion-fixed retinas the number of circulating cells and their activated forms were negatively correlated with their intracapillary concentration. This was significant for the number of circulating monocytes ($r = -0.59, P = 0.027$) and circulating activated monocytes ($r = -0.55, P = 0.068$), as well as for the number of circulating activated granulocytes ($r = -0.60, P = 0.039$).

Extravascular Monocytes, Macrophages, and Granulocytes

Extensive diapedesis and extravascular accumulation of monocytes and granulocytes were found in diabetic rats (Figure 4B to F), but not in control rats. Monocytes were the earliest extravascular blood cells observed, and 68% of the diabetic rat retinas showed interstitial monocytes.

Monocyte/macrophages were located within or beneath the lower capillary layer (84%) or in the superficial capillary layer (16%). Furthermore they were always observed in areas with capillary 'loss' and neovascularization (see below). Of the diabetic retinas, 85% showed preretinal monocytes (Figure 4D, E), and 47% showed preretinal granulocytes. Preretinal leukocytes were preferentially located around the optic disc. In 18% of the diabetic retinas, extravascular monocytes but no extravascular granulocytes were observed. By contrast, the presence of extravascular granulocytes was always associated with the presence of extravascular monocytes. In 74% of the retinas with extravascular monocytes, clusters of macrophages or foam cells were found (Figure 4F).

Capillary Occlusions by Leukocytes in Perfusion-fixed Retinas

Because *in situ* perfusion and fixation were carried out at physiologic pressure, blood cells retained in the capillaries represent cells that cannot be removed by normal pressure. Thus this method allows detection of occluded capillaries. After perfusion, the retinal capillaries of 2- and 5-month diabetic rats showed capillary occlusions by monocytes and granulocytes (Figures 8, 9). This phenomenon occurred almost exclusively in the deeper capillary layer. Not a single case of capillary occlusion was seen in perfusion-fixed control rats (Figure 9). In both diabetic and control groups, retinal arterioles and venules were free of red blood cells, whereas adherent leukocytes were observed in venules of diabetic retinas. Red blood cells were found only in capillaries occluded by leukocytes. No correlation between the intracapillary number of monocytes or granulocytes and their extravascular appearance was found.

A comparison of 2- and 5-month diabetic rats shows

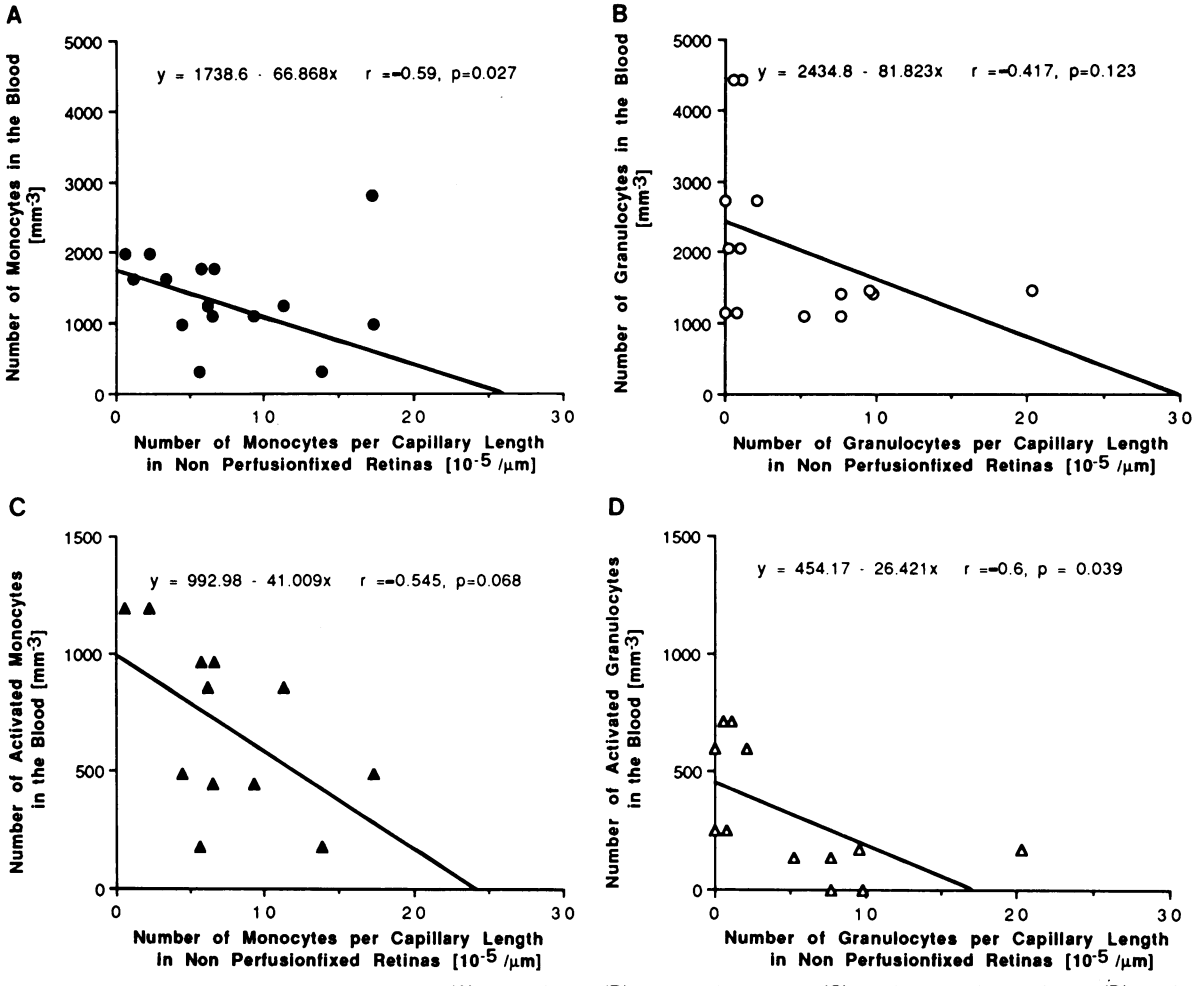


Figure 7. *Correlation of the number of monocytes* (A), *granulocytes* (B), *activated monocytes* (C), *and activated granulocytes* (D), *in the systemic blood with the intracapillary concentration of monocytes and granulocytes in the retinas of non-perfusion-fixed 2-, 5-, and 9-month diabetic rats. The regression equation, correlation coefficient, and probability for a random set* P *are shown in each case.*

that the number of occluding monocytes and granulocytes increased with the duration of diabetes (Figure 10). This was significant in the retina center for both cell types (monocytes: $P = 0.009$ and granulocytes: $P = 0.013$) and for monocytes in the midperiphery ($P = 0.022$). Furthermore a progressively higher number of leukocytes, especially monocytes, was observed in a direction from the center toward the periphery. The differences between the retina periphery and the center ($P = 0.031$) and between the periphery and midperiphery ($P = 0.029$) were significant for monocytes. In 2-month diabetic rats, capillary occlusions seemed to be preferentially caused by monocytes, whereas in 5-month diabetic rats granulocyte-mediated capillary occlusions seemed to predominate, but because of a large variation in absolute numbers of occluding cells between animals of the same group, this was not statistically significant. The individual data for each retina are listed in Table 1B.

