Involvement of Peripheral Polymorphonuclear Leukocytes in Oxidative Stress and Inflammation in Type 2 Diabetic Patients

OBJECTIVE — To determine the extent to which peripheral polymorphonuclear leukocytes (PMNs) contributed to oxidative stress (OS) and inflammation in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS — PMNs and plasma were separated from blood withdrawn from 18 type 2 diabetic patients and 16 age- and sex-matched normal control subjects. The rate of superoxide release from phorbol 12-myristate 13-acetate (PMA)-stimulated PMNs and the plasma glutathione (GSH) levels served as measures of OS. Inflammation was assessed by PMN recruitment, expressed by peripheral blood PMN counts, and the in vitro survival of PMNs, which reflects cell necrosis.

RESULTS — PMA-stimulated PMNs from diabetes released superoxide significantly faster, and plasma-reduced GSH was lower in diabetic patients than in normal control subjects. The rate of superoxide release from diabetic PMNs showed no correlation with the plasma glucose concentrations, whereas a positive linear correlation with HbA1c was found. The in vitro survival of diabetic PMNs was lower than normal control PMNs when each was incubated in its own serum. The in vitro survival of normal control PMNs was reduced when incubated with diabetic serum, whereas normal control sera promoted the survival of diabetic PMNs. Peripheral PMN counts were higher in diabetic patients than in normal control patients.

CONCLUSIONS — Type 2 diabetes is accompanied by a priming of PMNs, resulting in OS and increased self-necrosis. Necrosis starts a chain of inflammatory reactions that result in cell recruitment and in the long run, with OS, may result in endothelial dysfunction. Understanding the contribution of PMNs to OS and inflammation in diabetes may illuminate new mechanisms through which endothelial dysfunction evolves and causes angiopathy and atherosclerosis.

Diabetes Care 24:104–110, 2001

Diabetes constitutes a major independent cardiovascular risk factor, even when adjusting for age, hypertension, smoking habit, dyslipidemia, and left ventricular hypertrophy (1,2). Newly diagnosed patients with type 2 diabetes (and patients in the precursor phase) with impaired glucose tolerance have a higher risk for developing macrovascular and coronary heart diseases. Accelerated atherosclerosis in these patients was recently found to be associated with insulin resistance and endothelial dysfunction (2,3). Endothelial dysfunction precedes the occurrence of angiopathy and appears to play a central role in the pathogenesis of atherosclerosis in diabetic patients (4,5). In these patients, insulin resistance, oxidative stress (OS), and inflammation are among the mechanisms recently implicated to cause endothelial dysfunction (6–8). Recently, it has been shown that diabetic patients are exposed to increased OS. The existence of increased OS is based on decreased antioxidant capacity in diabetes, chronic exposure to increased levels of reactive oxygen species (ROS), increased plasma oxidizability, and increased peroxidation and glycoxidation (9–11). Indirect support for the involvement of OS was provided by the restoration of endothelial dysfunction in diabetic patients by vitamin C, a water-soluble antioxidant, and by desferoxamine, an iron chelator that prevents iron-catalyzed generation of hydroxyl radicals (12–14). It has been shown that the ubiquitous intra- and extracellular antioxidant, glutathione (GSH), neutralizes oxidants such as hydrogen peroxide and superoxide by converting them to other oxidized forms (15–20). In diabetes, it has been suggested that GSH is largely consumed, mainly because of the regeneration of vitamin C, which is extensively oxidized in diabetic patients (21).

The peripheral polymorphonuclear leukocyte (PMN) is one of the main inflammatory cells. Once activated, PMNs release ROS and mediators of proteolytic tissue degradation, contributing to OS, subsequent inflammation, and endothelial damage (22,23). We have previously shown that in clinical situations known to be associated with accelerated atherosclerosis and endothelial dysfunction, such as essential hypertension and uremia, PMNs are activated, contributing to OS and inflammation (24,25). The contribution of PMNs to OS in diabetes is unclear, because the data conflict (26,27) and no available data on PMN contribution to inflammation currently exist. Thus, the present study evalu-
Table 1—Clinical and chemical characteristics of diabetic patients and healthy control subjects

