

# Leukocyte Function in Chronic Myeloproliferative Disorders

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**ABSTRACT.** The myeloproliferative disorders (MPD) are clonal diseases that originate from a transformed stem cell and involve all myeloid lineage. The affected cells have both proliferative and functional impairment. Therefore, we evaluated and compared neutrophil function in 31 patients with polycythemia vera (PV), idiopathic myelofibrosis (MF), chronic myeloid leukemia (CML), and essential thrombocytosis (ET). Neutrophil chemotaxis, random migration, bactericidal activity and superoxide anion release in these patients were simultaneously compared to those of 31 healthy controls. In this study, chemotactic activity was significantly impaired in patients with PV and CML as compared to controls ( $M \pm SE$ :  $42 \pm 6$  vs.  $69 \pm 5$  cells/field;  $p < 0.005$  and  $47 \pm 7$  vs.  $68 \pm 5$ ;  $p < 0.05$ , respectively). The assessment of the bactericidal activity of neutrophils showed no impairment in most of the patients. In the CML group, the serum had a very strong "lytic" effect on bacteria, possibly due to the high levels of serum lysozyme ( $22 \pm 2$  ug/ml). The superoxide anion release was found to be normal in most of the patients. Nevertheless, in 25% of PV patients the superoxide production was impaired (less than 60% of the simultaneous controls). In ET most patients had normal neutrophil function. Regarding the effect of treatment, neutrophil chemotactic activity was found to be significantly reduced in the hydroxyurea-treated patients, as compared to the non-treated patients ( $p < 0.001$ ) or healthy controls ( $< 0.0001$ ).

We conclude that disturbances in neutrophil function are present in patients with various MPDs, except ET. This probably reflects abnormal maturation of precursors of the damaged stem cells. Nevertheless, we should keep in mind that therapy itself could affect neutrophil functions. This matter should be studied more extensively. Although infections are not common in MPD disorders, they occasionally occur. It is possible that impairment in the phagocytic function contribute to the development of infections in patients with myeloproliferative disorders.

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**Keywords:** myeloproliferative disorders, neutrophil chemotaxis, random migration, bactericidal activity, superoxide production, serum lysozyme

## INTRODUCTION

Chronic myeloproliferative disorders (MPD) are a family of malignant clonal diseases that originates from an abnormal pluripotent stem cell and are characterized by trilineage hyperplasia. These disorders include chronic myelogenous

leukemia (CML), polycythemia vera (PV), idiopathic myelofibrosis (MF), and essential thrombocytosis (ET). They usually run a chronic course but may transform into an aggressive phase. Bacterial and fungal infections occasionally complicate the course of these diseases. Infections could be related either to

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humoral, or leukocyte dysfunction (1-6). It has been suggested that neutrophil function is defective in MPD because the neutrophils are derived from the abnormal stem cell (1,5,6). However, different steps of neutrophil function including neutrophil margination and adherence to endothelial surfaces, neutrophil migration and chemotaxis, neutrophil recognition and ingestion of the opsonized bacteria, cell degranulation and the subsequent killing of the bacteria can be involved along these pathways (7,8). The purpose of this study is to further evaluate the phagocytic response of stimulated polymorphonuclear cells (PMNs) in patients with various chronic myeloproliferative disorders.

## PATIENTS AND METHODS

The study includes 31 patients: 12 patients with polycythemia vera (PV), 5 with idiopathic myelofibrosis (MF), 6 with chronic myeloid leukemia (CML), and 8 patients with essential thrombocytosis (ET). The control group comprises 31 healthy volunteers. Clinical characteristics of the patients are provided in table 1. Hydroxyurea (Hydrea) was the most common medication prescribed, but three patients with MF were treated with Naproxen (Naxyn) or Danazol and two patients with CML received Interferon alpha. The study was approved by the Helsinki Committee at Meir General Hospital, Sapir Medical Center, Kfar Saba, Israel.

