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Role of P-Glycoprotein in Evolution of Populations of Chronic Myeloid Leukemia Cells Treated with Imatinib

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Abstract—Imatinib mesylate (imatinib) is a new generation preparation that is now successfully used for treatment of cancer, particularly for chemotherapy of chronic myeloid leukemia (CML). Imatinib inhibits the activity of chimeric kinase BCR-ABL, which is responsible for the development of CML. The goal of this study was to investigate the role of a multidrug resistance protein, P-glycoprotein (Pgp), in the evolution of CML treated with imatinib. We demonstrate here that although imatinib is a substrate for Pgp, cultured CML cells (strain K562/i-S9), overexpressing active Pgp, do not exhibit imatinib resistance. Studies of CML patients in the accelerated phase have shown variations in the number of Pgp-positive cells (Pgp+) among individual patients treated with imatinib. During treatment of patients with imatinib for 6-12 months, the number of Pgp-positive cells significantly increased in most patients. The high number of Pgp+ cells remained in patients at least for 4.5 years and correlated with active Rhodamine 123 (Rh123) efflux. Such correlation was not found in the group of imatinib-resistant patients examined 35-60 months after onset of imatinib therapy: cells from the imatinib-resistant patients exhibited efficient Rh123 efflux irrespectively of Pgp expression. We also compared the mode of Rh123 efflux by cells from CML patients who underwent imatinib treatment for 6-24 months and the responsiveness of patients to this therapy. There were significant differences in survival of patients depending on the absence or the presence of Rh123 efflux. In addition to Pgp, patients' cells expressed other transport proteins of the ABC family. Our data suggest that treatment with imatinib causes selection of leukemic stem cells characterized by expression of Pgp and other ABC transporters.

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Key words: P-glycoprotein, chronic myeloid leukemia, imatinib, tyrosine kinase inhibitor, BCR-ABL

Imatinib (imatinib mesylate, STI 571, Gleevec) is currently successfully used for treatment of malignant tumors, particularly for chemotherapy of chronic myeloid leukemia (CML) [1, 2]. CML appears due to reciprocal chromosome translocation t(9;22), which originates from somatic mutation in hemopoietic stem cell and is cytologically expressed in formation of a shortened chromosome 22 (also known as Philadelphia chromosome or Ph chromosome). This translocation results in formation of a chimeric gene, *BCR-ABL*, which often encodes the protein $p210^{BCR-ABL}$, and rarely encodes chimeric proteins of 190 or 230 kD (in dependence on the breakpoint within the *BCR* gene) exhibiting increased tyrosine kinase activity [3]. Imatinib inhibits activity of BCR-ABL kinase and causes death of BCR-ABL+ cells. In more than 80% of CML patients in the chronic phase, treatment with imatinib caused disappearance of Ph+ cells (carrying Ph chromosome) known as complete cytogenetic response (CCR) [4]. Although in most patients this effect is stable during this treatment, nevertheless resistance to imatinib may be induced especially in patients in the accelerated phase (AP) or in CML blast crisis. One possible reason underlying the appearance imatinib resistance is the increased activity of proteins that belong to the ABC (ATP-binding cassette) superfamily [5-7]. Proteins of the ABC family bind ATP and

Abbreviations: AB) antibodies; AP) accelerated phase; CCR) complete cytogenetic response; CML) chronic myeloid leukemia; CR) cytogenetic response; MDR) multidrug resistance; Pgp) P-glycoprotein; Rh123) Rhodamine 123.

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use its energy for translocation of various substances through cell membranes. Proteins of this family (including those involved in drug resistance of tumors) are involved in important normal physiological functions [7, 8]. The increase in expression and activity of P-glycoprotein (ABCB1 or Pgp), a member of the ABC family, was the first identified mechanism determining multidrug resistance (MDR) of tumors [9]. MDR is a mechanism of cell protection against many drugs differing in chemical structure and mechanism of action (taxanes, anthracyclines, vinca alkaloids, podophylotoxins and camptotecines, etc.) [10]. Cultured cells exhibiting Pgp-mediated MDR are often characterized by simultaneous overexpression of several ABC transporters [11].

