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DETERMINATION OF THE OPTIMAL CHEMOTHERAPY DRUGS PRETREATMENT TIME THROUGH CULTIVATION OF HEMOPOIETIC CELLS IN CML-PATIENTS TREATED WITH TYROSINE KINASE INHIBITORS

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Background: **Targeted therapy drugs, including imatinib, are used for inhibiting the marker oncoprotein of chronic myeloid leukemia — BCR-ABL tyrosine kinase. However, in some patients the drug resistance can emerge too rapidly and a previous treatment with chemotherapy drugs can lead to formation of resistance.** ***Aim:*** **To evaluate the influence of drugs that were used prior to the imatinib on the performance of the functional activity of bone marrow cells from chronic myeloid leukemia patients and their individual responses to therapy.** ***Methods*****: Bone marrow aspirate from 57 patients, who were getting busulfan (19 patients) or hydroxycarbamide (38 patients) prior to imatinib was studied with cytogenetic and tissue culture methods *in vitro*.** ***Results*****: Obtained data suggested that pretreatment with busulfan, regardless of duration, negatively affects the response to further therapy with imatinib. Instead, after using hydroxycarbamide as a previous therapy for six month, there was optimal response to imatinib. In those cases when duration of pretreatment with hydroxycarbamide was increased to a year or more, there was a suboptimal response and a resistance to imatinib therapy. In addition, there was a positive correlation between the number of cell aggregates (colonies and clusters) in semisolid agar and the duration of a prior treatment with hydroxycarbamide, if previous therapy did not exceed 20 months. With an increase of pretreatment terms to 21 months or more, such a correlation was not observed.** ***Conclusions*****: These results suggest that chemotherapeutic agents (busulfan and hydroxycarbamide) may additionally contribute to the accumulation of mutations in the genome of leukemic cell clone affecting the behavior of these cells *in vitro*.**

Key Words**: chronic myeloid leukemia, imatinib, pretreatment, hydroxycarbamide, busulfan, cell culture *in vitro*.**

In recent years, tyrosine kinase inhibitors (TKI) such as imatinib, are used as effective therapeutic agents in chronic myeloid leukemia (CML). The action mechanism of this drug is based on the inhibition of the BCR-ABL tyrosine kinase, which is a product of the oncogene with a same name, formed as a result of reciprocal translocation between chromosomes 9 and 22 [1, 2]. Chromosome 22 with a shortened long arm is called Philadelphia chromosome, or Ph chromosome. This is a cytogenetic marker of CML [3].

However, the experience of imatinib monotherapy usage showed that about 25% of patients develop resistance after 12 months [4]. Most researchers believe that the most common causes of such resistance are point mutations in the gene BCR-ABL [2–4]. Moreover, the cause of resistance to therapy can also be attributed to amplification or increase of gene BCR-ABL expression, an additional chromosomal aberrations and expression of multiple drugs resistance proteins [3–5].

Center of Supervision for Cancer Patients Ontario developed a treatment program for patients with CML using TKI, which indicates that imatinib should be used as first-line therapy, or it can be used in those cases where patients have resistance to chemotherapeutic drugs (hydroxycarbamide, interferon, etc.) [6]. Furthermore, in everyday medical practice in Ukraine a large numbers of patients with CML were treated with chemotherapeutic agents, such as busulfan and hydroxycarbamide before the TKI therapy. The chemical nature of busulfan is derived from of disulfuronic acid with alkyl action that has a high tropism for DNA of myeloid progenitors of hematopoiesis [7]. The use of busulfan can reduce the size of leukemic clone, but it continues to dominate in hematopoiesis of the patient, and even during complete hematologic remission in all cells of bone marrow of patients the presence of the Philadelphia chromosome could be indicated [8]. Hydroxycarbamide is an inhibitor of ribonuclease. It is an enzyme that is essential for DNA synthesis. The drug can improve the survival rate of patients with CML by lengthening the chronic phase of the disease [9]. However, given the fact that the cells of leukemic clone are characterized by genomic instability, the use of chemotherapy drugs serves as an additional factor contributing to the selective accumulation of mutations, their application prior to imatinib therapy may accelerate the process of resistance development of leukemic cell clone [10, 11]. However, the question of influence of treatment duration with prior chemotherapeutic drugs on the formation of the resistance of bone marrow cells in CML to imatinib therapy still remains unresolved.