Figure 11 shows a correlation between the number of circulating cells and their activated forms in the blood and their intracapillary number per vessel length in the perfusion-fixed retinas. We found a significant inverse correlation between the number of activated monocytes ($r = -0.75$, $P = 0.005$), granulocytes ($r = -0.69$, $P = 0.014$), and activated granulocytes ($r = -0.62$, $P = 0.034$) in the systemic circulation and their intracapillary number in the retina.

Capillary Damage

Diabetic rats showed swelling of endothelial cells in retina capillaries occluded by leukocytes (Figure 8B). Endothelial swelling was observed in perfusion-fixed and non-perfusion-fixed diabetic rats, but never in control rats. Another phenomenon observed only in diabetic rats was 'vacuolation' of endothelial cells, which was limited to the lower capillary layer of the retina periphery in two of the 2-month diabetic retinas (perfusion-fixed and non-perfusion-fixed) and in five of the 5-month diabetic retinas

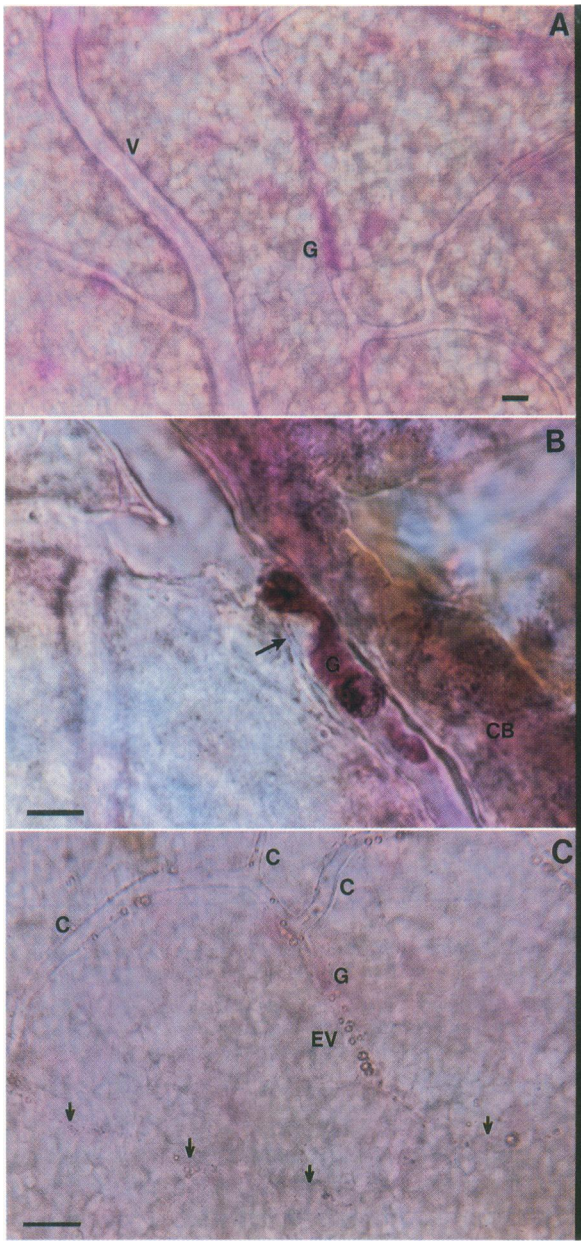


Figure 8. *Capillary occlusion by leukocytes in the deeper capillary layer of retinas of perfusion-fixed 5-month diabetic rats. Leukocytes were stained differentially with the specific esterase method.* **A**: *The lumen of a capillary in the midperiphery of the retina is completely filled by one or more granulocytes (G). (V) indicates a postcapillary venule.* **B**: *Occlusion of a capillary in the retina periphery adjacent to the ciliary body (CB). An endothelial cell protrusion into the lumen (arrow) constricts one of the three granulocytes (G) present in the capillary. Because endothelial swelling in perfusion-fixed retinas was only seen near occluding leukocytes, the leukocyte adhesion is likely to precede endothelial swelling.* **C**: *An example of capillary "loss" distal to an occluding granulocyte (G). Some segments of nonperfused capillaries (*) can still be traced from branch point to branchpoint. (C) denotes perfused capillaries. Endothelial vacuolation (EV) is often observed in the vicinity of occluding leukocytes. (bar = 10 μ in* **A** *and* **B**, *bar = 20 μ in* **C**)