During recent years, the active role of Pgp in resistance to imatinib has been actively investigated. There is evidence that active Pgp may cause cell resistance to the imatinib effect. First, Pgp binds imatinib [12]. Second, a subline of cultivated CML cells (K562 strain) characterized by the gradual increase in Pgp expression demonstrated corresponding decrease in intracellular accumulation of imatinib [13]. Third, it has been found that inhibition of Pgp decreases the level of cell resistance to imatinib [14, 15]. Insertion of the *MDR1* gene into cultivated CML cells (strain AR230) caused appearance of imatinib resistance [16]. However, opposite results have also been obtained. For example, K562 cells transfected with *MDR1* gene did not acquire imatinib resistance [17, 18]. Increased Pgp expression by mouse hemopoietic stem cells did not protect against the imatinib effect [18]. Conflicting experimental protocols may be responsible for some of these discrepancies.

In this study, we have investigated whether Pgp not always protects CML cells against imatinib; we also studied changes in the expression and functional activity of Pgp during treatment of patients in AP-CML with imatinib in order to investigate the effect of imatinib on reproduction of cell clones expressing functionally active Pgp *in vivo*.

MATERIALS AND METHODS

Cell cultivation. K562 cells and their resistant subline K562/i-S9 were cultivated using RPMI medium supplemented with 10% FBS and 50 μg/ml gentamicin. For antibody (AB) staining the cells were washed to remove cultivation medium, fixed, and treated with antibody following the protocol for staining of blood cells.

Clinical material. Patients with AP-CML were treated with imatinib at the Hematological Research Center of the Russian Academy of Medical Sciences from February 2001 to August 2005. Criteria for determination of AP-CML are given in [19]. All patients underwent karyotyping before treatment. The patients received 600 mg imatinib per day. Most patients were also pretreated (before the beginning of imatinib therapy) with other preparations employed for CML (interferon-α, hydroxyurea, cytarabine, and various courses of chemotherapy).

Immunocytochemical analysis of MDR proteins. The blood lymphocyte fraction was isolated using 1% gelatin. Remaining erythrocytes were lysed using the lysing buffer (Becton Dickinson, USA). The isolated lymphocytes were washed with phosphate buffer, pH 7.4. The following monoclonal antibodies (AB) (CHEMICON, USA) were used in the study: UIC-2 (anti MDR1), MAB 4126 (anti LRP), MAB 4146 (anti BCRP), and MAB 4122 (anti MRP). Conjugation with UIC-2 antibodies was carried out using non-fixed cells. In the case of staining with AB to LRP, BCRP, and MRP, cells were fixed in 4% paraformaldehyde in phosphate buffer for 20 min and then with 0.2% Triton X-100 in phosphate buffer for 20 min. These pretreated cells were then used for subsequent conjugation with AB according to the method described earlier [11]. Fluorescent intensity of cells was analyzed using a Becton Dickinson FACScan cytofluorimeter. Results were analyzed using CellQuest software.

UIC2-shift assay. For detection of antibody binding to functional Pgp, cells were pretreated (before conjugation) with 50 μM vinblastine or imatinib and then incubated with AB following the standard method.

Evaluation of functional activity of Pgp. Functional activity of Pgp was evaluated by the method described earlier [20]. Fluorescence in cells stained with Rh123 was evaluated using the cytofluorimeter. For evaluation of fraction of Rh123+ cells, we used the MAF coefficient [21], which was calculated using the following formula:

$$\mathrm{MAF} = \frac{\mathrm{Mean\ (inh)} - \mathrm{Mean\ (free)}}{\mathrm{Mean\ (inh)}},$$

where MAF is MDR Activity Functional, Mean(free) is the mean value of cell fluorescence without inhibitor, Mean(inh) is the mean value for cell fluorescence with inhibitor. Cells were defined as Rh123+ cells at MAF > 0.1.