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Abbreviations used: CFU-GM – colony-forming unit-granulocyte/macrophage; CIFU – cluster-forming unit; CML – chronic myeloid leukemia; PP – proliferative potential; TKI – tyrosine kinase inhibitors.

The aim of this work was to evaluate the influence of drugs that were used prior to the imatinib on the performance of the functional activity of bone marrow cells from CML patients and their individual responses to therapy based on the evaluation of *in vitro* functional activity of the hematopoietic cells.

MATHERIALS AND METHODS

228 bone marrow cultures from 57 patients who were taking busulfan and hydroxycarbamide in standard doses and standard scheme before treatment with imatinib (400 mg/day) were analyzed. All patients before the study signed a voluntary informed consent. All patient samples were obtained under Research Ethics Board. Wide variation in the duration of the aforementioned chemotherapeutic drugs prior to imatinib therapy made the direct assessment of this factor impact on the performance of the functional activity of bone marrow patients more complicated. Therefore, patients were divided into three groups, depending on the response to imatinib therapy: those with optimal response to imatinib (the bone marrow of patients had no cells containing the Philadelphia chromosome after 12 months of therapy), patients with suboptimal response (the bone marrow showed 35% of cells containing the Philadelphia chromosome after 12-month treatment), patients with resistance (after 12 months of treatment the bone marrow had more than 35% of cells containing the Philadelphia chromosome).

In order to observe the patient's response to imatinib therapy, we performed a cytogenetic analysis of bone marrow cells of patients with the direct method and using cell culture CFU-GM assay with even and G-differential staining, detecting the percentage of cells containing the Philadelphia chromosome.

Determination of the functional activity of bone marrow cells was done by culturing *in vitro* in semisolid agar with the addition of 20% fetal bone serum (Sigma, USA), 0.33% bacterial agar ("Difco", USA), 50 ng/ml GM-CSF (Sigma, USA), and antibiotics (50 IU/ml penicillin, 50 mg/ml streptomycin). Cultivation lasted for 14 days under conditions of absolute humidity and 5% CO_2. After the end of the cultivation the numbers of colonies and clusters were counted under an inverted microscope (Nikon, Japan).

For cluster (cluster-forming unit — CIFU) we took a cell aggregate, which included more than 40 cells (Fig. 1). A typical colony (colony-forming unit-granulocyte/macrophage — CFU-GM) included 40 to several hundred cells. Proliferative potential (PP) was calculated as a ratio between granulocyte-macrophage colonies and clusters.

Statistical analysis of the results was carried out using Mann — Whitney nonparametric comparison. Conclusion of the statistical significance of the results made at $p < 0.05$. For estimation of interrelations between obtained indexes, the correlational analysis with determination of Spearman's rank correlation coefficient has been conducted.

RESULTS AND DISCUSSION

The fact that previous therapy with cytotoxic drugs has a negative impact on overall survival of patients with CML [12], gives us an idea to investigate hematopoietic stem cells and their precursors in cell culture *in vitro* as a criterion for assessing the impact of chemotherapeutic agents on subsequent functional activity of bone marrow cells with CML and response to therapy TKI. For example, in previous studies it has been shown that the characteristics of the functional activity of hematopoietic cells in CML have predictive value for the likelihood of obtaining remission or progression of the disease [13, 14]. We used this phenomenon to study the impact of busulfan and hydroxycarbamide on leukemic clone cell sensitivity to further imatinib therapy.

