

Function of Neutrophils in Different Phases of Chronic Myelogenous Leukemia

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ABSTRACT

In chronic myelogenous leukemia (CML), the mature granulocytes originate from a stem cell line harboring an abnormal chromosome, therefore it is possible that metabolic-functional abnormalities occur in the morphologically mature cells. In the present study, the phagocytic activity including intracellular killing, nitro blue tetrazolium (NBT) reduction, and phagocytosis were studied in 37 CML patients in different stages of the disease. The results were compared with those of 37 normal controls. Patients' neutrophils display significantly lower intracellular killing ($P<0.01$), NBT reduction ($P<0.01$) and phagocytosis ($P<0.001$) than that of normal controls. Analysis of the results revealed an inverse correlation between phagocytic activity and leukocyte count or percentage of immature cells ($r = -0.3$, $P<0.01$). In conclusion, the results indicate that neutrophils of CML patients have impaired phagocytic activity. This defect is more prominent in patients in blastic phase, whereas patients in remission show normal values. *Iran. Biomed. J.* 6 (2 & 3): 83-88, 2002

Keywords: chronic myelogenous leukemia (CML), neutrophil, phagocytic function

INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal expansion of hematopoietic progenitor cells characterized clinically by myeloid hyperplasia, leukocytosis with basophilia, and splenomegaly [1]. Cytogenetic analysis reveals the classic 9; 22 translocation (Philadelphia chromosome, Ph) in 90-95% of the patients with CML. In many Philadelphia chromosome-negative patients, the characteristic chromosomal translocation can be demonstrated by molecular analysis [2]. The incidence of CML is 1:100000 populations, accounting for 15-20% of all cases of leukemia in adults [3]. Traditionally, CML is described as a phasic disease which most patients are in chronic phase [1]. The initial chronic phase is characterized by the presence of all neutrophilic series, with prominent peak frequencies in the segmented neutrophil and myelocyte categories. Promyelocyte and myeloblast usually make up less than 30% of the total number of cells [4]. The blastic phase develops in 75-85% of patients with CML and is associated with an extremely poor prognosis. The presence of more than 30% myeloblast plus promyelocyte is generally accepted as evidence of transmission to blast crisis [1]. Remission is

achieved when the leukocyte count reaches to normal, immature neutrophils usually disappear. However, in some patients low percent of immature cells persists in the blood [4].

Since mature neutrophils in CML are produced from a stem cell line containing an abnormal chromosome, it is possible that functional-metabolic abnormalities occur in these cells. Previous studies of phagocytic activity in CML patients showed defective [5, 6] or normal [7, 8] activity. To clarify these discrepancies, we attempted to define the phagocytic function of polymorphonuclears (PMN) in patients in different stages of CML. We were also interested in investigating any correlation between the function of neutrophils and leukocyte counts or particularly percentage of immature cells reflecting the severity of the disease.

MATERIALS AND METHODS

Study groups. CML patients were diagnosed on the basis of clinical status, peripheral blood and bone marrow findings. Thirty-seven patients included in the study; 21 patients were in chronic phase, 7 in blastic phase, and 9 in remission. The patients were at diagnosis or had not received any

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Table 1. Outline characteristics of CML patients.

Groups	No. of cases	Gender		Leukocyte count ($\hat{1} 10^3 /-1$)	% of immature cells*
		M	F		
Remission	9	5	4	3 - 10	0 - 4
Chronic	21	11	10	10 - 400	5 - 30
Acute	7	3	4	50 - 400	30 - 40
Normal	37	21	16	3 - 8	-

*myeloblast + promyelocyte

medication at least four months prior to the study. The peripheral white blood cell differential counts were routinely analyzed at the time of blood collection. Outline characteristics of the study groups are demonstrated in Table 1. Thirty-seven healthy medication-free volunteers matched for age and sex were studied as controls.

Isolation of granulocytes. Heparinized peripheral blood sample was collected from each case. The plasma was removed after centrifugation at $450 \times g$ for 10 min and kept at 4°C until used. Equal volumes of 6% dextran (Pharmacia, Uppsala, Sweden) and TC 199 medium (Sigma, Deisenhofen, Germany) were mixed (dextran/TC). The packed cells were resuspended in an equal volume of dextran/TC and incubated in upright position for 40 min at room temperature. The leukocyte rich supernatant was aspirated and pelleted by centrifugation at $450 \times g$ for 10 min. The cells were washed twice with TC 199 medium, then resuspended in this medium.