RNA isolation and RT-PCR. Total RNA was isolated from K562 and K562/i-S9 cells using TRI Reagent (Sigma, USA) following the supplier's recommendations. The reverse transcription reaction was carried out using random primers. The housekeeping gene encoding β-actin was used for normalization of cDNA in the amplified samples. Primers and amplification conditions have been described earlier [11].

Determination of sensitivity of K562 and K562/i-S9 cells by the MTT method. Cells were seeded into 96 well plates (10,000 cells/well in 100 μl of medium). Imatinib or vinblastine was added in the range of concentrations from 0.01 to 5 μM. After incubation of cells with the cytostatics for 72 h, they were then incubated with MTT (5 mg/ml), washed (to remove medium), and lysed with

dimethylsulfoxide. The results of the MTT assay were read using a plate photocolorimeter by evaluating the amount of formazan formed. The LD_{50} value (concentration of a substance causing 50% cell death) was graphically based on results of the MTT reaction.

RESULTS

Effect of imatinib on expression and functional activity of P-glycoprotein in cultured K562 cells. We used cultures of K562 cells (isolated from a patient with CML blast crisis) and also K562/i-S9 cells. The K562/i-S9 cell line was obtained by *MDR1* gene transfection into K562 cells followed by separation of cells expressing Pgp using cytofluorimetry (without using cytostatics) [22]. Figure 1 shows that K562/i-S9 cells contained increased amounts of *MDR1* mRNA compared with the parent variant and expressed increased Pgp content. Besides increased content of *MDR1* mRNA and Pgp, these K562/i-S9 cells were characterized by higher levels of mRNA and corresponding proteins of MRP1, BCRP, and LRP compared with the parent cells (Fig. 1).

K562/i-S9 cells were characterized by active efflux of Rh123; this suggests the presence of functional Pgp in these cells (Fig. 2, a and b). Imatinib inhibited Rh123 efflux from K562/i-S9; this effect was comparable to that of verapamil and vinblastine, the known substrates of Pgp (Fig. 2, c-e). This suggests that Pgp expressed by K562/i-S9 recognizes imatinib as well as some other of its substrates, and imatinib competes with other Pgp substrates for binding to this protein. For experimental validation of this suggestion, we used the UIC-2-shift assay [22, 23]. The UIC-2 monoclonal AB recognizes the Pgp confor-

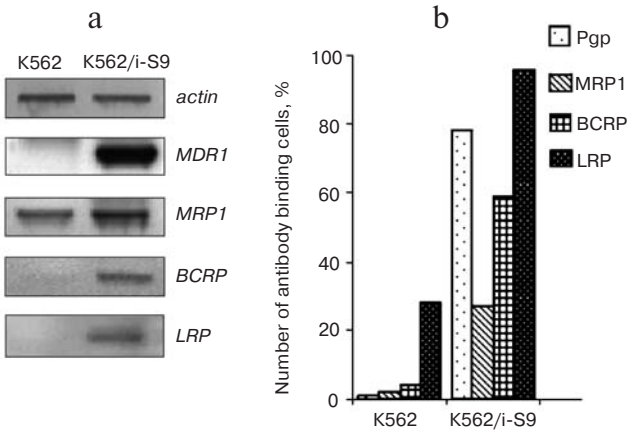

Fig. 1. Comparison of content of MDR mRNA (a) and proteins (b) in K562 and K562/i-S9. The content of mRNA of *MDR1*, *MRP1*, *BCRP*, *LRP* (and *actin* as a reference gene) was determined by RT-PCR; percent of cells with MDR proteins was determined by the cytofluorimetry after staining with corresponding antibodies.

