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# Evidence of Polymorphonuclear Neutrophils (PMN) Activation in Patients With Insulin-Dependent Diabetes Mellitus

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**Polymorphonuclear neutrophils' chemotaxis, surface charge, superoxide anions generation, NBT (nitro blue tetrazolium) reduction and intracellular lysozyme, and β-glucuronidase content were estimated in patients with type I diabetes mellitus in a similar state of metabolic control. The chemotaxis of diabetic cells toward bacterial chemotactic factors was similar to controls, whereas migration toward complement-derived chemoattractants was significantly reduced. Polymorphonuclear neutrophils isolated from diabetic patients, when unstimulated, produced significantly greater amounts of superoxide anions and reduced NBT more efficiently. They also revealed reduced surface charge and lower intracellular content of lysozyme, whereas β-glucuronidase content was similar to controls. The results obtained seem to indicate that neutrophils in patients with insulin-dependent diabetes manifest signs of being in the activated state. The possible mechanisms of such stimulation are discussed.**

**Key words: polymorphonuclear neutrophils, diabetes mellitus**

## INTRODUCTION

Reports concerning the unspecific host defense mechanisms in diabetic patients are fragmentary and very often controversial. Particularly inconsistent are opinions regarding the function of polymorphonuclear neutrophils (PMN) in diabetics. Bagdade et al. [2] reported the impaired phagocytosis in patients with poorly controlled diabetes. Bybee and Rogers [6] obtained similar results in diabetes with ketoacidosis. The normal phagocytosis in diabetics, however, was observed by Miller and Baker [15], Nolan et al. [19], and also in our previous studies [33]. Also different are the opinions concerning PMN bactericidal capacity [2,3,23] as well as chemotaxis [5,10,16,20].

These discrepancies seem to be the result of using various laboratory techniques, regardless of the type of diabetes or the current state of metabolic control. Nevertheless, the analysis of the most important published studies seems to indicate that PMN obtained from diabetic patients manifest several defects distinctly related to the range of metabolic disturbances. These suggestions are confirmed by the prospective studies of Rayfield et al. [22], who noticed the close relationship existing between the quality of diabetes control and the susceptibility to infection.

The main aim of our studies was to throw some light on the mechanisms leading to the impairment of PMN function in diabetics. Therefore, we evaluated the selected PMN functions in a group of patients with type I diabetes, being in a similar state of metabolic control.

## MATERIALS AND METHODS

Fifty patients, 22 males (15–42 years) and 28 females (18–48 years), with insulin-dependent diabetes mellitus were selected for the study. The known duration of disease was 1–11 years (mean 4.8 years). Eight patients were treated with a single insulin injection in the morning, consisting of either lente insulin alone or a combination of lente insulin plus neutral regular insulin injection. The mean insulin daily dose was 52 ± 8 U. Thirty-two patients received two daily insulin injections consisting of neutral regular insulin, lente or both, given in the morning and in the afternoon (mean daily dose, 64 ± 4 U). The fasting plasma glucose levels were less than 140 mg/100 ml (7,2 mmol/1), and for at least 10 days never exceeded 200 mg/100 ml (11,1 mmol/1), estimated 3 times a day. All the patients were poorly controlled previously, and normalized plasma glucose levels have been observed no longer than for 2 weeks. Mean $HbA1_c$

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level estimated in 17 patients was 10.2%. None of the patients showed evidence of renal failure, infection, or ketoacidosis in the course of the study. All patients were informed about the aim of the studies and gave their consent.

Fifty healty adults (blood donors and laboratory staff), aged 20 to 46, were used as control subjects.

The blood samples were always obtained from diabetic patients in the morning before insulin administration.

PMN were isolated employing the standard technique of dextran sedimentation, centrifugation on Ficoll/Hypaque, and hypotonic lysis [8].

PMN chemotaxis was estimated using the "under agarose" chemotaxis assay according to Repo [24]. Chemoattractants utilized were zymosan-activated plasma (ZAP), 5 mg/ml, 37°C, 30 min, incubated with cellophane according to Craddock et al. [8], supernate from an Escherichia coli culture prepared according to Ward et al. [29], and FMet-Leu-Phe (FMLP—Sigma Chemicals, St. Louis, MO) solution, $10^{-7}$ mol in phosphate-buffered saline (PBS). The results were expressed as a chemotaxis index, which was calculated as the ratio between the mean directional migration rates toward chemoattractant and toward inactivated control plasma, 56°C, 30 min.

PMN surface charge was measured by an electrophoretic mobility technique described previously [12]. The isolated cells were washed twice in Hanks' balanced salt solution (HBSS) and twice in 0.13 mmol phosphate-buffered, pH 7.2 5% mannitol, and the electrophoretic mobility then determined. The measurements were made with the use of a plastic chamber fitted with electrodes, which was placed and examined under the phase-contrast microscope equipped with a measuring ocular. The mean time of covering the constant distant (150 $\mu$m) by 20 PMN (also after reversal of polarity) was registered. The electronegative PMN surface charge was calculated and expressed as micrometers per second per volt per centimeter.

