Decreased Polymorphonuclear Leukocyte Deformability in NIDDM

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OBJECTIVE — To determine the rheological properties of polymorphonuclear leukocytes (PMN) from non-insulin-dependent diabetes mellitus (NIDDM) patients.

RESEARCH DESIGN AND METHODS — The deformability of PMN from 33 NIDDM subjects, 13 with impaired glucose tolerance (IGT), and 22 with normal glucose tolerance (NGT) was studied. A Cell Transit Analyzer that measures the transit time of PMN through 8-μm pores was used. Studies were performed under three different conditions: *1*) basal state; *2*) after incubation with cytochalasin B (20 μM) to dissociate f-actin from the cytoskeleton; and 3) following activation with N-formyl-methionyl-leucyl-phenylalanine (fMLP, 1 nM).

RESULTS — PMN from diabetic patients were more rigid (i.e., had longer transit time) than those from subjects with NGT or IGT under basal conditions and after cytochalasin B, but not after stimulation with fMLP. The deformability of PMN from subjects with IGT was similar to those of the NGT group. In the pooled data, basal transit time correlated with age; systolic and diastolic blood pressure; HbA_{1c}; and serum creatinine, cholesterol, and triglyceride concentrations (r = 0.29, 0.34, 0.37, 0.48, 0.25, 0.36, 0.29, respectively, $P < 0.05$ for each). Hypertensive diabetic patients had less deformable PMN than normotensive ones. No relation was found between PMN deformability and the duration of diabetes, type of treatment, or the presence of retinopathy.

CONCLUSIONS — These data indicate increased rigidity of PMN in NIDDM that may contribute to development of microcirculatory disturbances and microangiopathy.

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NIDDM, non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; PMN, polymorphonuclear leukocytes; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; FPG, fasting plasma glucose; BP, blood pressure; sBP, systolic blood pressure; dBP, diastolic blood pressure; fMLP, formyl-methionyl-leucyl-phenylalanine; PBS, phosphate-buffered saline; CTA, Cell Transit Analyzer; TT, transit time; DMSO, dimethyl sulfoxide; TG, triglyceride; RIA, radioimmunoassay; ANOVA, analysis of variance; NT, normotensive; HT, hypertensive.

The microcirculatory disturbances seen in diabetes mellitus have prompted numerous studies of blood rheology; salient findings are discussed in recent reviews (1–3). Increased whole blood and plasma viscosity (4–7) and enhanced erythrocyte aggregation (7,8) have been well documented, whereas alterations of erythrocyte mechanical properties remain somewhat controversial (8–12). Much less is known, however, about the rheological behavior of leukocytes in diabetes. Leukocytes are larger and much more rigid than erythrocytes (13) and have been shown to strongly influence microvascular blood flow (14). Moreover, polymorphonuclear leukocytes (PMN), which comprise the largest fraction of leukocytes, are capable of causing direct microvascular damage by the release of proteases and toxic oxygen radicals (15). Ernst and Matrai (16) and Vermes et al. (17) have reported abnormal leukocyte rheology in diabetes, yet interpretation of their data is hampered by the use of either erythrocyte/leukocyte mixtures (16) or unfractionated leukocyte suspensions (17). We have, therefore, studied the rheological properties of PMN from patients with non-insulin-dependent diabetes mellitus (NIDDM) using pure PMN suspensions and a newly developed micropore filtration method.

RESEARCH DESIGN AND METHODS

— This study included 68 Mexican-American subjects (23 men and 45 women) who were 19–66 years of age (mean 44.3 years). Twenty-seven NIDDM subjects were recruited from those attending the Diabetes Outpatient Clinic. Diabetes was diagnosed on the basis of a fasting plasma glucose (FPG) ≥7.8 mM or a random plasma glucose ≥11.1 mM on at least two previous clinical visits. The remaining 41 subjects were recruited from family members and friends accompanying the patients to the clinic or from hospital employees. The

latter 41 subjects underwent an oral glucose tolerance test with a 75-g glucose-equivalent carbohydrate load.