### *Isolation of Polymorphonuclear Leukocytes*

Human purified PMNs (98%) were isolated from heparinized venous blood. After sedimentation with 3% dextran (MW 250.000, Sigma), the leukocyte enriched-plasma was layered on to a Ficoll-Hypaque gradient (Lymphoprep, Nycomed Pharma AS, Oslo) and centrifuged at 400 g for 30 min, as previously described (9). The supernatant was discarded and the pellet was subjected to hypotonic lysis for 20 sec to free the PMNs from contaminating red blood cells. The PMNs were resuspended for

chemotaxis in M-199 medium (Earle's salt with L-glutamine, Biological Industries, Kibutz Beth Haemek, Israel). For superoxide anion release, PMNs were suspended in Hanks' Buffered Salt Solution (HBSS - Sigma) with 10mM Hepes (Sigma), and for the bactericidal assay in Phosphate-Buffered Saline with 0.2% glucose and 1% bovine serum albumin (PBS/glucose-albumin). The percentage of immature white blood cells was less than 10% in CML patients and less than 2% in the other MPD.

### *Chemotaxis Assay*

A 48-well chemotactic microchamber (Neuro Probe, Inc., Bethesda, MD) was used to determine random migration and chemotaxis (10). Either the chemoattractant N-formylmethionyl-leucyl-phenylalanine (fMLP, Sigma) at a concentration of 1  $\mu$ M or the M-199 medium was added to the bottom wells. A polycarbonate membrane filter, PVP free, with 3  $\mu$ m pores (Nucleopore Corp., Pleasanton, CA), was placed on top of the wells in the bottom plate. The gasket and the top plate were affixed, and 50  $\mu$ l of  $10^6$  PMNs/ml were added to the upper wells. The assembly was incubated for 60 min in humidified air. After incubation the filter was wiped off and stained with May-Grunwald-Giemsa dye. The average number of cells in nine fields was counted under light microscopy with a 20 x objective and an optical grid at 10x magnification. Net chemotaxis was calculated by subtracting the random migration from the chemotactic activity. Experiments were performed in duplicate.

### *Bactericidal Activity*

The quantitation of maximal bactericidal activity was measured as the decrease in the number of viable bacteria after incubation of bacteria and PMNs in the presence of serum, as described previously (11). Bacteria (*Staphylococcus Aureus* American type Culture) were freshly grown before each experiment and

allowed to enter an early stationary phase (4 h shaking, at 37°C). The final concentration of bacteria was ascertained by spectrophotometry at 640 nm and by plating and counting the Colony Forming Units (CFU). A suspension of PMNs at a final concentration of  $10^6$ /ml was incubated with bacteria at a ratio of 1:3, and either autologous or a pool of homologous AB serum obtained from healthy controls, at 37°C, for 90 min with continuous shaking. After washing, the PMNs were then lysed with 5 volumes of distilled water, suspensions were diluted and plated in triplicate in broth agar plates for 18 h, at 37°C. Each experimental setup included two controls which were comprised of PBS/G-A and bacteria or serum and bacteria. The colony-forming units (CFU) were assessed and the log of decrease of bactericidal activity was calculated and compared with that of the control counts.

#### *Superoxide Anion Release*

This assay was performed as previously reported (12). The PMNs were suspended in 1 ml of HBSS at a final density of  $1 \times 10^6$  cells/ml, with 60  $\mu$ M of ferricytochrome C (Sigma), with or without 214 U of superoxide dismutase (Sigma). To initiate the reaction 0.1  $\mu$ M of fMLP (Sigma) or 100 ng of PMA (Sigma) was added, and the rate of superoxide anion release was measured at 550 nm for 5 and 10 min respectively, at 37°C, in a UV-260 Shimadzu spectrophotometer. The results of duplicate tests were averaged, and the superoxide anion release calculated using the Massey extinction coefficient of  $2.1 \times 10^4$  M<sup>-1</sup> cm<sup>-1</sup>.

#### *Lysozyme Assay*

Lysozyme activity was determined by measuring the lysis of *Micrococcus lysodeikticus* (13). In each experiment controls were simultaneously tested. Analysis of patients and controls was performed simultaneously.