Test organism. *Candida albicans* was cultured in a medium containing 1% bactopecton and 2% glucose. A portion of this culture was autoclaved at 120°C for 20 min to prepare dead organisms.

Intracellular killing test. Intracellular killing was assessed according to the procedure of Ghavami-Nejad and Zandieh [9]. Briefly, 10^6 PMN cells isolated from CML patients were mixed with 10^6 live *C. albicans* in final volume of 0.4 ml in a tube

(Nunc, Roskilde, Denmark) and 0.2 ml plasma was added. For each patient, five sets of tubes, each set in triplicate, were arranged as Table 2. The tubes were placed on a rotamixer and incubated at 37°C for 90 min. Then, 0.2 ml of 2.5% sodium deoxycolate (Difco, Heidelberg, Germany) and 0.1 ml of 50 mg/ml DNase (Sigma, Deisenhofen, Germany) were added to each tube and vortexed vigorously. In a 96-well microplate, triplicate set of wells was used for contents of each tube (0.2 ml each). The cells were pulsed with $0.1 \mu\text{Ci}$ of $[^{14}\text{C}]$ uridine (Amersham, Little Chalfont, UK) per well for 30 min and incorporation of labeled uridine was assessed by liquid scintillation counting. The mean of counts per minute (cpm) of each triplicate was calculated and intracellular killing was estimated according to the method of Yamamura *et al.* [10] by using the following formula:

$$\% \text{Killing} = 100 - [\text{cpm} (\text{Candida} + \text{PMN}) : \text{cpm} (\text{Candida})] \times 100$$

Nitro blue tetrazolium (NBT) reduction test. Oxidative pathway was evaluated by NBT reduction test after stimulation of the cells with opsonized *C. albicans*. In a tube, 2×10^6 PMN, 2×10^6 heat killed *Candida*, 0.2 ml plasma, and 0.2% NBT (Sigma, Deisenhofen, Germany) were mixed together. After 15 min incubation at 37°C a drop of this mixture was examined under microscope. The percentage of the cells containing reduced blue formazan was determined.

Table 2. Protocol for studying intracellular killing. Five sets of tubes, each in triplicate, were arranged for each sample. Each tube contains PMN cells, autologous or heterologous plasma, and live *Candida*.

	Patient PMN	Patient plasma	Normal PMN	Normal plasma	Live <i>Candida</i>
Set 1	+	+	-	-	-
Set 2	+	-	-	+	-
Set 3	-	-	+	+	+
Set 4	-	+	+	-	+
Set 5	+	+	+	+	+

Table 3. Comparison of phagocytic parameters between CML patients and normal controls. The data represent mean \pm SD. All P values are < 0.01 .

Groups	Intracellular killing (%)	NBT reduction (%)	Phagocytic index	Phagocytosis (%)
CML patients	60.9 \pm 16.6	72.8 \pm 11.0	2.1 \pm 0.4	82.7 \pm 9.0
Normal controls	73.3 \pm 13.0	88.2 \pm 5.9	2.5 \pm 0.4	92.3 \pm 4.4

Phagocytosis. Purified PMN from patients and controls were adjusted to $10 \times 10^6/\text{ml}$ in culture media. Cell suspension (1 ml) was distributed to each well of a 12- well migration plate (Nunc, Roskilde, Denmark) and each well covered with 22×22 mm cover slips. This plate was covered with another plate to support the cover slips. Then, the plates were gently turned upside down and incubated at 37°C for 1 h. The cover slips were then removed and gently washed with saline and then incubated at 37°C for 30 min with 10×10^6 live *C. albicans* in T.C 199 medium containing 10% either autologous or heterologous plasma. After the incubation, the cover slips were removed, washed, fixed with methanol and stained with Geimsa stain. The number of *C. albicans* ingested by 100 PMN, was determined microscopically and the mean of organisms per cell was calculated (phagocytic index); the percentage of PMN ingesting microorganisms was considered as the phagocytosis percent.