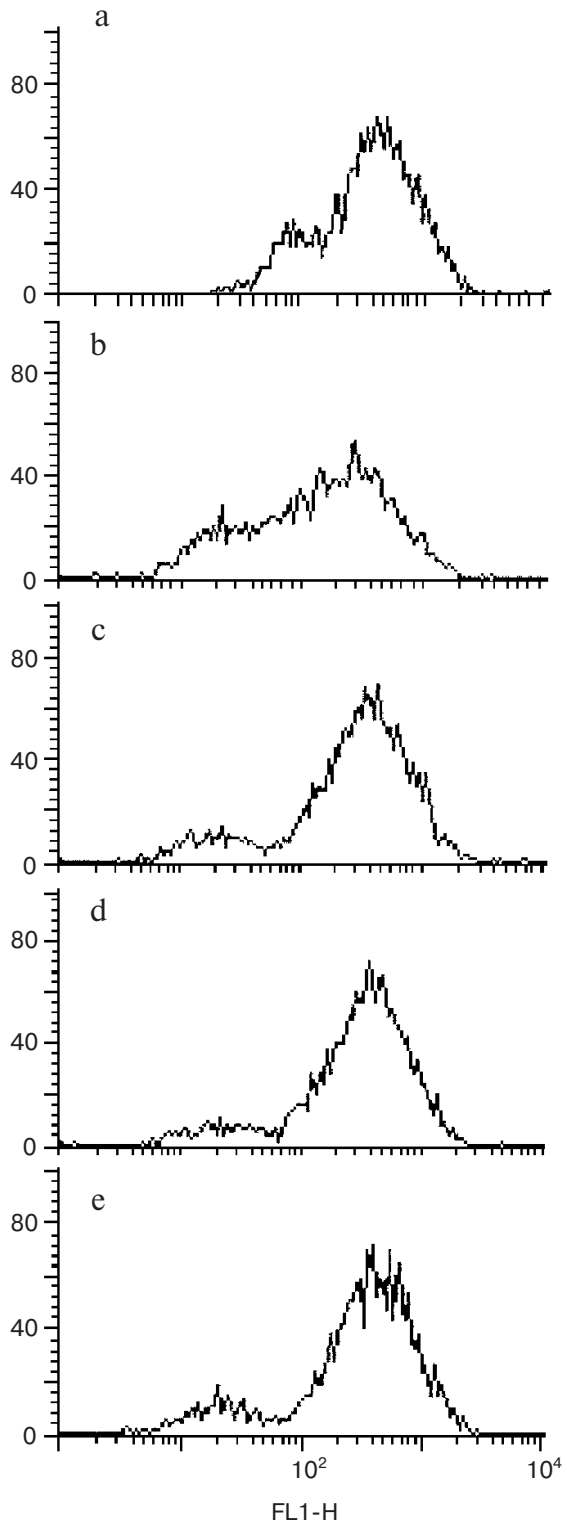

Fig. 2. Effects of verapamil (50 μM), imatinib (50 μM), and vinblastine (50 μM) on Rh123 efflux by K562/i-S9 cells. Histogram of distribution of fluorescent cells after incubation with Rh123 (a) and subsequent incubation in Rh123 free medium for 20 min (b) and wash with verapamil (c), imatinib (STI 571) (d), and vinblastine (e) for 20 min. The abscissa shows fluorescence intensity of cells, the ordinate shows the number of cells with a certain level of fluorescence.