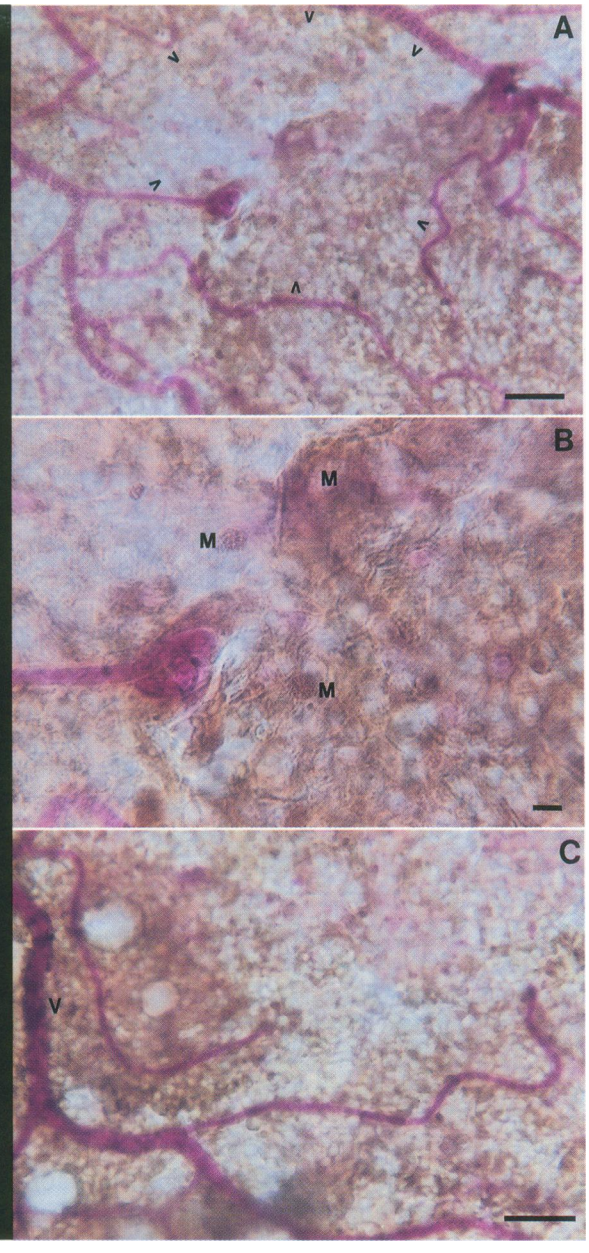


Figure 14. *Midperiphery of the non–perfusion-fixed retina of a 2-month diabetic rat, showing a typical picture of circumscribed capillary "loss" in the deeper capillary layer and monocyte/macrophage accumulation.* **A**: *An area of capillary "loss" is delimited by arrows. A loop-shaped neovascularization can be seen at the end of a capillary extending toward this area.* **B**: *A higher magnification of the neovascularization shows a number of extravascular monocyte/macrophages (M).* **C** *shows the "loss" of capillaries near a posicapillary venule (V). Macrophage accumulation and areas of cell death are found in the extravascular space, which are typical of the waxy exudates observed in diabetic retinopathy (bar = 50 μ in* **A**, **C**; *bar = 10 μ in* **B**).

(perfusion-fixed and non–perfusion-fixed) (Figure 8C). In the 2-month diabetic group, 2.3% of the peripheral capillary segments showed endothelial vacuolation, compared with 8.9% in the 5-month group. In two retinas, vacuolation of endothelial cells was also seen in postcapillary venules. Furthermore endothelial vacuolation was often observed in capillaries occluded by leukocytes. In circumscribed areas, the process of capillary destruction presented a picture of highly vacuolated capillary walls without visible lumen, or complete capillary 'loss' distal to the occluding leukocyte (Figure 8C).

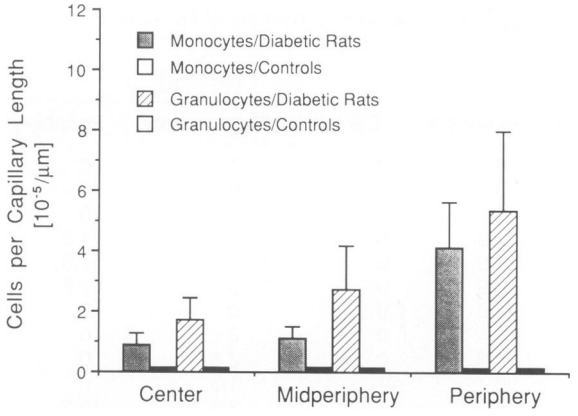


Figure 9. *Number of monocytes and granulocytes per capillary length in the center, midperiphery, and periphery of retinas of perfusion-fixed diabetic and control rats. For this figure, the data for 2- and 5-month diabetic rats and control rats, respectively, were pooled. Note that there were no monocytes and no granulocytes in the control retinas. (mean ± SEM, n = 16 for diabetic, and n = 8 for control retinas)*

Inner Diameter, Wall Thickness, and Cross-sectional Area of Capillaries

The inner diameter of retinal capillaries was not significantly influenced by perfusion fixation, as shown by a comparison with non–perfusion-fixed diabetic rats and control rats (Table 2A). Figure 12 compares the mean inner capillary diameter in the three regions of the retinas of perfusion-fixed and non–perfusion-fixed diabetic and control rats. Wall thickness and wall cross-sectional areas are listed in Table 2A. Although the average of all three parameters was higher in diabetic retinas than in controls, the differences were not significant. Clearly the localized endothelial swelling in occluded capillaries had little influence on the mean inner diameter. There was, however, a significant increase of the inner diameter from the center toward the periphery in both diabetic and control retinas. In perfusion-fixed and non–perfusion-fixed diabetic retinas, the differences between the retina center, midperiphery, and periphery were all significant ($P \leq 0.05$). In the controls, only the periphery had significantly greater capillary diameters than the midperiphery or the center ($P \leq 0.05$).

Capillary Length

The total length of capillaries and the number of capillary segments per area measured in the different regions of the retinas in diabetic and control rats is reported in Table 2B. There were no significant differences in the mean capillary length or in the number of segments between diabetic rats and controls. In general, capillary length decreased significantly from the retina center toward the periphery in both diabetic and control rats ($P \leq 0.05$; Figure 13). This was not the case in 9-month diabetic