#### *Statistical Analysis*

Both the Student's T test and the Mann-Whitney non-parametric test were used for data analysis of all leukocyte functions. For comparing granulocyte function in treated vs. untreated MPD patients, the Fisher's exact test was used.

## RESULTS

#### *Chemotaxis*

The results of the net chemotaxis in the various MPD and control groups are provided in table 2. Net chemotaxis was significantly lower in PV and CML patients ( $42 \pm 6$  cells/field in PV compared to  $69 \pm 5$  in healthy controls;  $p < 0.005$  and  $47 \pm 7$  cells/field in CML patients compared to  $68 \pm 5$  in healthy controls;  $p < 0.05$ ). Random migration was normal in all MPD patients.

Comparing neutrophil net chemotaxis of hydra-treated patients ( $40 \pm 3.75$  cells/field) versus untreated patients ( $66 \pm 5.06$  cells/field) or hydra-treated versus healthy controls ( $65 \pm 2.75$  cells/field), significant reduced net chemotaxis was recorded ( $p < 0.001$ ) (Table 3). Therefore, the treatment with hydra exerts a negative effect on neutrophil chemotaxis.

#### *Bactericidal Activity (BA)*

BA was not significantly different from normal controls in patients with CML, PV, and ET, irrespective of whether autologous or homologous serum was used in the test (Table 2). Patients with CML showed marked lytic effect of their serum on bacteria, most probably due to the high levels of serum lysozyme. Two patients with MF (patients 2 and 5) demonstrated a significant bactericidal defect: patient 2, showed correction of the BA when their cells were incubated with homologous sera, indicating a humoral defect; while patient 5 did not show any correction with homologous sera, supporting a cellular defect. Indeed, in this patient superoxide anion generation was very low (28% of control).

**Table 1.** Characteristics of the study patients

Patient No.	Age (Years)	Sex	Disease	Follow up (Years)	Treatment
1	75	F	PV	15	Hydrea
2	65	F	PV	6	Hydrea
3	85	M	PV	16	Hydrea
4	60	M	PV	9	Hydrea
5	65	F	PV	3	Hydrea
6	83	F	PV	12	Hydrea
7	79	M	PV	4	Hydrea
8	67	F	PV	4	Hydrea
9	79	M	PV	10	-
10	68	F	PV	12	-
11	81	M	PV	12	-
12	100	F	PV	3	-
13	28	M	CML	3	Interferon
14	30	F	CML	3	Interferon
15	45	F	CML	4	Hydrea
16	63	M	CML	8	Hydrea
17	75	F	CML	0.5	Hydrea
18	72	M	CML	9	-
19	43	M	MF	1.5	-
20	46	F	MF	1.5	-
21	68	M	MF	4	Naxyn
22	81	M	MF	3	Naxyn
23	82	M	MF	1.5	Danazol
24	57	M	ET	0.8	Hydrea
25	71	M	ET	2	Hydrea
26	74	F	ET	2	Hydrea
27	78	F	ET	3	Hydrea
28	82	M	ET	1.5	Hydrea
29	82	M	ET	1	-
30	43	F	ET	0.5	-
31	64	M	ET	0.3	-