Intracellular PMN lysozyme content was estimated after disrupting of isolated cells by incubation with Triton X-100 solution (vol/vol). The lysozyme activity was determined in supernates by measuring the rate of lysis of Micrococcus lysodeicticus according to a turbidimetric method [26]. The results were expressed as micrograms egg white lysozyme equivalents, $10^7$ cells.

Intracellular PMN $\beta$-glucuronidase content was estimated after disruption of cells by measuring spectrophotometrically (540 $\mu$m) the release of phenolophtalein from its $\beta$-glucuronate after a 1-hr incubation at pH 4.5 [28]. Activity was expressed as micrograms of phenolophtalein per $10^7$ neutrophils per hr.

Superoxide anions ($O_2^-$) generation by PMN was measured by determining superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C (both Sigma Chemicals, St. Louis, MO) according to Bellavite et al. [4]. PMN samples were analyzed in the unstimulated state and after addition of serum opsonized zymosan 5 mg/ml, 37°C, 30 min. The results were expressed as nmol $O_2^-$, $10^6$ PMN, 10 min.

Nitrobluetetrazolium (NBT) reduction by PMN was estimated spectrophotometrically according to Müller et al. [17]. Unstimulated PMN as well as the cells stimulated with opsonized zymosan were evaluated. The results were expressed as nmol $H_2$, $5 \times 10^5$ cells.

Statistical evaluation of the data was done in accordance with Student's t-test for unpaired observations.

**TABLE 1. Chemotaxis of Polymorphonuclear Neutrophils (PMN) Isolated From Patients with Type I Diabetes Mellitus and From Controls Toward Zymosan-Activated Plasma (ZAP), Plasma Incubated With Cellophane (PC), FMLP, and Supernate From E. Coli Culture (Index of chemotaxis)[a]**

| | Controls (n = 50) | Diabetic patients (n = 30) | Significance of differences |
|---|---|---|---|
| ZAP | 2.09 ± 0.15 | 1.84 ± 0.12 | $p < 0.001$ |
| PC | 1.76 ± 0.11 | 1.60 ± 0.14 | $p < 0.001$ |
| FMLP | 2.11 ± 0.21 | 2.03 ± 0.13 | $p > 0.05$ |
| E. coli | 1.87 ± 0.26 | 1.88 ± 0.17 | $p > 0.05$ |

[a]mean values ± SD.

## RESULTS

### PMN Chemotaxis

PMN isolated from patients with insulin-dependent diabetes manifested the impaired chemotaxis toward ZAP and plasma incubated with cellophane in comparison with chemotaxis of control cells, $p < 0.001$ (Table 1). The directed migration of neutrophils toward FMLP and especially toward supernate from E. coli culture was similar in diabetic patients and healthy persons, $p > 0.05$.

### PMN Surface Charge

The electrophoretic mobility of diabetic PMN was significantly decreased in comparison with PMN obtained from controls $p < 0.001$. This reflects the serious reduction of negative surface charge of diabetic cells (Table 2).

### Intracellular Lysozyme and $\beta$-Glucuronidase Contents

The amounts of lysozyme liberated from disintegrated diabetic PMN were significantly smaller than those observed in healthy persons (Table 2). The PMN intracellular $\beta$-glucuronidase activity was similar in both evaluated groups.

**TABLE 2. Intracellular Lysozyme and β-Glucuronidase Activity, Superoxide Anion/$O_2^-$/ Production, NBT Reduction, and Surface Charge of Polymorphonuclear Neutrophils Isolated From Patients With Type I diabetes Mellitus and From Controls[a]**

| | Controls | Diabetic patients | Significance of differences |
|---|---|---|---|
| Lysozyme activity | (n = 15) | (n = 15) | |
| (μg/$10^7$ cells) | 10.60 ± 1.71 | 7.75 ± 2.02 | $p < 0.001$ |
| β-glucuronidase | (n = 15) | (n = 15) | |
| activity (U/$10^7$ cells) | 17.01 ± 2.23 | 17.46 ± 1.67 | $p > 0.05$ |
| $O_2^-$ production | (n = 15) | (n = 15) | |
| (nmol/$10^6$PMN/10 min) | | | |
| unstimulated | 4.15 ± 1.24 | 11.69 ± 2.53 | $p < 0.001$ |
| stimulated | 23.67 ± 4.36 | 22.52 ± 2.94 | $p > 0.05$ |
| NBT reduction | (n = 50) | (n = 30) | |
| (nmol $H_2$/5 × $10^5$PMN) | | | |
| unstimulated | 3.52 ± 1.36 | 5.71 ± 1.44 | $p < 0.001$ |
| stimulated | 32.05 ± 12.02 | 26.40 ± 7.82 | $p < 0.001$ |
| Surface charge | (n = 50) | (n = 30) | |
| (μm/sec/V/cm) | 1.90 ± 0.31 | 1.48 ± 0.23 | $p < 0.001$ |

[a]mean values ± SD.