According to the World Health Organization criteria (18), 22 of the 41 subjects had normal glucose tolerance (NGT) (FPG and 2-h postload plasma glucose concentration <7.8 mM), and 13 subjects had impaired glucose tolerance (IGT) (FPG <7.8 mM and 2-h postload plasma glucose concentration ≥7.8 and <11.1 mM). Of the 41 subjects, 6 were found to have diabetes (FPG ≥7.8 mM or 2-h postload glucose concentration ≥11.1 mM) and were placed in the diabetic group that therefore included 33 subjects. The duration of the disease in the 27 subjects with previously known diabetes ranged from 3 months to 28 years (median 8 years). Of these subjects, 13 were receiving oral hypoglycemic drugs and 14 were taking insulin. Of the diabetic patients, 11 had hypertension that was clinically diagnosed previously (blood pressure [BP] >140/90 mmHg) and were receiving enalapril (9 patients), captopril (1 patient), or nifedipine (1 patient).

A medical history was obtained and a physical examination was performed on all subjects. Blood samples were collected after a 12-h fast for determination of concentrations of FPG, HbA_{1c}, blood urea nitrogen, and serum creatinine, cholesterol, and triglyceride (TG) concentrations. A random urine sample was collected to determine the albumin/creatinine ratio in milligrams of albumin per gram of creatinine (19). Subjects with insulin-dependent diabetes mellitus (IDDM), cardiac or hepatic disease, acute or chronic inflammatory conditions, infections, or renal impairment (serum creatinine > 180 μM) and those receiving medications other than antidiabetic or antihypertensive drugs and smokers were excluded. The study was approved by the Los Angeles County-University of Southern California Medical Center Institutional Review Board, and all subjects gave informed consent.

PMN preparation

Whole blood was drawn into a syringe containing sodium heparin (10 U/ml blood) using a 19 gauge needle. The tourniquet was removed immediately after insertion of the needle. Pure PMN suspensions were prepared by the method of Boyum (20). In brief, 10 ml of whole blood was mixed with 5 ml of 6% dextran 70 (Macrodex 70, 6% wt/vol in normal saline, Pharmacia, Piscataway, NJ) and allowed to stand for 90 min at room temperature. The leukocyte-rich plasma was layered onto 4 ml of Histopaque 1.077 medium (Sigma, St. Louis, MO) and centrifuged at 400 *g* for 30 min. The PMN-rich pellet was resuspended, and the residual erythrocytes were removed by hypotonic lysis with distilled water for 30 s. Tonicity was restored with an equal vol of 1.8% wt/vol saline. The PMN were washed and maintained at room temperature in calcium and magnesium-free phosphate-buffered saline (PBS) (PBS, pH 7.4, 285 mOsm/kg). Purity was assessed by optical microscopy (>95% PMN), whereas viability was confirmed either visually by trypan blue or with propidum bromide using a flow cytometer (≥98%). Mean PMN volume was determined with a 76-μm aperture impedance counter and a multichannel analyzer (Particle Data, Elmhurst, IL). A shape factor of 1.50, equivalent to that for a rigid sphere, was assumed for all PMN volume measurements. Leukocyte count was determined with an automated hematology analyzer (Roche Diagnostic Systems, Branchburg, NJ). To minimize artifacts caused by sample handling and processing, sterile tissue-culture-grade endotoxin-tested media and sterile disposable plasticware were used for all procedures.

PMN deformability measurement

PMN deformability was assessed using the Cell Transit Analyzer (CTA) (ABX, Montpellier, France) (21–23). The CTA measures the time taken for individual cells in a dilute suspension to deform and pass through 1 of 30 identical pores in a polycarbonate micropore filter, which represents a simple, yet geometrically stable, in vitro model of a capillary bed. The use of the CTA for assessing PMN deformability has been described previously in detail (13,21). Briefly, a dilute suspension of PMN in PBS (10^5 cells/ml) is placed into reservoir A (Fig. 1) on one side of the membrane, while PBS alone is placed in reservoir B on the other side. The cell suspension is forced through the pores by the pressure difference applied across the membrane. The transit time (TT) for each complete cell passage is obtained by monitoring the transient change in electrical resistance as each cell passes through a pore; an increased TT indicates decreased PMN deformability (13,21). The basic static mode of the CTA gives the mean and median TT from ≥1,000 cell passages averaged over an ~5-min period. We have recently extended the basic capabilities of the CTA by adding a new kinetic operating mode, in which PMN deformability can be monitored during each of 6 consecutive 20-s intervals (21). The purpose of this modification is to follow the time course of rapid changes in TT after PMN are exposed to a stimulus (typically a chemotactic agent).