PV: Polycythemia Vera

CML: Chronic Myeloid Leukemia

MF: Myelofibrosis

ET: Essential Thrombocytosis

**Table 2.** Results of Neutrophil Chemotaxis, Superoxide Generation and Bactericidal Activity in MPD-patients

MPD - patients	Net Chemotaxis (cells/field)*	Superoxide generation		Bactericidal Activity	
		fMLP (nmolO <sub>2</sub> /10 <sup>6</sup> PMNs/min)	PMA	Autol. serum	Homol. sera
				log decrease of CFU	
PV (12)**	42 ± 6	2.23 ± 0.31	3.15 ± 0.28	0.78 ± 0.03	0.89 ± 0.04
control (12)	69 ± 5.4	2.80 ± 0.40	4.00 ± 0.30	0.80 ± 0.05	0.84 ± 0.05
p- value	< 0.005	NS	NS	NS	NS
CML (6)	47 ± 6.6	2.55 ± 0.43	4.79 ± 0.28	1.07 ± 0.33	0.73 ± 0.14
control (8)	68 ± 4.8	3.17 ± 0.61	4.14 ± 0.37	0.91 ± 0.10	0.84 ± 0.06
p- value	< 0.05	NS	NS	NS	NS
MF (5)	63 ± 9	1.97 ± 0.52	3.75 ± 0.17	0.64 ± 0.17	0.71 ± 0.10
control (10)	77 ± 7	2.95 ± 0.35	4.09 ± 0.33	0.83 ± 0.09	0.82 ± 0.05
p- value	NS	NS	NS	NS	NS
ET (8)	53 ± 5.8	2.45 ± 0.45	4.34 ± 0.48	0.87 ± 0.06	0.85 ± 0.05
control (11)	64 ± 2.7	2.28 ± 0.37	4.54 ± 0.25	0.84 ± 0.04	0.79 ± 0.04
p- value	NS	NS	NS	NS	NS

\* mean ± standard error

\*\* (n): number of patients

NS: not significant

CFU: Colony Forming Units

PV: Polycythemia Vera

CML: Chronic Myeloid Leukemia

ET: Essential Thrombocytosis

**Table 3.** Effect of hydra treatment on neutrophil functions of MPD-patients

	Net Chemotaxis (cells/field)*	Superoxide generation		Bactericidal Activity	
		fMLP (nmolO <sub>2</sub> /10 <sup>6</sup> PMNs/min)	PMA	Autol. serum	Homol. sera
				log decrease of CFU	
treated (16)	40 ± 3.75	2.25 ± 0.23	4.12 ± 0.31	0.86 ± 0.04	0.89 ± 0.05
untreated (10)	66 ± 5.06	2.76 ± 0.4	3.76 ± 0.36	0.71 ± 0.06	0.78 ± 0.04
p- value	< 0.001	NS	NS	NS	NS
treated (16)	40 ± 3.75	2.25 ± 0.23	4.12 ± 0.31	0.86 ± 0.04	0.89 ± 0.05
control (16)	65 ± 2.75	2.79 ± 0.36	4.53 ± 0.23	0.8 ± 0.07	0.86 ± 0.04
p- value	< 0.0001	NS	NS	NS	NS
untreated (10)	66 ± 5.06	2.76 ± 0.4	3.76 ± 0.36	0.71 ± 0.06	0.78 ± 0.04
control (16)	65 ± 2.75	2.79 ± 0.36	4.53 ± 0.23	0.8 ± 0.07	0.86 ± 0.04
p- value	NS	NS	NS	NS	NS

\* mean ± standard error

NS: not significant

CFU: Colony Forming Units

No significant difference in BA was found between the Hydrea-treated and untreated patients.

### *Superoxide Anion Release*

The results of superoxide release experiments are shown in table 2. Using either fMLP or PMA stimulation, no significant differences from control were detected in the various groups of MPD. Nevertheless, in 25% of PV patients, the superoxide anion release was below 60% of healthy simultaneous controls.

Treated or untreated MPD patients did not show any significant difference in superoxide anion generation.

### *Serum Lysozyme Measurements*

In four patients with CML, serum lysozyme was measured and found to be  $22 \pm 2$  ug/ml (normal  $< 7$  ug/ml).

## DISCUSSION

In the current study, which is one of the largest of its kind, we have demonstrated impaired granulocyte function in patients with MPD. The magnitude of impairment, and the specific function that is affected varied, depending on the diagnostic category of the patient. There was no relationship between impaired neutrophil function and patient age. Further discussion of our results and comparison with published studies, is best done by analysis of specific diagnostic categories of MPD.

### *Polycythemia Vera (PV)*

Previous studies both in vitro and in vivo could not demonstrate a chemotactic defect in patients with PV (14,15). In contrast, Corberand (1), found reduced random migration in these patients, a finding that we could not confirm. In our study, PV patients as a group, showed

significantly impaired chemotactic responses, although about half of the patients in this group had responses that fell within the normal range. The controversial results reported by the investigators could be related to small sampling; nevertheless, it is important to note that treatment with hydrea significantly affected the chemotactic activity of our patients.