<table>
<thead>
<tr>
<th></th>
<th>Healthy control subjects</th>
<th>Diabetic patients</th>
<th>P</th>
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<tbody>
<tr>
<td>n</td>
<td>16</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>47 ± 8.7</td>
<td>51.5 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>7/9</td>
<td>9/9</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.9 ± 0.1</td>
<td>7.12 ± 0.18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>91.5 ± 2.3</td>
<td>155 ± 7.2</td>
<td>&lt;0.004</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>17.4 ± 1.6</td>
<td>19 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>24.3 ± 4.5</td>
<td>24 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>197 ± 44</td>
<td>210 ± 8.4</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dl)</td>
<td>83.4 ± 14.7</td>
<td>112 ± 9</td>
<td>NS</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>4.7 ± 0.3</td>
<td>4.7 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Transferrin (mg/dl)</td>
<td>286 ± 36</td>
<td>291 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>Blood urea nitrogen (mg/dl)</td>
<td>13.7 ± 0.6</td>
<td>13.5 ± 0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>PMN (×10^9/ml)</td>
<td>3.2 ± 0.3</td>
<td>5.0 ± 0.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GSH (µmol/l)</td>
<td>1.21 ± 0.09</td>
<td>0.6 ± 0.12</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are n or means ± SEM, unless otherwise indicated. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

RESEARCH DESIGN AND METHODS

Study population
A total of 18 (9 men and 9 women) non-smoking, non-sedentary, non-diabetic patients (duration of disease 36–216 months, mean 96 ± 14), aged 34–73 years (average 51.5 ± 2.5), with normal kidney function, no actual infection, and BMI <30 kg/m² were included in the study.

Patients with known cardiovascular risk factors, significant dyslipidemia (LDL cholesterol ≥130 mg/dl, HDL cholesterol ≤35 mg/dl, and triglycerides ≥400 mg/dl), hypertension (blood pressure >130/85 mmHg), and severe obesity (BMI ≥30 kg/m²), as well as patients with evidence for microvascular or macrovascular diseases, were excluded from the study.

The diabetic patients were compared with an age- and sex-matched normal control group. The characteristics of the subjects are shown in Table 1.

Blood withdrawal and PMN separation
Blood was withdrawn from all of the above subjects and patients after an overnight fast for biochemical and hematological parameters and PMN isolation.

PMN isolation was carried out according to the method of Klebanoff and Clark (28) and modified according to Kristal et al. (24,25). The separated PMNs (>98% pure) were resuspended in a minimal volume (0.1–0.3 ml) of cold Hank’s balanced salt solution (HBSS) (Biological Industries, Beit Haemek, Israel), immediately counted, and diluted to a final volume of 1 ml with HBSS with 0.5% glucose, according to the different experimental needs. All sera were saved for in vitro survival assays.

Oxidative stress
Determination of the rate of superoxide release from PMNs. The rate of superoxide release was assayed for 90 min under basal conditions, after stimulation with 0.32 × 10⁻⁷ mol/l PMA (Sigma, St. Louis, MO), and after stimulation with zymosan.

The assay was based on the superoxide dismutase inhabitable reduction of 80 µmol/l cytochrome C (Sigma) to its ferrous form (29) with slight modifications (24,25).

Zymosan was prepared by an incubation for 30 min at 37°C of 4 mg zymosan particles (Sigma), with 1 ml sera, pooled from 10 healthy donors. This solution was washed twice with 154 mmol/l NaCl, resuspended in 154 mmol/l NaCl at a concentration of 10 mg/ml, and stored at −80°C until it was used. In our assays, the particle-to-cell ratio was 15 (30).

In a separate set of experiments, PMNs from seven randomly chosen diabetic patients and seven age- and sex-matched normal control subjects were preincubated with 100 and 500 mg/dl glucose for 20 min at 37°C, PMA was added, and the assay was carried out as previously described.

Determination of GSH levels in plasma.
GSH determinations were carried out for all subjects. To avoid the spontaneous oxidation of GSH in plasma, the plasma was separated within 4 min of blood withdrawal and acidified (1 ml plasma with 50 µl 50% 5-sulfosalicylic acid) (24,31). At this point, the acidified plasma was frozen for a maximum of 3 weeks. Before the determination of GSH, the plasma was thawed and back-titrated to pH 7 with 0.2 mmol NaOH. The determinations of plasma GSH were carried out according to Adams et al. (32) and Griffith (33), using oxidized GSH (GSSG)-reductase with slight modifications (24). Briefly, two separate evaluations were carried out: assessment of the total GSH level in the plasma ([GSH] + [2(GSSG)]) and assessment of the oxidized form of GSSG. The plasma level of GSH was calculated as the difference between the total GSH and the oxidized 2(GSSG). The total GSH concentration was determined using 10 mmol/l 5,5’-dithiobis, after reduction of the GSSG concentration by GSSG-reductase. The resulting amount of GSH was determined according to a standard curve (0–2.5 mol/l GSSG), recorded in a spectrophotometer (spectronic 1201; Milton Roy, Rochester, NY) at 412 nm, and expressed as the change in absorbency per minute during the enzymatic reduction with GSSG-reductase. To measure GSSG in the plasma, the initial alkylation of the sulphydryl groups was carried out with 10 mmol/l freshly prepared N-ethylmaleimide (NEM). The excess of NEM was removed by separation on Supelclean (LC18; Sigma) columns, and the GSSG concentration was determined enzymatically in the effluent, as previously described.