Statistical analysis. Data were analyzed statistically by student's t -test and Pearson correlation.

RESULTS

Intracellular killing test. Intracellular killing test was performed to assess microbicidal capacity of PMN to kill ingested microorganisms. As shown in Figure 1 and Table 3 the mean percentage of killing for CML patients was significantly lower than that of normal individuals ($P \leq 0.01$). There were significant differences in mean percentage of killing between normal controls and patients in chronic phase ($P = 0.04$) or blastic phase ($P \leq 0.01$). However, the difference in mean percentage of intracellular killing between patients in remission and normal controls was not significant (Table 4). Analysis of the results showed an inverse correlation between killing capacity and leukocyte count ($r = -0.33$, $P = 0.02$), and between killing capacity and the percentage of immature cells

($r = -0.33$, $P = 0.01$). There was no significant difference in microbicidal capacity of normal PMN incubated with either normal or patient plasma (data not shown).

NBT reduction test. This method allows measurement of the percentage of PMN producing respiratory burst. PMN from CML patients showed significantly lower NBT reduction than PMN from normal controls ($P \leq 0.001$), as demonstrated in Figure 1 and Table 3. The differences between normal donors and patients in remission ($P = 0.02$), and patients in chronic and acute phases ($P \leq 0.001$) were significant (Table 4). A negative correlation was found between NBT reduction and leukocyte count ($r = -0.4$, $P = 0.004$), and between NBT reduction and percentage of immature cells ($r = -0.3$, $P = 0.007$). There was positive correlation between NBT reduction and intracellular killing ($r = 0.5$, $P = 0.001$).

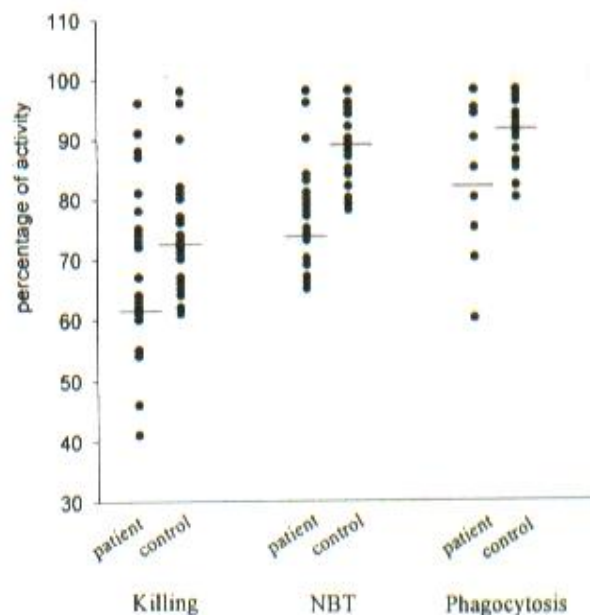


Fig. 1. Results of intracellular killing, NBT reduction, and phagocytosis for CML patients and normal controls. The means are indicated.

Table 4. Phagocytic parameters for CML patients in different phases of disease. Values represent the mean \pm SD.

Groups	Intracellular killing (%)	NBT reduction (%)	Phagocytic index	Phagocytosis (%)
Remission	70.4 \pm 9.2	81.6 \pm 10.3	2.0 \pm 0.1	83.3 \pm 12.6
Chronic phase	67.8 \pm 13.4	75.1 \pm 8.8	2.2 \pm 0.4	85.1 \pm 8.2
Blastic phase	59.0 \pm 12.5	69.8 \pm 4.8	1.9 \pm 0.0	75.7 \pm 8.4
Normal controls	73.3 \pm 13.0	88.2 \pm 5.9	2.5 \pm 0.4	92.3 \pm 4.4

Phagocytosis. The phagocytic capacity of PMN was assessed by measuring the ingestion of live yeasts. The data presented in Table 3 show that phagocytic index of PMN from patients was significantly decreased ($P \leq 0.01$); that is, each PMN of patients ingest less number of *Candida* than PMN from normal individuals. The differences between mean phagocytic index for normal controls and that for patients in chronic and acute phases were also significant ($P = 0.016$ and $P \leq 0.001$, respectively). However, there were not any significant differences between normal individuals and patients in remission (Table 4).