As in previous studies (1,16), the bactericidal activity of the patients was found to be normal. The oxidative burst stimulation was found to be normal in most patients. Part of the patients showed decreased superoxide production, although the magnitude of the defect was mild to moderate, a fact that could explain the normal BA. Samuelsson reported defective oxygen burst in fMLP-stimulated phagocytes in most PV patients (6,15,17,18), but not in the PMA-stimulated cells. He suggested that the reduction of phospholipase D-mediated generation of diacylglycerol, could explain the reduced superoxide generation in fMLP-stimulated neutrophils (18).

### *Chronic Myelogenous Leukemia (CML)*

Abnormal neutrophil function has been reported in many studies on patients with chronic myelogenous leukemia, and various biochemical (2,4,5,19,20), functional (20-24) and membrane defects (25) have been attributed to explain the functional defect. In our study, patients with CML had significant decreased chemotaxis. There are various explanations for the chemotactic defect in CML. These include impaired fMLP interaction with its receptor (24), low expression of fMLP or  $LTB_4$  specific receptors with low synthesis of  $LTB_4$  (20), and aberrant sialylation of granulocyte membrane (19). The common pathway of all the proposed mechanisms is alteration at the level of the cell membrane receptors.

The bactericidal activity of CML patients in this study has been found to be normal. While some authors reported abnormal BA in CML patients, linking this finding to impaired

oxidative burst (22,26), others found normal cytochrome c reduction (6,21,25).

We found as others that in CML patients have high levels of serum lysozyme with very high bactericidal effect. Indeed, our experiments showed that incubation of the bacteria with the autologous serum alone caused their complete lysis. Thus, in the BA assay, the bacteria added to the incubation mixture were lysed by the patients' serum and were therefore not available for ingestion and killing by neutrophils. This could explain the low rate of infections in these patients. Additionally, the presence of a high number of mature granulocytes in CML patients could compensate for the cellular dysfunction, probably related to the concomitant presence of immature granulocytes.

#### *Myelofibrosis (MF)*

Studies of leukocyte function have been only rarely reported in idiopathic MF patients, and there are no reports on neutrophil chemotactic activity. In this study, no significant differences were found in the chemotactic activity of MF patients as compared to healthy controls (Table 2). Nevertheless, two hydrea-treated patients showed significant decreased chemotaxis. Bactericidal activity was found to be normal in 60% of the patients. Low levels of NBT reduction have been reported elsewhere (1,16,27). Similarly, we found impaired fMLP-stimulated superoxide production in 3 of the 5 patients studied, which correlated with their low bactericidal activity. Another possible explanation for the defective bactericidal activity could be the impaired phagocytosis (16,27) or low levels of myeloperoxidase (2), as previously reported.

#### *Essential Thrombocytosis (ET)*

There have been few reported studies of neutrophil functions in ET. Our results are in agreement with the finding of normal leukocyte function reported in most of these patients. Bactericidal activity and superoxide anion release

were found to be normal. It could be that the basic impairment in this MPD condition does not affect the myelopoietic line, and therefore, do not cause leukocyte dysfunctions.

We conclude that phagocytic dysfunctions, particularly the chemotactic activity and the oxidative burst, are found in PV, CML and MF. The linkage between these dysfunctions and the development of infections has not been well established. Since MPD patients only occasionally develop infections, it seems that the magnitude of the disorder is well compensated by other mechanisms, as is the case in CML patients, in whom we found strong bactericidal effect of their serum, possibly because of its high lysozyme content.

It is possible that some medications routinely used in these diseases correct part of the abnormalities, but other could alter cell functions, increasing patient's susceptibility to develop infections. We found that most patients treated with hydrea showed mainly significant deterioration of net chemotaxis. Other studies reported no effect (1,23) or improvement of functions after treatment with myleran and interferon gamma (17,19,21,25). Therefore, the effect of treatment with different medications and their molecular mechanism should be further evaluated in order to find the optimal treatment for these conditions.

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