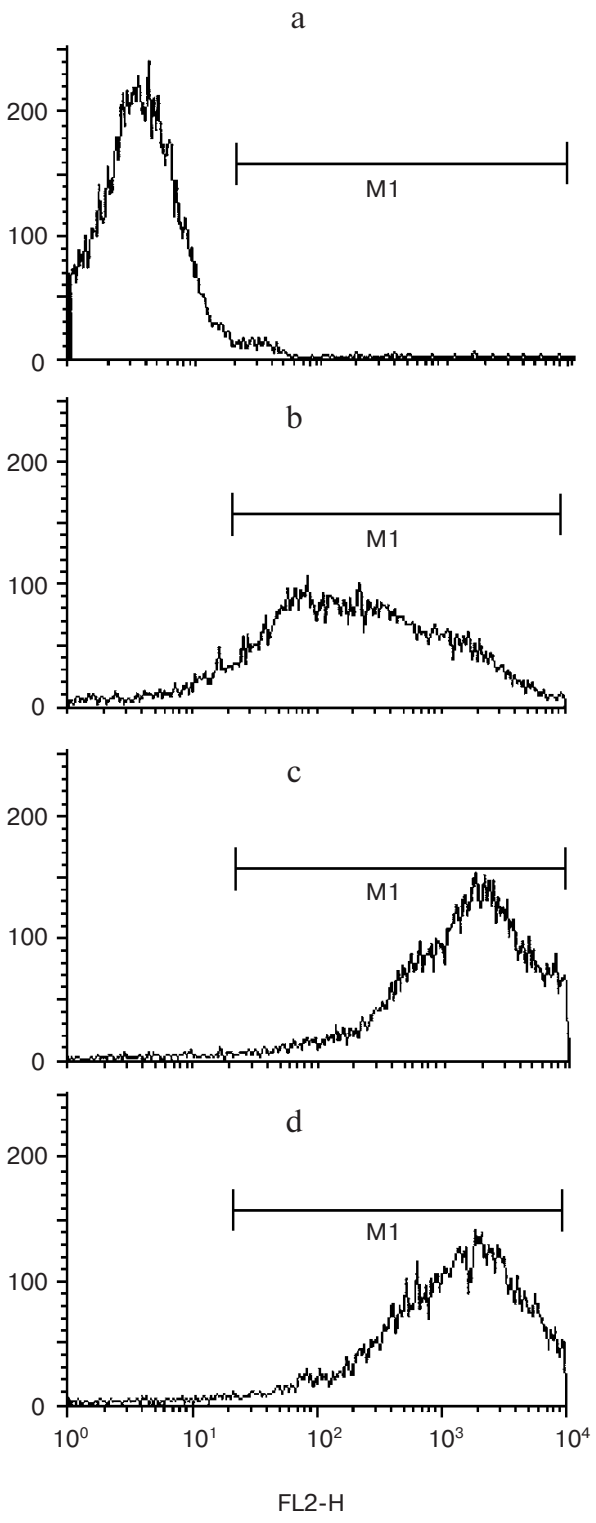

Fig. 3. UIC2-shift assay in K562/i-S9 cells induced by vinblastine (50 μM) and imatinib (50 μM). Binding of UIC-2 antibody to Pgp by K562/i-S9 cells under standard conditions (b), after preincubation of cells with vinblastine (c), or with imatinib (STI 571) (d). An irrelevant antibody of the same isotype (a) served as control. The marker M1 shows the histogram regions with UIC-2 positive cells.

mation, which appears during ATP hydrolysis when Pgp binds its substrate. The method can simultaneously determine Pgp content and its functional activity. Figure 3a shows distribution of cells bound to control isotypic antibodies. Figure 3b shows that more than 90% of K562/i-S9 cells are UIC-2 positive; the "flattened" shape of the plot suggests heterogeneity of population of K562/i-S9 cells: there are cells with higher and lower levels of Pgp expression. Pretreatment of cells with Pgp substrates (e.g. vinblastine) significantly increased amount of Pgp per cell recognized by subsequent conjugation of such cells with antibody (Fig. 3c). Use of imatinib gave a similar result (Fig. 3d). Thus, results of these experiments suggest that imatinib causes similar conformational change of Pgp as its typical substrate, vinblastine.

Comparison of sensitivity of K562 and K562/i-S9 cells to vinblastine and imatinib revealed that although resistance of K562/i-S9 cells to vinblastine was more than 15 times higher than that of K562 (LD_{50} of 6 nM and 10 μM for K562 and K562/i-S9 cells, respectively) (Fig. 4a), not only they lacked resistance to the cytotoxic effect of imatinib but they also exhibited 3-fold higher sensitivity to imatinib compared with K562 cells (Fig. 4b).