Likewise, the phagocytosis percent of CML patients was significantly lower than that of controls ($P \leq 0.01$) (Fig. 1, Table 3). There were significant differences between the mean percentage of phagocytosis for normal controls and for patients in remission ($P = 0.01$) and also for patients in chronic or acute phases ($P \leq 0.001$).

DISCUSSION

The aim of the present study was to clarify the function of peripheral blood PMN leukocytes in different phases of CML patients. There are some reports on phagocytic function of patients with CML. However, in most reports all understudied patients were in one of the phases of the disease. Considering different clinical and hematological status of patients in various phases of CML, study and comparison of neutrophil function in all three phases of the disease seem to be more informative. In this study, intracellular killing of *Candida albicans* and stimulated NBT reduction test were used to evaluate the microbicidal capacity of PMN. The results of both tests were significantly impaired in patients with CML. These results are in accordance with those of others that have reported impaired oxidative pathway in formyl-methionyl-leucyl-phenylalanine- and phorbol-myristate-acetate- activated neutrophils of patients [5, 6]. On the contrary, there are other studies reported normal

killing activity and production of oxygen metabolites by PMN of CML patients [7, 8]. In these studies, microorganisms other than *Candida* have been used and shown that different microorganisms have various degrees of sensitivity to several microbicidal mechanisms of granulocytes [11]. Therefore, the discrepancy in results may be due to the different microorganisms used for killing. Furthermore, in studies that reported normal phagocytic activity for CML patients, the understudied patients were in remission [5, 6, 12]; whereas, studies that reported decreased phagocytic activity included patients in chronic or blastic phases. In our study, patients who were in remission showed normal intracellular killing and NBT reduction, whereas the values of patients in chronic and blastic phases were impaired.

It has been suggested that during remission, granulocytes mainly derived from normal stem cells [13-15], although abnormal clone persists in bone marrow [16]. Therefore, mature granulocytes appear in peripheral blood of patients in remission show normal phagocytic function. Our results show microbicidal impairment is more severe in blastic phase than the chronic one.

There was negative correlation between microbicidal assays and leukocyte count or immature cells e.g., by increasing leukocyte count or percentage of immature cells, NBT reduction and intracellular killing would decrease. Lanza and Castoldi [17] found similar correlation between the expression of complement receptor type 1 (CR1) and the disease activity. They showed that patients who developed blastic crisis expressed lower levels of CR1 antigens. Likewise, more pronounced defective bactericidal function of PMN in the stage of blastic crisis of CML patients has been reported [18].

There was a positive correlation between NBT reduction test and intracellular killing ($r = -0.5$, $P = 0.001$) indicating that decreased microbicidal capacity of leukemic neutrophils could at least be partly related to the impairment of oxidative pathways.

Decreased phagocytic index of CML patients' neutrophils indicates that the ingestion capacity of each cell is diminished, and decreased phagocytic percentage shows that the number of neutrophils that can ingest *Candida* has been decreased. This impaired phagocytosis could be the result of the defect in cytoskeleton. It has been shown that the amount and polymerization of actin is significantly lower in CML neutrophils when compared with neutrophils from normal subjects [19, 20].

Absence of difference in function of normal PMN incubated with either normal or patient plasma indicates that there was not any phagocytic inhibitory factor in the plasma of CML patients.

In conclusion, the data presented in this study demonstrate that neutrophil function is different in various phases of CML. Phagocytic function is defective in chronic and acute phases and is almost normal in patients in remission. Furthermore, decreased neutrophil function is more prominent in patients in acute phase than in chronic phase. The results also indicate as leukocyte count and percent of immature cells increase, the function of PMN decreases.