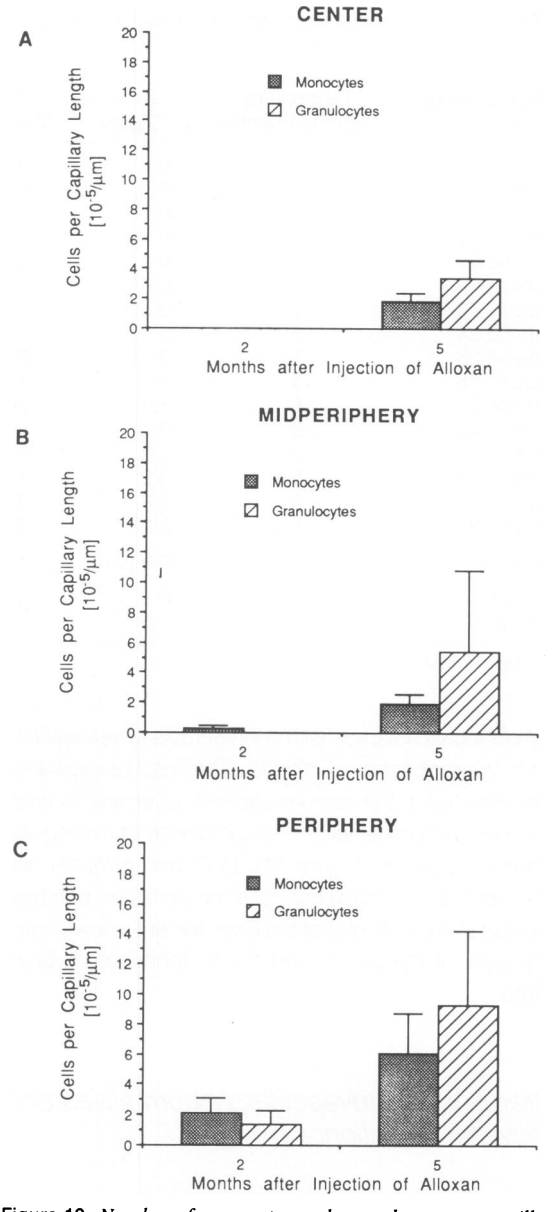


Figure 10. *Number of monocytes and granulocytes per capillary length in the center, midperiphery, and periphery of retinas of perfusion-fixed 2- and 5-month diabetic rats (mean ± SEM).*

retinas. Furthermore the capillary length in the midperipheral region of the 2-month control retinas was not lower than in the center. The number of capillary segments per area decreased significantly from the center toward the periphery in both groups ($P \leq 0.05$). A significant change in capillary length with duration of diabetes was only observed in the retina center between 9-month diabetic and 2-month diabetic animals ($P = 0.023$). Likewise, the age-matched controls showed no time-dependent differences in capillary length, with the exception of the midperiphery between 2 and 5 months ($P = 0.01$).

Although the mean capillary length in each group of diabetic rats did not change in time, in individual diabetic

Table 1B. *Number of Monocytes (M/l) and Granulocytes (G/l) per Capillary Length in Perfusion-fixed Rat Retinas*

Perfusion-fixed retina	Duration of diabetes (months)	M/l [10^{-5}/μ]			G/l [10^{-5}/μ]		
		Center	Midperiphery	Periphery	Center	Midperiphery	Periphery
Diabetic 17	2	0	0	0	0	0	0
Diabetic 18	2	0	0	0	0	0	0
Diabetic 19	2	0	0	0	0	0	0
Diabetic 20	2	0	0	7.8	0	0	1.6
Diabetic 21	2	0	0	1.6	0	0	1.6
Diabetic 22	2	0	1.1	3.9	0	0	7.7
Diabetic 23	2	0	0	4.0	0	0	0
Diabetic 24	2	0	1.2	0	0	0	0
Diabetic 25	5	0	0.9	1.3	0	2.8	0
Diabetic 26	5	0	1.3	0	1.1	1.3	0
Diabetic 27	5	3.9	3.3	4.1	7.7	3.3	12.2
Diabetic 28	5	0	1.1	1.6	9.0	23.3	16.3
Diabetic 29	5	2.8	2.5	3.6	3.7	1.2	0
Diabetic 30	5	3.9	1.2	6.1	0	0	0
Diabetic 31	5	1.8	0	3.7	1.8	2.7	6.5
Diabetic 32	5	2.2	5.5	23.2	4.3	8.8	39.6
Diabetics*		0.9 (0.4)	1.1 (0.4)	4.1 (1.5)	1.7 (0.7)	2.7 (1.5)	5.3 (2.6)
Controls* (n = 8)		0	0	0	0	0	0

* Mean (SEM).

animals localized areas with considerably lower capillary length were observed (Table 3). The 'loss' of capillaries was detected in the deeper capillary layer and in each case was accompanied by accumulation of monocytes and macrophages (Figure 14). In 2- and 5-month diabetic retinas, the capillary 'loss' occurred in patches, whereas in 9-month diabetic retinas, the entire lower capillary layer of the center and the midperiphery was involved.

Intraretinal Microvascular Abnormalities and Neovascularizations

Intraretinal microvascular abnormalities (IRMA) were observed in the lower capillary layer of six retinas, and close to areas of capillary 'loss.' In each case, the abnormal vessels were spacially correlated with extravascular and intravascular monocytes and macrophages and were not observed in controls or in retinas of rats in which the injection of alloxan did not result in a diabetic condition. We assume that most of these abnormalities represent genuine neovascularizations, rather than dilated and distorted retinal capillaries. The temporal sequence of events in the retinal pathology seemed to indicate that 'loss' of capillaries precedes the appearance of convoluted retinal capillaries, in the same regions of the retina. Furthermore, in circumscribed retinal areas, the microvascular network and capillary branching pattern showed distinct rearrangement that could not be accounted for by mere capillary distortion. In the following, the term 'neovascularizations' is therefore used to describe these vascular convolutes.

Only a selected number of the diabetic animals showed neovascularization, and none of the controls. Figures 14 and 15 provide examples of neovascularizations in 2- and 9-month diabetic retinas. In the 2-month specimens, neovascularization was limited to circumscribed areas of the midperiphery and in the 5-month specimens to areas of the midperiphery and the center, whereas all regions of the 9-month diabetic retinas were involved. Table 3 lists the length of neovascular capillaries per area in only those regions that showed neovascularization. Compared with normal capillaries, the new capillaries showed significantly greater inner diameters in all regions of the retina (data not shown).

Discussion

The destruction of islet cells by alloxan is mediated through the generation of oxygen-containing radicals, a mechanism similar to that of streptozotocin.[27,28] The toxicity of alloxan to the pancreatic B cells is presumably attributable to the sensitivity of these cells to peroxides, resulting from low intracellular glutathione peroxidase, an enzyme catalyzing the reduction of peroxides, which depends on the availability of nicotinamide-adenine dinucleotide phosphate (reduced form) (NADPH)/ nicotinamide-adenine dinucleotide (reduced form) (NADH).[27,29] Poly-(adenosine diphosphate [ADP]-ribose)-synthetase inhibitors, such as nicotinamide, pro-