Changes in the number of cells expressing P-glycoprotein during evolution of CML in patients. We investigated changes in Pgp expression by peripheral blood cells from AP-CML patients treated with imatinib. Sequential pilot studies of the same patients ($n = 11$) at certain time intervals after the beginning of imatinib therapy demonstrated individual mode of changes of Pgp expression (Table 1). Four patients treated with imatinib for 3 months still had Pgp+ cells; after 6-12 months one of these four (Pgp+) patients became Pgp-negative, whereas cells from six of seven "Pgp−" patients became "Pgp+".

Thus, in spite of individual variability of Pgp expression by blood cells, most patients demonstrated time-dependent tendency in the increase of Pgp+ cells in peripheral blood (Table 1). This is consistent with the results of studies of 41 AP-CML patients at various time intervals after the beginning of treatment (in contrast to above mentioned 11 patients not all these patients were investigated several times) (Table 2). These patients were observed for at least 4.5 years. Examination of patients three months after the beginning of treatment revealed how differences in the number of Pgp+ patients compared with the group "before treatment", but imatinib treatment for 6-12 months caused a significant increase of Pgp+ cells in patients from 33.3% (five of 15 patients) to 87.5% (14 of 16 patients), $p < 0.01$. Subsequent studies also revealed high proportion of patients with Pgp+ cells (Table 2). The last row of Table 2 summarizes data on 11 patients treated with imatinib for 2-6 years (eight patients of this group were treated with imatinib for five years). Repeated examinations revealed the presence of BCR-ABL transcripts in bone marrow cells of all 11

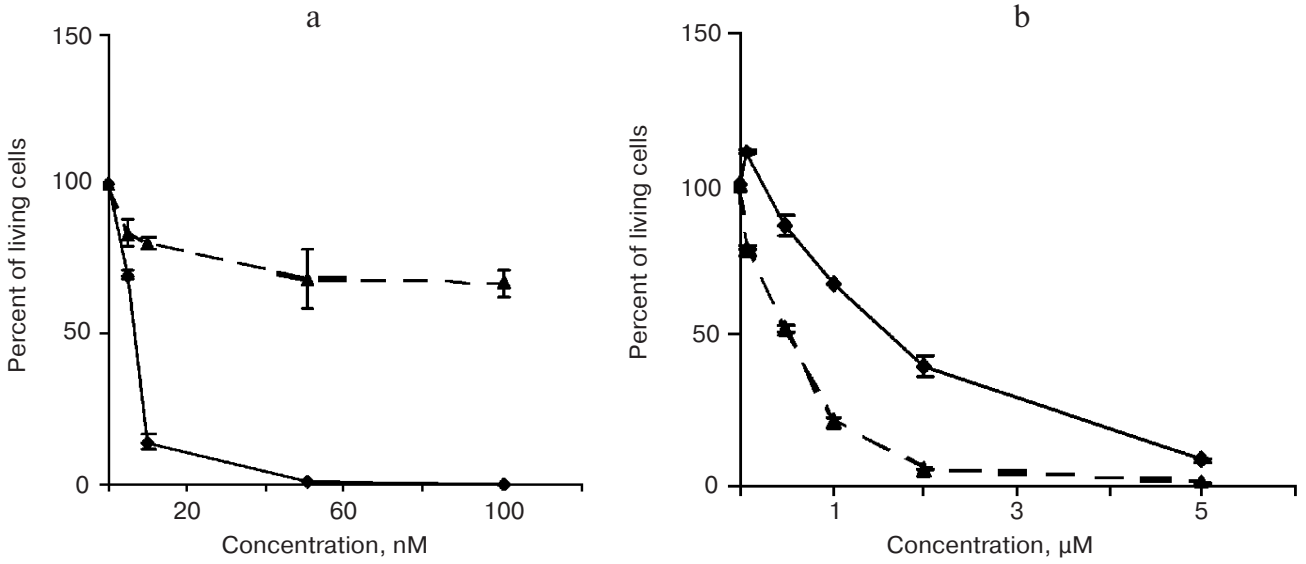

Fig. 4. Effects of vinblastine (a) and imatinib (b) on survival of K562 (solid lines) and K562/i-S9 (broken lines) evaluated by the MTT test. The number of cells grown in the drug-free medium was defined as 100%. Data represent the mean of three experiments.

patients. This means that imatinib does not totally suppress pathological clones. Indeed, all these patients exhibited resistance to imatinib (primary or secondary resistance) and so these patients were subsequently treated with new preparations. The table shows no relative increase of Pgp+ cells in the group of these patients compared with previous examinations (treatment with imatinib for 6-24 months).