For each subject, PMN deformability was assessed as follows: To examine the cells in their basal state, an aliquot of the PMN suspension was diluted in sterile PBS to a final concentration of 10^5 cells/ml, and static mode CTA analysis was performed in triplicate. The median TT of a minimum of 1,000 cell passages was recorded each time, and the mean of the three median values was taken as the final result. A similar aliquot of the PMN suspension was incubated with 20 μM cytochalasin B (Sigma) for 5 min, and the median TT was subsequently measured. Cytochalasin B aborts and rapidly reverses increases in PMN content of f-actin resulting from exposure to chemotactic agents (24). Because f-actin is the primary determinant of PMN rigidity (21,24), treatment with cytochalasin B should thus eliminate any differences in PMN deformability caused

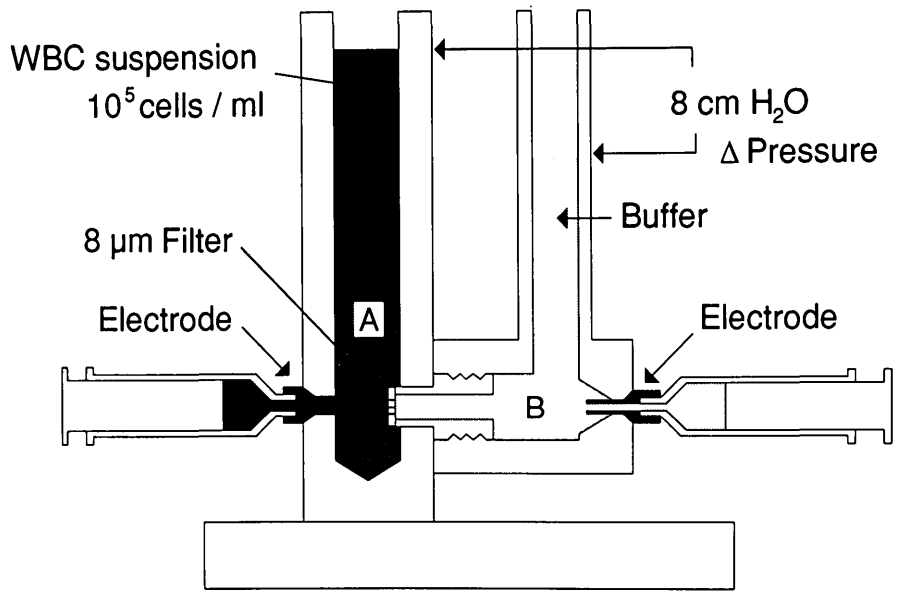

Figure 1—*Schematic representation of CTA. A dilute suspension of PMN in PBS buffer is placed in reservoir A, with PBS alone in reservoir B; the pressure gradient (8 cm H_2O) resulting from the difference in height between the two fluid columns forces the suspension through the 8-μm pores of the filter. The two needle electrodes are connected to an AC conductimeter that measures the resistance of the filter, digitizes this signal, and outputs the digital information to a computer for computation of PMN pore transit time.*

by acute activation. Finally, the response of the cells to a chemotactic stimulus was measured using the kinetic mode of the CTA. The chemotactic tripeptide N-formyl-methionyl-leucyl-phenylalanine (fMLP, 1 nM, Sigma) was added to an aliquot of the diluted cell suspension, and TT measurements were made immediately for six consecutive 20-s intervals.

Because PMN rigidity rapidly increases, peaks at ~60 s, and then decreases following fMLP stimulation, the integrated value of TT over the first 60 s was used to quantitate the response to fMLP (21). All CTA experiments, for all subjects, were conducted at 25°C using the same polycarbonate filter (8-μm diameter by 21-μm-long pores) and at a driving pressure of 8 cm H_2O within 4 h of venipuncture and 2 h of separation of PMN. Unstimulated PMN showed no change in basal TT for up to 2 h following isolation from blood or during the 120-s period of the kinetic mode analysis. Both cytochalasin B and fMLP were initially dissolved in dimethyl sulfoxide (DMSO) then later diluted with PBS to give <0.1% final concentration at the given cytochalasin B and fMLP concentrations. This concentration of DMSO has no effect on PMN deformability (21).