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REFERENCES

- Enright, H. and McGlave, P.H. (2000) Chronic myelogenous leukemia. In: *Hematology Basic Principles and Practice*. (Hoffman, R., Benz, E.J., Shattil S.J., Furie, B., Cohen, H., Silberstem, L.E. & McGloave P.H., eds.), Churchill Livingstone, U.S.A., pp. 1155-1171.
- Cortes, J., Talpas, M., Beran, M., O'Brien, S.M., Rios, M.B., Stass, S. and Kantargian, H.M. (1995) Philadelphia chromosome-negative chronic myelogenous leukemia with rearrangement of the breakpoint cluster region. *Cancer* 75: 464-470.
- Brincker, H. (1982) Population-based age- and sex-specific incidence rates in the four main types of leukemia. *Scand. J. Haematol.* 29: 241-249.
- Athens, J.W. (1993) Chronic Myeloid Leukemia. In: *Wintrobe's Clinical Hematology* (Lee, G.R., Bithell, T.C., Foerster, J., Athens, J.W. & Lukens, J.N., eds.) Lea & Febiger, U.S.A., pp. 1969-1998.
- Ghavami Nejad, M. and Zandieh, T. (1988) Inhibition of ^{14}C uptake by *Candida albicans*. A new method. *Med. J. IRI.* 2: 237-242.
- Yamamura, M., Boler, J. and Valdimarsson, H. (1977) Phagocytosis measured as inhibition of uridine uptake by *Candida albicans*. *J. Immunol. methods* 14: 19-24.
- Wakita, H, Asai, T., Oh, H., Itoh, K. and Yoshida, S. (1993) chemoluminescence of neutrophils in patients with myeloproliferative or myelodysplastic hematologic diseases relation to neutrophil alkaline phosphatase activity. *Int. Med.* 32: 204-209.
- Kaplan, S.S., Berkow, R.L., Joyce, R.A., Basford, R.E. and Barton, J.C. (1992) Neutrophilic leukemia: defective respiratory burst in response to phorbol esters. *Acta Haematol.* 87: 16-21.
- Samuelsson, J., Forslid, J., Hed, J. and Palmblad, J. (1994) Studies of neutrophil and monocyte oxidative responses in polycythemia vera and related myeloproliferative disorders. *Br. J. Haematol.* 87: 464-470.
- Wolach, B., Gavrieli, R., Manor, Y. and Lishner, M. (1998) Leukocyte function in chronic myeloproliferative disorders. *Blood Cells Mol. Dis.* 24: 544-551.
- Olofsson, T. and Odeberg, H. (1976) Granulocyte function in chronic granulocytic leukemia II. Bactericidal capacity, phagocytic rate, oxygen consumption and granule protein composition in isolated granulocytes. *Blood* 40: 581-592.
- Tsurumi, H., Shimazaki, M., Takahashi, T., Moriawaki, H. and Muto, Y. (1993) Flow cytometric determination of active oxygen (hydrogen peroxide) produced by peripheral blood neutrophils in patients with hematological disorders. *Acta Haematol.* 57: 213-219.
- Bernstien, R. (1988) Cytogenetics of chronic myeloid leukemia. *Sem. Hematol.* 25: 20-34.
- Wysocky, H., Wierusz-Wysocka, B., Siekierka, H., Szczepanic, A., Klimas, R. and Wykretowicz, A. (1988) Polymorphonuclear neutrophils function in untreated patients with chronic myeloid leukemia. *Oncol.* 45: 79-83.
- Naik, N.R., Adavani, S.H. and Bhisey, A.N. (1989) PMN cells from chronic myeloid leukemia patients show defective chemotaxis in remission. *Leuk. Res.* 13: 959-965.
- Talpaz, M., Kantarjian, H.M., Kurzrock, R. and Gutterman, J. (1988) Therapy of CML: chemotherapy and interferons. *Sem. Hematol.* 25: 62-73.
- Lanza, F. and Castoldi, G. (1992) Complement receptor 1 expression in chronic myeloid leukemia. *Leuk. Lymphoma* 8: 35-41.
- Romanova, A.F., Fedorovskaia, E.A. and Pesotskaia, L.A. (1991) The immunological indices of patients with chronic myeloid leukemia at the blast crisis stage. *Vrach Delo* 4: 65-67.
- Tarachandani, A., Advani, S.H. and Bhisey, A.N. (1993) Chronic myeloid leukemia granulocytes have

- lower amounts of cytoplasmic actin. *Leuk. Res.* 17: 833-838.
20. Radhika, V., Naik, N.R., Advani, S.H. and Bhisey, A.N. (2000) Actin polymerization in response to different chemoattractants is reduced in granulocytes from chronic myeloid leukemia patients. *Cytometry* 42: 379-386.