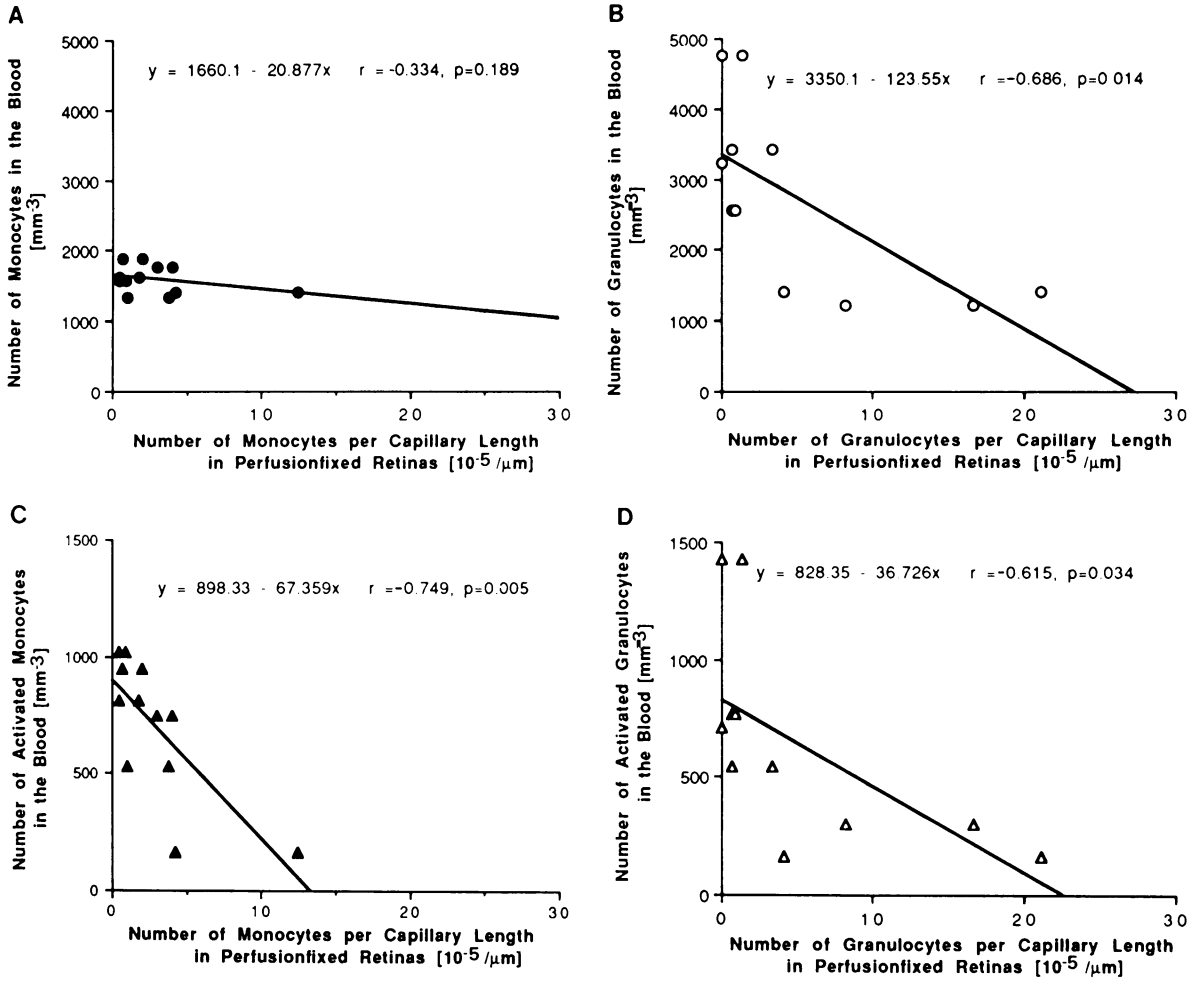


Figure 11. *Correlation of the number of monocytes* (A), *granulocytes* (B), *activated monocytes* (C), *and activated granulocytes* (D), *in the systemic blood with the intracapillary concentration of monocytes and granulocytes in the retinas of perfusion-fixed 2- and 5-month diabetic rats.*

tect against the alloxan-induced islet damage by inhibiting NAD degradation through poly(ADP-ribose).[30] Interestingly nicotinamide also prevents the onset of diabetes in genetically diabetic nonobese mice by inhibiting the antibody-dependent cell-mediated cytotoxicity of mononuclear cells, which requires the Fc-receptor of mononuclear cells.[31] Another indication that mononuclear cells, in particular monocyte/macrophages, contribute to the

Table 2A. *Capillary Dimensions in Rat Retina*

	Inner diameter D_i [µ]		Wall thickness D_o-D_i[µ]		Cross-sectional area of capillary wall $\bar{A}$ [mm^{-2}]	
	Diabetics	Controls	Diabetics	Controls	Diabetics	Controls
Center						
Non–perfusion-fixed*	3.8 (0.1)	3.7 (0.2)	1.5 (0.1)	1.5 (0.1)	11.3 (0.7)	10.5 (1.1)
Perfusion-fixed*	3.9 (0.2)	3.5 (0.2)	1.7 (0.1)	1.6 (0.1)	13.6 (0.8)	11.2 (1.3)
Midperiphery						
Non–perfusion-fixed*	4.2 (0.1)	3.9 (0.1)	1.5 (0.1)	1.5 (0.2)	11.7 (0.8)	10.9 (1.4)
Perfusion-fixed*	4.3 (0.1)	3.8 (0.2)	1.8 (0.1)	1.7 (0.1)	14.7 (0.6)	12.8 (0.9)
Periphery						
Non–perfusion-fixed*	4.8 (0.1)	4.8 (0.2)	1.5 (0.9)	1.3 (0.1)	13.1 (0.9)	10.8 (1.4)
Perfusion-fixed*	5.1 (0.9)	4.6 (0.3)	1.8 (0.1)	1.6 (0.2)	16.7 (1.1)	13.7 (1.5)

* Mean (SEM)
n = 16 for non–perfusion-fixed and perfusion-fixed diabetics.
n = 12 for non–perfusion-fixed and n = 8 for perfusion-fixed controls.