Indirect measurements of inflammation
PMN counts. The PMN counts of the blood withdrawn in EDTA were performed by using a Coulter STKS (Coulter, Miami, FL) apparatus in both the normal control subjects and the diabetic patients.
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Measurement of PMN survival in vitro.
Separated PMNs (10^7/ml) from 10 randomly chosen diabetic patients and 10 age- and sex-matched normal control subjects were incubated with the individual autologous serum (25% vol/vol diluted with HBSS) for 90 min at 37°C. In a second set of experiments, cross incubations were carried out with heterologous sera. PMNs were counted before and after 90 min of incubation. Cell viability was confirmed by trypan blue (0.1% wt/vol) exclusion. Survival was expressed as the ratio of cell counts before and after the 90-min incubation with sera (%).

Statistical analysis
Data are expressed as means ± SEM. Data are expressed as whiskers (median and range) for the description of the in vitro effect of glucose on the rate of superoxide release. The differences between the study parameters of the two groups were compared by Student’s t test. Serum glucose levels, HbA_1c levels, and rate of superoxide release by PMA-stimulated PMNs were correlated by linear regression analysis. P < 0.05 was considered statistically significant.

RESULTS

Study population
Table 1 shows that normal control and diabetic patient groups were similar concerning age and sex distribution, blood cholesterol, triglycerides, and normal liver and kidney functions. Blood glucose and HbA_1c were significantly higher in the diabetic subjects than in normal control subjects (Table 1), as expected.

Oxidative stress
Superoxide release. No superoxide release from resting PMNs could be detected under basal conditions during 90 min. The superoxide release from PMA-stimulated PMNs from diabetic patients was significantly greater than the superoxide release of PMA-stimulated PMNs from normal control subjects (Fig. 1). This indicates that the PMNs from diabetic patients are primed. In contrast, stimulation with zymosan, a physiological stimulant used to assess the phagocytic potential of PMNs, resulted in a significant decrease in the rate of superoxide release from diabetic PMNs (Fig. 1).

Figure 2 shows the in vitro effect of two concentrations of glucose on the rate of superoxide release from PMA-stimulated PMNs. No significant effect on glucose was found for either normal control or diabetic PMNs when compared with cells without glucose. It should be emphasized that within each group, the range of superoxide rates of release was similar, with faster rates in the diabetic patient group.

Plasma GSH levels. The plasma levels of GSH were significantly lower in diabetic patients than in normal control subjects (Table 1).

Relationships between OS and diabetic parameters. Figure 3 shows that in diabetes, no linear correlation could be found between the rate of superoxide release from

![Figure 1](image1.png)

![Figure 2](image2.png)
PMA-stimulated PMNs and the same individual's blood glucose levels \((n = 14, r = 0.001, P = 0.995)\) (Fig. 3A); however, HbA1c and the rate of superoxide release were positively correlated \((n = 11, r = 0.6, P < 0.05)\) (Fig. 3B).

**Indirect measurements of inflammation**

**PMN counts.** Diabetic patients had significantly \((P < 0.001)\) higher peripheral PMN counts than normal control subjects, although all of the values were within the upper quartile of the accepted normal range (Table 1).

**In vitro survival of PMNs.** A significant \((P = 0.001)\) reduction in diabetic PMN survival after an incubation of 90 min in diabetic serum was evident when compared with 90 min of incubation of normal control PMNs in normal control sera. In fact, normal control sera promoted the survival of diabetic PMNs \((P = 0.001)\), whereas diabetic sera significantly killed normal control PMNs \((P < 0.001)\) (Fig. 4).

**CONCLUSIONS** — We studied the properties and activities of PMNs and their possible contributions to OS and inflammation in diabetic patients. Our results show that type 2 diabetic patients are exposed to OS and chronic inflammation (partially because of the primed state of their PMNs) before any clinical evidence of angiopathy exists (Fig. 5). When separated, PMA-stimulated diabetic PMNs release superoxide significantly faster than normal control PMNs and concomitantly with a reduced level of the plasma antioxidant GSH, predisposing these patients to OS. Freedman and Hatchell (34) and Cantero et al. (26) have previously shown similar results of significantly higher levels of ROS release from stimulated diabetic PMNs in both cats and patients. Methodological differences may account for the discrepancy between these reports and that of Fuller et al. (27), who reported no changes in the rate of superoxide release from PMNs in type 2 diabetic subjects without vascular disease. In diabetes, the decrease in plasma GSH may be an outcome of greater GSH consumption by ROS. This reduced plasma GSH and the augmented rate of superoxide release showed a negative linear correlation (data not shown), which suggested that superoxide reacts directly with GSH, a phenomenon that was also supported by Thomas et al. (35). It has also been suggested that in diabetes, the antioxidant GSH is largely consumed, mainly because of a regeneration of vitamin C, which is extensively oxidized in diabetic patients (21). Lower amounts of GSH may also be explained by glycation of the enzyme \(\gamma\)-glutamyl cystein synthetase, which generates decreased amounts of GSH in diabetes (36).