Biochemical analysis

Plasma glucose concentration was measured by the glucose oxidase method with a Beckman glucose analyzer (Beckman Instruments, Fullerton, CA). HbA_{1c} was determined by high-performance liquid chromatography (25). Serum cholesterol (26) and TGs (27) were measured by enzymatic methods. Urinary creatinine was determined by a modification of the kinetic Jaffe reaction (28) and urinary albumin by a double antibody radioimmunoassay (RIA) (Diagnostic Products, Los Angeles, CA).

Statistical analysis

All data are presented as means ± SE. Comparisons among the three groups were performed by the χ^2 method or analysis of variance (ANOVA). The Tukey test was used for intergroup comparisons. The relations between variables were analyzed by Pearson simple correlation.

RESULTS— Table 1 shows the characteristics of the subjects. NIDDM subjects were on average slightly older than those with NGT or IGT. In addition, they had higher BP and serum TG concentrations. Of the diabetic patients, 11 had hypertension whereas all members of the other two groups were normotensive. Seven diabetic subjects had microalbuminuria (albumin/creatinine ratio >30 mg/g), but none had clinical renal disease. Background or proliferative retinopathy was found in 14 patients (7 each).

No difference was observed among the three groups in the total leukocyte or PMN counts or PMN volume. The basal TT of PMN from diabetic patients was significantly longer (i.e., PMN were less deformable) than in the other two groups (Table 2). The difference still persisted after incubating PMN with cytochalasin B. No difference was found in the TT among the three groups up to 60 s after stimulation with fMLP. However, the rate of recovery of deformability of diabetic PMN was significantly slower during the subsequent 60 s (Fig. 2). Basal and post-cytochalasin B TT were slightly longer for PMN from subjects with IGT than NGT, but the differences were not statistically significant. In the pooled data from all subjects, basal TT correlated significantly with age, systolic and diastolic blood pressure (sBP, dBP), HbA_{1c}, and serum creatinine, cholesterol, and TG concentrations (r = 0.29, 0.34, 0.37, 0.48, 0.25, 0.36, 0.29, respectively, $P < 0.05$ for each).

Among diabetic patients, PMN from hypertensive subjects had a longer basal TT than normotensive ones (11.5 ± 0.9 vs. 9.6 ± 0.5 ms, $P < 0.05$) (Fig. 3).

Table 1—*Clinical characteristics of subjects*

	Subject groups			
	NGT	IGT	NIDDM	*P**
n	22	13	33	
Age (years)	39.8 ± 2.2	43.7 ± 2.8	47.6 ± 2.0†	<0.05
Sex (M/F)	9/13	3/10	11/22	NS
Body mass index (kg/m^2)	29.4 ± 1.4	29.6 ± 1.4	31.9 ± 1.3	NS
sBP (mmHg)	117 ± 3	124 ± 4	131 ± 2‡	<0.01
dBP (mmHg)	67 ± 2	69 ± 3	81 ± 2§	<0.001
FPG (mM)	5.2 ± 0.1	5.3 ± 0.2	11.2 ± 0.8§	<0.001
HbA_{1c} (%)	5.2 ± 0.2	5.2 ± 0.1	8.7 ± 0.4§	<0.001
Serum creatinine (μM)	97 ± 9	88 ± 8	97 ± 9	NS
Serum cholesterol (mM)	4.99 ± 0.21	5.28 ± 0.23	5.74 ± 0.23	NS
Serum TG (mM)	1.46 ± 0.22	1.69 ± 0.25	2.37 ± 0.23‡	<0.01
Urinary albumin/creatinine ratio (mg/g)	4.6 ± 1.1	5.1 ± 1.1	25.5 ± 6.3‡	<0.01

Data are means ± SE.

*P values are for comparison among the three groups by ANOVA.

†$P < 0.05$ between NIDDM and NGT using the Tukey procedure.

‡$P < 0.01$ between NIDDM and NGT using the Tukey procedure.

§$P < 0.001$ for comparison between NIDDM and NGT using the Tukey procedure; differences between IGT and NGT were not significant.