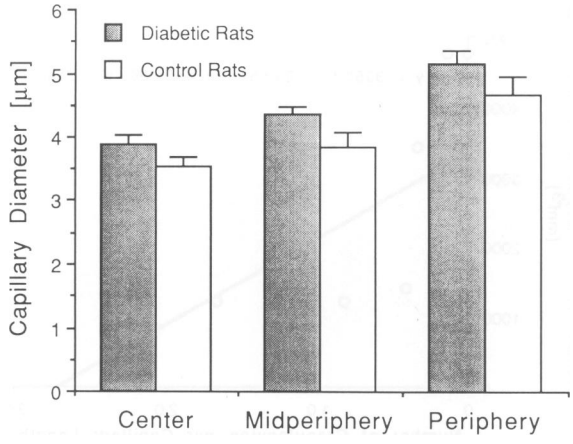


Figure 12. *Capillary diameters in the center, midperiphery, and periphery of retinas of perfusion-fixed 5-month diabetic and control rats (mean ± SEM, n = 8 in the diabetic group, and n = 4 in the control group)*

pathogenesis of diabetes comes from the observation that macrophages can spontaneously attack and destroy islet cells *in vitro* without prior stimulation by T lymphocytes.[32] In both streptozotocin diabetic mice and in genetically diabetic mice, the development of diabetes has been shown to be macrophage dependent.[33,34] Monocytes in their activated form also may be involved in B cell destruction. Monocytes and granulocytes have insulin receptors.[35,36] In physiologic concentrations, insulin inhibits the antibody-dependent cytotoxicity of macrophages,[37] and in high concentrations and over a relatively long period (5×10^{-5} mol/l, 72 hours), insulin decreases the number of Fc-receptors on peritoneal macrophages.[38] This implies that once the monocytes are activated and destroy B cells, the potential inhibitory effect of insulin on monocyte/macrophages might decrease together with insulin production. Hyperglycemia itself could contribute to this process. When leukocytes from normal rats are exposed to high blood glucose levels, their production of free oxygen radicals increases (Figure 1).

Finally human and rat diabetic subjects have increased plasma lipid peroxides, and their low-density lipoprotein fractions contain more lipid peroxidation products.[39,40] Macrophages are capable of oxidizing lipoproteins *in vitro*; oxidized lipoproteins are avidly taken up by macrophages through their scavenger receptors. Oxidized lipid–protein adducts are spacially correlated with monocyte/macrophages in atherosclerotic lesions,[41] especially early lesions characterized by both lipid and macrophage accumulation.[42] Considering that waxy exudates and the presence of numerous interstitial macrophages are characteristic components of the diabetic retinopathy (Figure 14),[43] similarities in the pathogenesis of both processes may exist.

Monocytes and neutrophils release free radicals and may generate lipid peroxides. Both products are toxic for endothelial cells.[20,44,45] Our results indicate that leukocytes in general, and monocyte/macrophages in particular, may not only be involved in the early diabetic retinopathy, but may also—at least in part—initiate the microvascular pathology observed at later stages. The increase of activated monocytes in the systemic circulation of 2-month diabetic rats and in the retinal circulation of 2- and 5-month diabetic rats was larger, compared with activated granulocytes. Monocytes were the first leukocytes observed extravascularly. Occluding monocytes or granulocytes were found only in diabetic retinas. Their number showed individual variability, with some animals displaying impressive vascular pathology and high leukocyte numbers, whereas others had low numbers of occluding cells and appeared almost normal. This is in agreement with clinical observations that the extent of diabetic retinopathy is difficult to correlate with parame-

Table 2B. *Capillary Dimensions in Rat Retina*

	Length/tissue area L/A [µ/mm²]		Number of segments⁺/ tissue area N_s/A [mm⁻²]	
	Diabetics	Controls	Diabetics	Controls
Center				
Non–perfusion-fixed*	52060 (2244)	52997 (1765)	484 (33)	503 (26)
Perfusion-fixed*	50603 (1170)	51234 (1057)	484 (14)	476 (39)
Midperiphery				
Non–perfusion-fixed	48168 (1971)	45305 (1770)	430 (27)	418 (21)
Perfusion-fixed*	44745 (1308)	40524 (2674)	422 (18)	368 (10)
Periphery				
Non–perfusion-fixed*	31923 (1560)	27563 (549)	233 (13)	205 (8)
Perfusion-fixed*	30588 (1390)	31282 (778)	232 (17)	218 (15)

⁺ Capillaries between branching points.
* Mean (SEM)
n = 16 for non–perfusion-fixed and perfusion-fixed diabetics.
n = 12 for non–perfusion-fixed and n = 8 for perfusion-fixed controls.

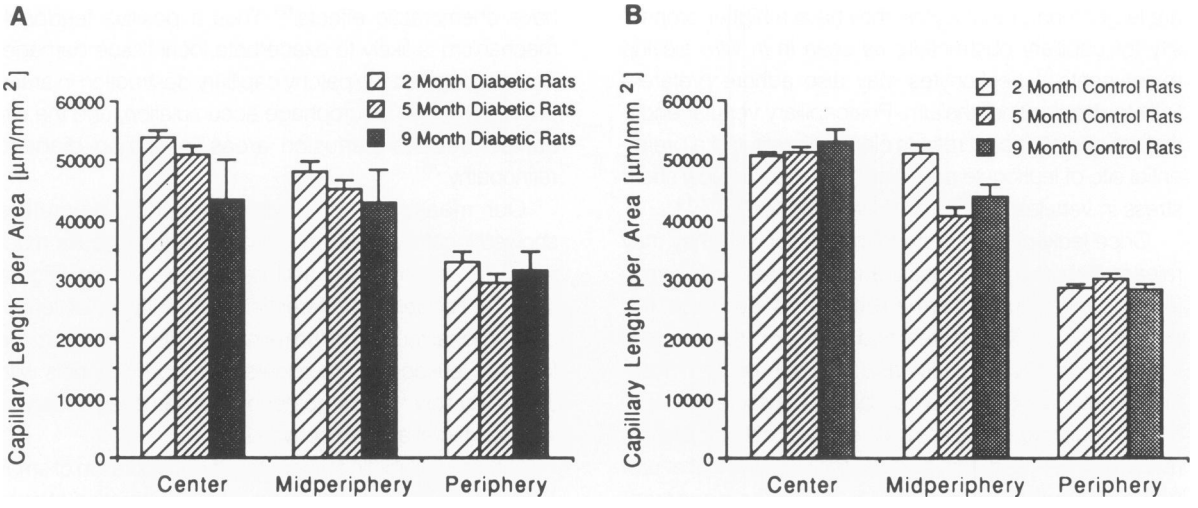


Figure 13. *Capillary length in the center, midperiphery, and periphery of retinas of 2-, 5-, and 9-month diabetic* **(A)** *and control rats* **(B)** *(mean ± SEM).*

ters such as glucose level or duration of diabetes. This variability complicates comparison between controls and diabetic animals in terms of mean numbers of leukocytes per capillary length. Although this parameter was statistically significant only in selected areas of the retina, strong correlation exists in each retina between local leukocyte accumulation and other local vascular pathology, such as endothelial cell damage, nonperfusion areas, and extravascular leukocytes.