### Superoxide Anions ($O_2^-$) Generation by PMN

The neutrophils obtained from diabetic patients, when unstimulated, produced significantly greater amounts of $O_2^-$ than did control cells, $p < 0.001$ (Table 2). Stimulation of cells due to phagocytosis of opsonized zymosan particles led to increased $O_2^-$ generation. The stimulated production of superoxide anions by control PMN was higher than diabetic cells; however, the differences were not statistically significant, $p > 0.05$.

### NBT Reduction by PMN

The diabetic PMN produced significantly greater amounts of hydrogen ($H^+$), reducing nitrobluetetrazolium to formazan, in comparison with control cells, $p < 0.001$ (Table 2). Stimulation of neutrophils increased NBT reduction, but the control cells produced $H^+$ in significantly greater amounts. Because the main source of reducing substances ($H^+$) in PMN is the hexosemonophosphate shunt (HMPS), in this situation NBT reduction reflects the activity of this glucose metabolism pathway [1,17].

## DISCUSSION

The circulating PMN are the cells manifesting small metabolic activity. In the course of phagocytosis or under the influence of certain membrane perturbing agents, the intracellular glucose metabolism rapidly increases, mainly via HMPS [1]. This "respiratory burst" results in the production of the large quantities of superoxide anions ($O_2^-$), reduction of the electro-negative surface charge, as well as degranulation of secondary granules [31].

In our patients with type I diabetes, PMN manifested the features resembling those noticed when stimulated cells were evaluated. The unstimulated PMN from diabetic patients reduced NBT much more intensively than those obtained from controls, indicating the primary enhancement of HMPS activity. We also noticed the increased unstimulated $O_2^-$ production, similar to observations of Shah et al. [25]. The reduced PMN surface charge and the decreased intracellular lysozyme content with the concomitant unchanged β-glucuronidase activity additionally confirmed the activation of circulating neutrophils in diabetics.

Among many possible mechanisms theoretically responsible for the stimulation of PMN circulating in diabetic patients, hyperglycemia seems to be the most important. The above concept is suggested by several reports indicating the close relationship existing between the quality of metabolic control of diabetes and the impairment of PMN function [2, 22]. Hill et al. [11], who evidenced the activation of circulating monocytes in diabetic patients, also suspected hyperglycemia of the participation in the pathomechanism of observed events. Some clinical observations have provided additional evidence for a role of hyperglycemia in the stimulation of PMN [7,30].

It is impossible to offer a definite explanation at this time, but it seems that the increased plasma glucose level may activate neutrophils by the influence on the intracellular carbohydrate metabolism. Esman [9] evidenced, in diabetic PMN, the inhibition of oxidative glycolysis with simultaneous increase of glucose metabolism via HMPS. He postulated that it may be due to the decrease of the activity of insulin-dependent enzymes: phosphofructokinase and pyruvate kinase. Another mechanism of oxida-

tive glycolysis inhibition could be the respiratory modulation of glucose metabolism—Crabtree-Pasteur effect [13,14].

It is well known that the rise of HMPS activity leads to the production of large amounts of nicotinamide adenine nucleotides (NADPH). This is the substrate for membrane bound NADPH-oxidase, responsible for the $O_2^-$ production by PMN [1]. The increased production of superoxide anions and most probably the generation of subsequent oxygen-derived radicals—OH, $H_2O_2$, $^1O_2$—cannot be discounted as other mechanisms leading in turn to PMN activation. These highly reactive oxygen species can react with plasma lipids [21] and IgG molecules [32] supplying neutrophils with the potent soluble stimuli.

The PMN obtained from diabetic patients manifesting the signs of selective degranulation certainly are the source of C5 activator released from secondary granules [35]. The impairment of chemotaxis of diabetic PMN toward complement-derived chemotactic factors (chemotactic deactivation?) [18] may indicate the generalized intravascular complement activation. Our previous studies concerning certain properties of diabetic plasma, although performed without direct complement estimation, confirm the presence of circulating C5a [34]. Recently, Sundsmo et al. [27] have provided direct evidence for the complement activation in diabetic patients.

The changes of certain PMN functions in the evaluated group of diabetic patients were distinct in spite of normalization of plasma glucose levels. Probably the duration of normoglycemia in these cases was too short for removing the cellular metabolic consequences. The PMN precursors already activated in bone marrow probably retain their characteristic feature through the whole life of these cells. It should be supposed that only the "long enough" duration of good metabolic control enables diabetic patients to preserve the normal functions of neutrophils. Because that is difficult to achieve, the reports demonstrating the normal functions of diabetic PMN are relatively rare.

The proposed mechanisms of PMN disturbances in diabetic patients and subsequent events seem to be of clinical importance. They could partly explain the impairment of host defense in diabetics. Moreover, the heightened production and release of reactive oxidizing agents, as well as the exocytosis of enzymes from degranulating cells, might contribute to the development of microangiopathic changes.

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