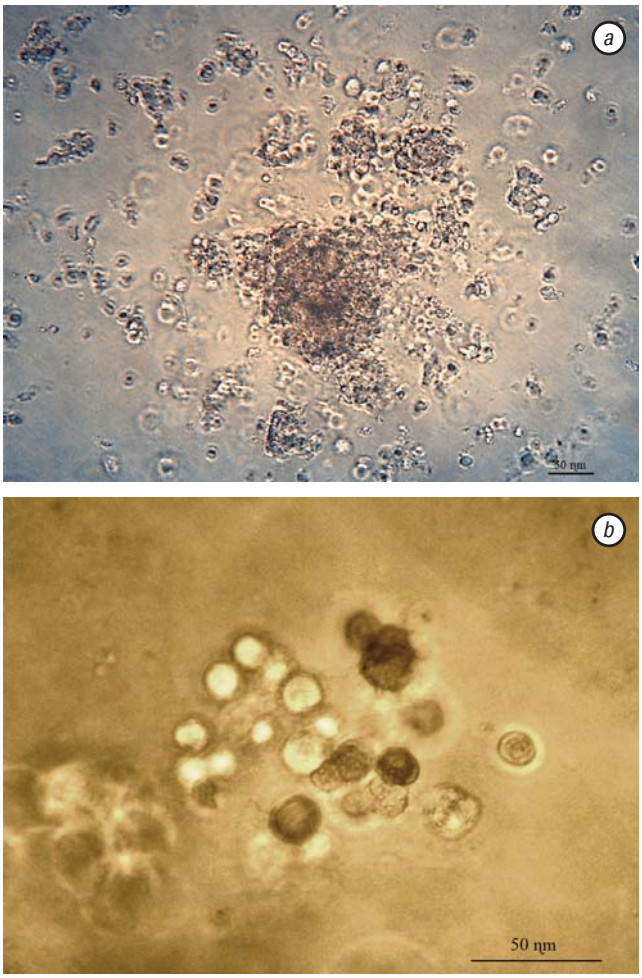

Fig. 1. Types of bone marrow cell aggregates on day 14 of cultivation in semisolid agar *in vitro*: *a* — colony, *b* — cluster

Based on the experimental data it was shown that patients, who received busulfan as basic therapy, regardless of the number of months of the admission, showed no optimal response to further therapy with imatinib that resulted in high PP. In addition, there was no statistically significant difference between the numbers of colonies and clusters in the two groups. Thus, patients with suboptimal response to imatinib therapy had proliferative capacity index of 1.92 ± 0.2 and the number of colonies and clusters reached 125.0 ± 7.3 and 51.0 ± 3.2 on $1 \cdot 10^5$ explanted mononuclear cells respectively. In turn, patients, who were characterized by the lack of response to imatinib therapy, had a PP index of 3.21 ± 0.1, the number of colonies and clusters in the cell culture

in vitro was 188.0 ± 5.8 and 27.0 ± 4.5 for $1 \cdot 10^5$ explanted cells respectively (Table 1).

Table 1. Characteristics of the functional activity of bone marrow cells of patients with busulfan prior therapy

Response	The number of colonies	The number of clusters	The number of Ph^+	PP
Optimal response	–	–	–	–
Suboptimal response	125.0 ± 7.3	51.0 ± 3.2	45.0 ± 0.4	1.92 ± 0.2
Resistance	188.0 ± 5.8	27.0 ± 4.5	81.0 ± 1.1	3.21 ± 0.1

These results may indicate that administering busulfan as basic therapy increases the risk of mutations in the cells of leukemic clone because of its affinity to the cell DNA. This is the reason for the formation "cross-linking" in the DNA molecules [15], which leads to genomic instability and development of the resistance to subsequent therapy with TKI group, in particular, to the imatinib.

Minimum term of hydroxycarbamide usage in a studied cohort of patients before imatinib therapy ranged from 4 to the maximum of 90 months. But in patients with an optimal response to imatinib therapy, the average hydroxycarbamide pretreatment term was 6.5 ± 2.1 months. In turn, patients who had a suboptimal response to therapy with hydroxycarbamide was 12.0 ± 0.4 months and for patients with acquired resistance the pretreatment, this term reached 22.0 ± 3.6 months; there were also 2 patients, who received imatinib therapy with hydroxycarbamide for 54 and 90 months. Such long pretreatment had a negative effect on the formation of response to imatinib, because these patients are characterized by initial resistance to the drug.

When comparing the functional activity of bone marrow cells of patients, who were taking imatinib therapy before hydroxycarbamide, it was observed that increase in the duration of the hydroxycarbamide therapy leads to the increased functional activity of bone marrow cells of patients. Thus, in the group of patients, who were taking hydroxycarbamide for 6.5 ± 2.1 months, the numbers of colonies in semisolid agar *in vitro* was 16.1 ± 5.5 CFU per $1 \cdot 10^5$ explanted mononuclear cells. In patients with doubled period of hydroxycarbamide pretreatment, the numbers of colonies was 4.8 times higher than in patients, who received hydroxycarbamide for 6.5 ± 2.1 months and was 78.0 ± 2.2 CFU per $1 \cdot 10^5$ explanted cells. With increasing duration of hydroxycarbamide pretreatment up to 22.0 ± 3.6 months, the number of CFU reached 82.0 ± 1.2 on $1 \cdot 10^5$ explanted cells, indicating a disproportional increase in the numbers of cloned cells with unlimited PP, which are able to form colonies in semisolid agar, depending on the hydroxycarbamide pretreatment duration [16].