Our observations emphasize that the oxidative metabolism of PMNs depends on the nature or type of the stimulant. By using the same assay system, we have shown that the same cell may respond in opposite directions to different stimuli. PMA stimulation of diabetic PMN caused a faster release, whereas zymosan stimulation caused a slower release of superoxide from the same diabetic PMN preparation when compared with a normal control PMN. Because zymosan is used to assess the phagocytic function of PMN, our findings also support the increased susceptibility to infections in diabetic patients, as previously described (37–39).

However, glucose per se did not modulate the rate of superoxide release from both diabetic and normal control PMNs in vitro. In addition, no correlation between the rate of superoxide release and blood glucose concentrations of the same individual was found. This implies that in vivo, as well as in vitro, blood glucose concentration does not directly modulate the oxidative metabolism of diabetic PMNs. In this regard, several authors have shown similar findings (40), whereas others have shown that glucose alone modulates PMN activity (41). Nevertheless, we have shown a close relation between OS and the metabolic control of

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**Figure 3**—Linear correlation between the rate of superoxide release and both blood glucose levels, expressed as \(\text{nmol} \cdot 10^{-11} \text{ min} \cdot 10^{-6} \text{ cells} (n = 14)\) (A) and HbA1c \((n = 11)\) (B).
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Furthermore, we found a significant positive correlation between the rate of superoxide release and blood levels of HbA1c, showing that they are closely related processes. The causative effect of OS on HbA1c in diabetes was previously reported by Young et al. (14) and Ruiz et al. (42). The strong positive correlation between the rate of superoxide release and blood levels of HbA1c, which to our knowledge is reported for the first time, further strengthens the relations between OS and enhanced glyco-oxidation of proteins in diabetes. Rodriguez-Manas et al. (43) have shown a close relation among endothelial dysfunction, levels of HbA1c, and production of superoxide in an animal model of diabetic rats. Our findings and those of Rodriguez-Manas et al. suggest a close relation between the levels of HbA1c and diabetes-associated endothelial dysfunction, underlined by OS.

The second important outcome of this study is the PMN contribution to chronic inflammation in diabetes. The existence of chronic inflammation in diabetes is mainly based on the increased plasma concentrations of C-reactive protein, fibrinogen, interleukin-6, interleukin-1, and tumor necrosis factor (44,45). Our in vivo data of increased diabetic PMN counts, combined with the decreased in vitro PMN survival rate, is evidence that primed PMNs contribute to chronic inflammation in diabetes. The leukoclastic effect of the diabetic sera suggests that extracellular factors are involved in the cellular priming of diabetic PMNs. Primed PMNs die by self-necrosis, and at the same time, they actively recruit more PMNs into circulation. The increase in diabetic PMN count, although within the upper quartile of the normal range, adds an indirect inflammatory parameter. Interestingly, the increase in leukocyte counts has been suggested as a predictor of myocardial infarction, even when the counts lie within the normal range (46). We have already described the fate of a primed PMN (inducing OS concomitantly with cell necrosis) in clinical disorders known to be associated with high prevalence of atherosclerosis and cardiovascular morbidity, such as essential hypertension and uremia (24,25). This study adds diabetes to these disorders, and these relationships are summarized in Fig. 5. The findings of Abu El-Asrar et al. (40), which show that diabetic sera increased superoxide release from PMNs, in combination with our findings, imply that diabetic sera leukoclastic activity may be responsible for the increased superoxide release. The nature of these factors remains unknown. However, intracellular factors should not be ruled out; the magnitude of necrosis in separated PMNs, regardless of their origin, correlated significantly with the rate of their superoxide release, indicating that intracellular factors are also involved in PMN priming (24). High cytosolic calcium, as was found in diabetic PMNs, can explain such priming (47).

An understanding of the contribution of PMNs to OS and inflammation in diabetic patients may illuminate new mechanisms through which endothelial dysfunction evolves and thereby results in angiopathy and atherosclerosis. In the future, a better understanding of the functions of PMNs may be effective in evaluating different treatment modalities, long before clinical evidence of angiopathy appears.
Acknowledgments—This study was partially supported by a grant donated by the Israel Society of Nephrology and Hypertension and by Technion VPR Fund (WK. Langfan Diabetes Research Fund).

References


27. Shurtz-Swirski and Associates
Neutrophils in type 2 diabetes