Our results showed that monocytes and granulocytes cause capillary obstruction in the rat model of diabetic retinopathy, followed by destruction of the capillary bed. Capillary obstruction occurred almost exclusively in the lower capillary layer of the retina, which in the rat is known to be connected more closely to the retinal venules[46,47] and therefore presumably is the site of a major decrease in vessel wall shear stress and preferential site of leukocyte adhesion, as observed in many other organs.[11,14]

The increased numbers of intracapillary monocytes and granulocytes in diabetic retinas compared with controls and continued intracapillary accumulation during the progression of diabetes were not due to an increased number of circulating leukocytes, as the concentrations of monocytes and granulocytes, as well as their activated forms, in diabetic blood were inversely correlated to their intracapillary numbers (Figures 7, 11). A lower number of circulating (activated) monocytes and granulocytes indicates their net 'loss' in the microcirculation, which may be due to microvascular retention. The increased number of monocytes and granulocytes observed in retinal capillaries of diabetic rats also may be accompanied by an elevated passage time of the leukocytes through the capillary network, which depends on the rigidity of the cells, their adhesion to the endothelium, and the capillary lumen dimensions.[15,48] Increased leukocyte rigidity and increased monocyte adhesiveness both have been described in human diabetes[49,50] and constitute important risk factors for capillary obstruction. Because monocytes

Table 3. *Capillary "Loss" and Neovascularization in Diabetic Rat Retinas*

Retina *	Duration of diabetes (months)	Area of neovascularization*	L/A normal [µ/mm²]	L/A neovascularized [µ/mm²]	L/A mean of age-matched controls [µ/mm²] n = 8 (2, 5 months) n = 4 (9 months)
5	2	Midperiphery	25706	8834	50936
6	2	Midperiphery	22313	1325	50936
24	2	Midperiphery	27307	4488	50936
12	5	Center	18067	5045	51380
		Midperiphery	29463	4049	40605
15	9	Center	28337	7654	52897
		Midperiphery	19905	9672	43722
		Periphery	27981	1214	28131
16	9	Center	36764	5940	52897
		Midperiphery	38955	3780	43722
		Periphery	26002	4048	28131

are larger than granulocytes, they have a higher propensity for capillary obstruction, as seen in *in vitro* sieving experiments.[51] Leukocytes may also adhere preferentially to venular endothelium. Postcapillary venular endothelium of diabetic rat retinas clearly constituted a preferential site of leukocyte adhesion. The relatively low shear stress in venules may be a contributing factor.[11,14]

Once leukocytes adhere to the vessel wall, they may release free oxygen radicals and enzymes, which may increase vascular permeability and damage the endothelium.[20] Monocytes and granulocytes are well known to produce elastase and collagenase.[52–55] Neutrophil granules contain a number of constituents capable of mediating vascular injury, such as cationic lysosomal proteins,[56] and acid and neutral proteases of azurophilic granules, which digest vascular basement membranes.[57,58] Proteolytic enzymes released from macrophages can degrade proteoglycans, fibronectin, and glycoproteins and thereby destroy the basement membrane and extracellular matrix of capillaries.[54,55] Endothelial vacuolation was seen in the lower layer of capillaries of the retina periphery, an area characterized by higher leukocyte concentration. Endothelial vacuolation was not a generalized phenomenon, but occurred only in areas of preferential leukocyte circulation.

Endothelial cell damage caused by leukocyte activation may depend on the degree of protection against lysis by secreted hydrogen peroxide.[59] The glutathione redox cycle, which depends on the availability of NADPH/NADH, is an essential cytoprotective mechanism.[59,20] Hyperglycemic periods in diabetes lead to the intracellular generation of sorbitol by aldose reductase, a process that consumes NADPH and therefore may weaken the defense mechanism against free radical injury. In the past, the development of endothelial damage and basement membrane thickening in diabetic microangiopathy of the retina, as well as of other organs, has been attributed predominantly to the increase in sorbitol, which could be prevented by aldose reductase inhibitors.[60–62]

Macrophages are a source of interleukin-1 and tumor necrosis factor,[63] which stimulate leukocyte migration and adherence to the vascular endothelium.[64–67] Furthermore tumor necrosis factor enhances the macrophage capacity to secrete reactive oxygen intermediates,[68] and induces the expression of adhesion proteins on endothelial cells.[64,65] Breakdown products of endothelial cells damaged by adhering leukocytes also have chemotactic effects.[69] Thus a positive feedback mechanism is likely to exacerbate local tissue damage. This may explain the patchy capillary destruction in areas of extravascular macrophage accumulation, and the circumscribed nonperfusion areas in human diabetic retinopathy.[70]