Numbers of clusters in culture *in vitro* for different groups of patients changed similarly to changes in the numbers of colonies in semisolid medium. Thus, in patients with an optimal response to imatinib therapy the number of clusters was 56.0 ± 9.0 CIFU $1 \cdot 10^5$ explanted cells and in patients with suboptimal response to the drug the number of clusters was 2.3 times higher, reaching 130.0 ± 5.6 CIFU $1 \cdot 10^5$ explanted mononuclears. In the group of patients with acquired resistance, where hydroxycarbamide pretreatment term was about 22.0 ± 3.6 months, the CIFU number in culture was 1.5 times higher and reached 198.0 ± 1.3 to $1 \cdot 10^5$ explanted cells, compared to patients whose hydroxycarbamide pretreatment term was about 12.0 ± 0.4 months (Table 2).

Table 2. Characteristics of the functional activity of bone marrow cells of patients with CML with hydroxycarbamide therapy before using imatinib

Response	Duration, months	The number of colonies	The number of clusters	The number of Ph^+	PP
Optimal response	6.5 ± 2.1	16.1 ± 5.5	56.0 ± 9.0	0	0.39 ± 0.1
Suboptimal response	12.0 ± 0.4	78.0 ± 2.2	130.0 ± 5.6	18.0 ± 0.8	1.23 ± 0.1
Resistance	22.0 ± 3.6	82.0 ± 1.2	198.0 ± 1.3	72.0 ± 7.1	1.34 ± 0.2

We were interested in the fact what hydroxycarbamide pretreatment duration resulted in improved survival of the CML patients. The question was focused on the identification of the pretreatment duration period with hydroxycarbamide that in patients would show optimal response to TKI therapy without any kind of suboptimal response and no acquisition of resistance to the drug. In previous studies it was found that the value of PP in bone marrow cells, when cultured in semisolid agar *in vitro*, has a prognostic value for the imatinib treatment [17]. If the value after six months of therapy is lower than PP 1 — there is an optimal response to imatinib therapy; if the value is higher than the PP 1 — there is a suboptimal response to therapy with the threat of the drug resistance [17]. Therefore, we determined the PP of different patients groups and found that patients with an optimal response to imatinib therapy the PP value was 0.39 ± 0.1 and in patients with suboptimal response to therapy and with resistance to imatinib the value of PP was 1.23 ± 0.1 and 1.34 ± 0.2. Thus, based on the value of bone marrow cells PP among different groups of patients, an optimal period to use hydroxycarbamide as a base treatment of CML before applying the imatinib was 6.5 ± 2.1 months. In case of extension of previous hydroxycarbamide treatment the risk of adverse impact on the formation of leukemic cell clone response to further imatinib therapy increases.

In bone marrow samples of patients treated with hydroxycarbamide there is a correlation (R = 0.85331) between indicators of colony forming activity and the duration of pretreatment with hydroxycarbamide in period between 1 and 20 months (Fig. 2). Patients, who had a hydroxycarbamide pretreatment for 21 months or more, did not show such correlation. In addition, there was also a correlation (R = 0.8744) between the indices of cluster forming activity and the duration of hydroxycarbamide pretreatment in bone marrow samples of patients taking the drug in the 1–20 months time interval. Patients with hydroxycarbamide pretreatment duration more than 20 months did not show such correlation, same as in the case of colony forming activity. However, between the value of PP bone marrow cells of patients and hydroxycarbamide pretreatment duration the correlation was found (R = 0.2143).

Given the fact that in previous studies, we have demonstrated a direct relationship between the translocation t(9,22)(q4;q11) in leukemic progenitor cells of the bone marrow and their functional activity [18], we can assume that with increasing duration of hydroxycarbamide treatment, the leukemic clone cells genome accumulates mutations that can affect the sensitivity of BCR-ABL tyrosine kinase to imatinib therapy. This can directly affect the functional activity of both cells with unlimited PP in semisolid agar, which are able to form colonies and more differentiated leukemic cells clone with limited PP in semisolid agar, which are able to form clusters [16].