Even with the hypertensive subjects excluded, the basal TT of PMN from normotensive diabetic patients (9.6 ± 0.5 ms) was significantly higher than for subjects with NGT (8.0 ± 0.2) or IGT (8.4 ± 0.2, $P < 0.05$ for each comparison). Despite receiving antihypertensive medications, diabetic patients with hypertension had higher BP than normotensive ones (144 ± 4/88 ± 2 vs. 125 ± 2/78 ± 2 mmHg, $P < 0.01$). The two diabetic subgroups did not differ significantly, however, in age, FPG concentration, or HbA_{1c} level. Diabetic subjects with hypertension had a higher albumin/creatinine ratio than normotensive (NT) ones (44 ± 12 vs. 16 ± 7 mg/g, $P < 0.05$), but no significant correlation was observed between this ratio and the basal TT. No significant relations were detected between TT and duration of diabetes, type of treatment, or presence of retinopathy.

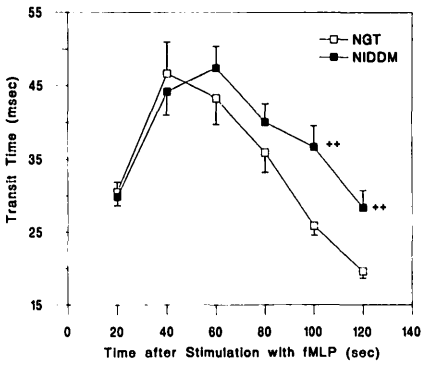

Figure 2—*PMN transit times following stimulation with 1 nM fMLP. The differences between the NGT and NIDDM groups are significant at 100 and 120 s post-stimulation (P < 0.01). The data for the IGT group are similar to those for the NGT and not shown to simplify the figure.*

CONCLUSIONS— The present data, obtained with the CTA system, clearly indicate that PMN are abnormally rigid in NIDDM. The CTA micropore filtration system (23) represents a novel approach to the measurement of PMN deformability. Compared with other filtration techniques, it has the advantage of providing

Table 2—*Characteristics of PMN in the three groups*

	Subject groups			
	NGT	IGT	NIDDM	*P**
Leukocyte count (10^9/L)	6.0 ± 0.3	5.8 ± 0.4	6.0 ± 0.3	NS
PMN count (10^9/L)	3.1 ± 0.3	3.4 ± 0.3	3.2 ± 0.2	NS
PMN vol (fL)	322 ± 3	334 ± 5	325 ± 3	NS
Basal TT (ms)	8.0 ± 0.2	8.4 ± 0.2	10.2 ± 0.5†	<0.001
Cytochalasin B TT (ms)	4.6 ± 0.1	4.8 ± 0.2	5.3 ± 0.1†	<0.001
fMLP TT (ms)	40.2 ± 2.8	41.0 ± 3.5	40.5 ± 2.0	NS

Data are means ± SE.

*P values are for comparison among the three groups by ANOVA.

†$P < 0.001$ for comparison between NIDDM and NGT using the Tukey procedure; differences between IGT and NGT were not significant.

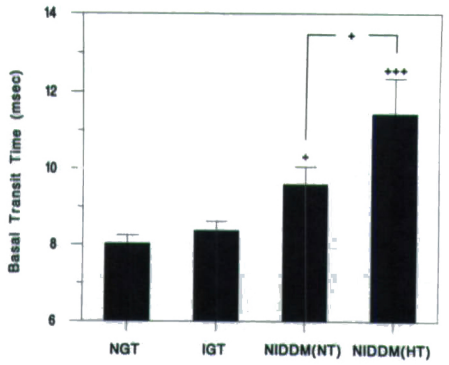

Figure 3—*Basal PMN transit time for NGT and IGT subjects and for NIDDM patients divided into NT and hypertensive (HT) subgroups. Both the normotensive and hypertensive diabetic patients have PMN that are significantly less deformable than those from the NGT subjects ($P < 0.05$ for NT, $P < 0.001$ for HT). In addition, PMN from HT diabetic patients are more rigid than those with normal BP ($P < 0.05$).*

information on the pore passage time of individual cells, with >1,000 PMN measured per test. Furthermore, it allows rapid kinetic analysis of the mechanical response to stimuli such as fMLP, which is beyond the capability of other systems because of the short time course of this response.