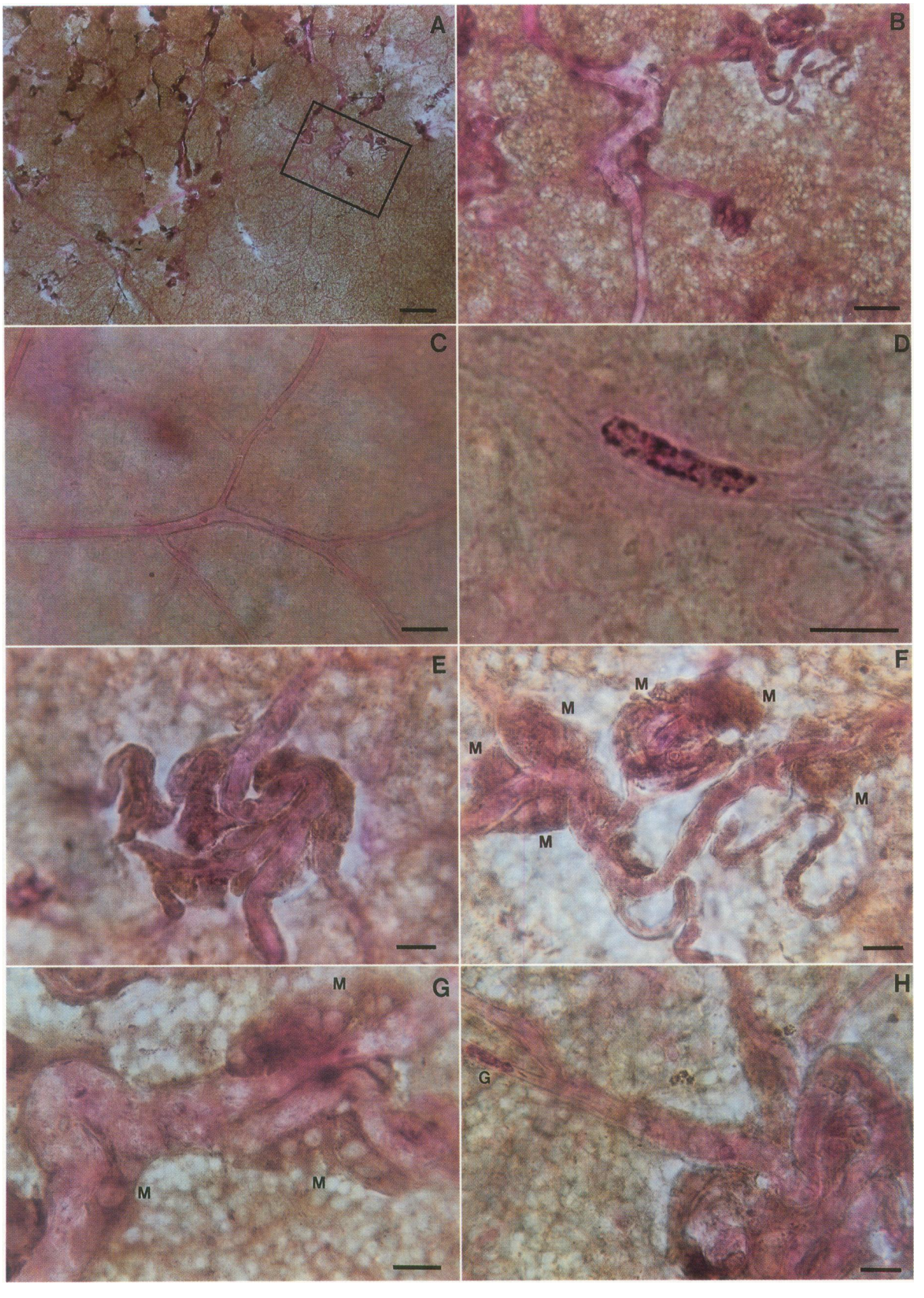
Our measurement of microvascular diameters showed local vasodilation in areas of extravascular macrophage accumulation and neovascularization (Figure 15). Consequently preferential accumulation of leukocytes in local regions of the retina may also result from the fact that leukocytes are shunted through channels with the fastest flow,[71] a phenomenon that has been observed in other microvascular networks.[72,73]

In human diabetic retinopathy, the association of small areas of capillary nonperfusion with the formation of capillary microaneurysms and IRMA, both in terms of appearance time and in terms of spacial correlation, is so striking that the production of an angiogenic factor by ischemic tissue has been postulated.[2,3] We found newly formed intraretinal capillaries in the lower capillary layer of the retinal network, which in rats is closely connected to postcapillary venules. Similar early neovascularization in the vicinity of postcapillary venules was reported in human diabetic retinopathy.[74]

In the rat model, without exception, areas of capillary 'loss' and neovascularization were associated with the presence of monocytes or macrophages (Figures 14, 15). Phagocytes are known to be connected with proliferative retinopathy, but they were in the past only considered to be necessary for the removal of pathologic products. Numerous studies demonstrated the modulatory effect of macrophages and their secretory products on endothelial cell proliferation[55,75–77] and angiogenesis.[78,79] Specifically Polverini et al[80] reported that macrophages activated *in vitro* and *in vivo* induce vascular proliferation in guinea pig cornea, whereas neutrophils or activated lymphocytes did not. Capillary occlusion by leukocytes is likely to cause localized low oxygen tension, which is considered a primary stimulus for diabetic neovascularization. Low oxygen tension in turn causes macrophages to release vasoproliferative factors.[79]

In conclusion, diabetic retinopathy in humans is characterized by vasodilation, endothelial injury and loss of pericytes, increased capillary permeability, capillary occlusions, and microaneurysms, followed by neovascularization, hemorrhages, and finally degeneration and dis-

Figure 15. *Advanced intraretinal neovascularization and its association with monocyte/macrophages in the midperiphery of nonperfusion-fixed 9-month diabetic retinas.* **A**: *A dense network of newly formed capillaries covers the greater part of this visual field (bar = 100 μ). Higher magnifications of the marked area in two focal planes* **(B, C)** *show that the new capillaries originate in the lower capillary layer of the retina* **(C)**, *whereas the upper capillary layer* **(B)** *is free of neovascularization (bar = 50 μ). Intracapillary leukocytes frequently are found in areas of neovascularization* **(D)**, *(bar = 20 μ). Higher magnification of the neovascularizations* **(E–H)** *show highly convoluted new capillaries* **(E)**. *Monocyte/macrophages (M) are usually attached in dense groups to the neovascularizations* **(F)**. *Areas of capillary dilation are frequent within the convoluted new vessels, in proximity to extravascular macrophages (M)* **(G)**. *Increased numbers of intracapillary leukocytes are found within capillaries connected to neovascular convolutes; (G) indicates an intracapillary granulocyte* **(H)**, *(bar = 20 μ in* **E–H**).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organization of the retinal tissue.[81–83] Patchy capillary occlusion in humans is usually accompanied by capillary dilation and neovascularization, which have been interpreted to be a 'compensatory response' to local ischemia and hypoxia.[4–6] Capillary nonperfusion has been attributed to disruption and disappearance of the endothelium.[84] Thus there are a number of similarities to the current observations in the rat. The rat model indicates that leukocytes may act in a variety of ways during the progression of the pathology. Leukocytes show an increased adhesion to the endothelium and cause capillary occlusion and endothelial damage. Leukocytes secrete free radicals, proteases, and vasoactive agents, and increase vascular permeability. Monocytes and macrophages play a key role in the pathogenesis of diabetic retinopathy in the rat model. Monocyte blood counts are increased in short-term diabetic rats, and their circulation in the retinal vessels is elevated. They are the first leukocytes to appear extravascularly and mediate early capillary occlusions. Monocytes and macrophages, known to release angiogenic factors, are present in regions of neovascularization. Finally macrophages are known to release chemotactic factors, which can attract further leukocytes. The conclusions drawn from the mechanisms we found in the rat model do not directly translate to human diabetic retinopathy, but the parallels of circumstances and reactions are sufficient to warrant further exploration of the role of leukocytes in the pathogenesis of human diabetes.

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