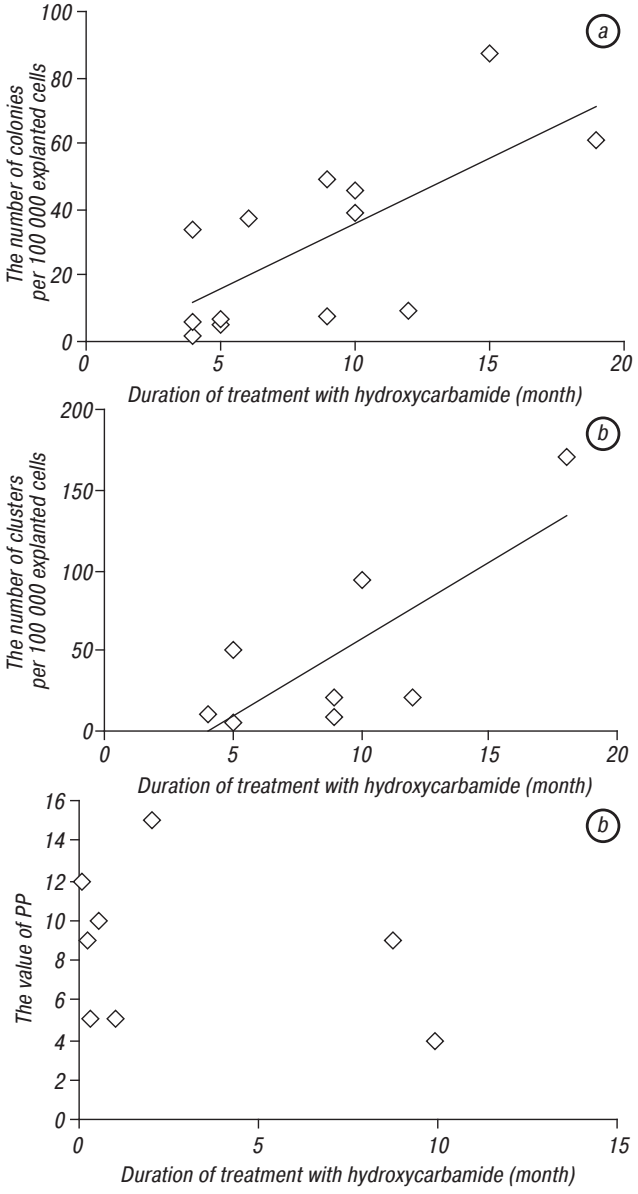

Fig. 2. Correlation analysis colony forming activity of bone marrow cells from CML patients and the duration of pretreatment with hydroxycarbamide: *a* — the number of colonies, *b* — the number of clusters, *c* — PP

However, some authors hold on to the idea that one of the significant causes of mutations in the genome of leukemic cells is increased activity of BCR-ABL oncoprotein [19]. It is believed that the BCR-ABL tyrosine kinase is able to induce genomic instability through the involvement of several mechanisms, one of which is oxidative stress. Reactive oxygen species, produced as a result of increased activity of the BCR-ABL tyrosine kinase mutations can cause mutations even in the BCR-ABL gene. That is why the duration of BCR-ABL oncoprotein activity exposure on genes in leukemic cell clones is important. In fact, with the increasing duration after the formation of the translocation t(9,22)(q4;q11) and fusion of BCR-ABL tyrosine kinase, the possibility of mutations in the BCR-ABL gene and additional chromosomal translocations raises, that can be the reason for the insensitivity of a particular cells to imatinib therapy. The use of additional stress factors, such as chemotherapeutic agents (busulfan and hydroxycarbamide) may additionally contribute to the accumulation of mutations in the genome of leukemic cell clone.

In conclusion, we have found a relationship between the increasing duration of chemotherapeutic drugs pretreatment and indicators of functional activity of bone marrow cells of patients with CML. In particular, the use of busulfan as a preliminary treatment agent before using TKI is objectionable due to the fact that regardless of the number of months of its admission, patients showed the lack of optimal response to subsequent imatinib therapy and high PP. In addition, it was found that prolonged treatment with hydroxycarbamide (over 6 months) promotes resistance to TKI. In case of extension of previous treatment with hydroxycarbamide the risk of adverse impact on the formation of leukemic cell clone response to further imatinib therapy increases. In addition, there was a positive correlation between the numbers of clusters and colonies and the duration of previous hydroxycarbamide treatment between 1 and 20 months. Patients, who were getting hydroxycarbamide pretreatment for 21 months or more, did not have such correlation.

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