Our findings are in general agreement with two previous studies that measured leukocyte deformability indirectly using a pore-clogging technique (16,17). However, in neither of these studies was PMN separated from other leukocyte types, and thus the cell types responsible for the differences in pore clogging could not be determined. MacRury et al. (29), using PMN suspensions, found no differences in PMN deformability between NIDDM patients and control subjects but stressed the need to consider leukocyte activation in studies of their mechanical behavior. Thus, in addition to measurements made on the freshly prepared PMN suspension, we also examined PMN deformability both after stimulation with fMLP and after incubation with cytochalasin B. Treatment with cytochalasin B aborts and rapidly reverses acute increases in PMN f-actin content caused by exposure to chemotactic and other stimuli (24) and should mitigate artifacts caused by acute activation. Because the difference in PMN TT persisted after treatment with cytochalasin B, the decrease in basal PMN deformability seen in NIDDM appears to represent a chronic phenomenon and is unlikely to be caused by activation during in vitro handling of the cells.

The mechanism of decreased PMN deformability in diabetes is unknown. This could be caused by changes in membrane lipid composition resulting in altered membrane fluidity, because decreased PMN membrane fluidity has been described in streptozocin-induced diabetes in rats (30). Alternatively, increased glycation of the cell membrane or intracellular proteins may cause stiffening of cells, especially as the basal TT correlated significantly with HbA_{1c}. A further possibility is the presence of a state of chronic low-grade PMN activation that could increase basal cytoskeletal f-actin leading to a reduction in PMN deformability (31–33). Indeed, diabetic patients show a decrease in PMN lysozyme content (31) and increased plasma PMN elastase concentration (32,33) and superoxide production (34–36), all of which suggest chronic PMN activation.

The identical initial response to stimulation exhibited by PMN in the NIDDM and NGT groups (Table 2, Fig. 2) implies no difference in functional capacity between PMN isolated from the two groups. Although previous reports have suggested a chemotactic defect in diabetes (37), a more recent study (38) has shown that chemotaxis is suppressed only in response to complement-derived factors but not to bacterial products or their analogues (e.g., fMLP). Our results with fMLP stimulation are consistent with this latter study. Presently, we are unable to explain the slower recovery of PMN deformability in NIDDM after stimulation with fMLP. It is plausible that such behavior reflects slower depolymerization of f-actin to g-actin (24).

Abnormally rigid PMN may impede or temporarily obstruct flow in capillaries (14). This may not be particularly damaging to the microcirculation, because shunting would occur around the affected vessels and even rigid PMN are expected to be eventually released. However, if increased PMN rigidity in diabetes is associated with activation, the risk for microvascular injury may be enhanced. Activated PMN have increased surface expression of the adhesion receptor CD11b/CD18 that promotes their adhesion to endothelium (39). PMN adhesion to endothelium is also promoted by hyperglycemia (40). Adherent PMN can degranulate in situ, releasing various potent cytotoxic agents that include proteolytic enzymes and oxygen radicals capable of significant local tissue destruction (15). PMN proteases are active against elastin, collagen, fibrinogen, and proteoglycans and can destroy vascular basement membrane (41). Leukotriene B4, oxygen radicals, and other cationic proteins released from PMN can increase vascular permeability either directly or through activation of complement (38). Thus, increased PMN rigidity coupled with activation could contribute to the development of diabetic microangiopathy.

Hypertensive diabetic patients had less deformable PMN than those from patients with normal BP (Fig. 3). Although rheological abnormalities, including increased whole blood and plasma viscosity and decreased erythrocyte deformability, have been reported in hypertension (42–45), PMN mechanics have not been studied. Evidence exists, however, of increased leukocyte activation in patients with hypertension (46,47) and in the spontaneously hypertensive rat (48). The mechanism of this abnormality is unknown, but it is thought to contribute to hypertension development and its complications either directly by increasing capillary vascular resistance (14) or indirectly by releasing

proteolytic enzymes that act on the angiotensin system (49) or free radicals that interfere with endothelium-derived relaxing factor (50). In addition, the decreased PMN deformability in diabetes may contribute to the development of hypertension that is commonly present even in the absence of nephropathy. The marked increase in PMN rigidity in hypertensive diabetic patients may also explain, at least in part, the detrimental effect of hypertension on the development of diabetic complications such as nephropathy and retinopathy (51,52).

In conclusion, PMN rigidity is increased in diabetes and is correlated with BP and HbA_{1c} levels. Reduced PMN deformability and increased PMN activation may contribute to the pathogenesis of diabetic microangiopathy. The mechanisms responsible for these changes in PMN mechanics remain to be clarified.

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