Expression of MDR proteins by peripheral blood cells of patients. In seven patients, we investigated binding of various MDR proteins by peripheral blood cells. Figure 5 shows that cells isolated from all patients expressed not only Pgp but also other MDR proteins. Frequently, the number of cells expressing other MDR proteins exceeded that of Pgp+ cells.

Changes in the number of cells exhibiting Rho123 efflux during CML evolution in patients. Rh123, a fluorescent dye, is a known substrate for Pgp. The flow cytofluorimetry tests can evaluate efflux of fluorescent dyes (e.g. Rh123, $DiOC_2$, Calcein AM) or fluorescent drugs (e.g. daunomycin) from Pgp+ cells [24]. Intensity of efflux of these substances reflects Pgp activity in these cells [23]. Although Rh123 is widely used for evaluation of functional activity of Pgp, it should be noted that Rh123 efflux is not highly specific for Pgp. For example, it has been shown that Rh123 is also a substrate for the MRP1 (ABCC1) membrane pump [25].

Table 1. Changes in Pgp expression in patients with chronic myeloid leukemia (accelerated phase) during imatinib therapy (results of repeated examinations of 11 patients)

Time intervals for repeated examinations	Number of patients with corresponding Pgp expression	
Study I, imatinib therapy for 3 months	Pgp+ *	Pgp– **
	4	7
Study II, imatinib therapy for 6-12 months	Pgp+ 3 / Pgp– 1	Pgp+ 6 / Pgp– 1
Study III, imatinib therapy for 12-16 months	Pgp+ 2	Pgp+ 3 / Pgp– 1

* >10% of peripheral blood cells from patients bind UIC-2 antibody.
** <10% of peripheral blood cells from patients bind UIC-2 antibody.

Table 2. Number of patients with cells exhibiting Pgp expression and Rh123 efflux at various time intervals after beginning of imatinib therapy of AP-CML patients

Duration of imatinib therapy (months)	Frequency of Pgp expression (Pgp+)* and efflux of Rh123 (Rh123+)**		
	number of Pgp+/number of examined patients (I)	number of Rh123+/number of examined patients (II)	significance of changes (p) between groups I and II
0 (before treatment)	8/21	6/13	$p > 0.1$
3	5/15	3/10	$p > 0.1$
6-12	14/16	8/12	$p > 0.1$
⩾16-24	6/12	5/11	$p > 0.1$
35-60	6/11***	11/11	$0.01 < p < 0.02$

* >10% peripheral blood cells from patients bind UIC-2 antibody.
** Peripheral blood cells from patients exhibit Rh123 efflux sensitive to inhibition by verapamil (MAF > 0.1).
*** Patients resistant to imatinib.

Our data show that the treatment with imatinib for up to two years is characterized by a correlation between the numbers of Pgp+ cases and the numbers of Rh123+ cases: there were insignificant differences between the numbers of Pgp+ and Rh123+ cases (Table 2). Such correlation was not found in the group of imatinib resistant patients examined 35-60 months after the beginning of therapy with imatinib: in all 11 patients irrespectively to Pgp level there was Rh123 efflux by blood cells and there were statistically significant differences between the numbers of Pgp+ and Rh123+ cases (Table 2). Figure 6 shows examples of Rh123 efflux by cells isolated from patients. Cells from patient 1 were Pgp+, Rh123+ (37% of cells bound UIC-2, MAF = 0.74). Cells from patient 2 were Pgp–, Rh123+ (3.6% of cells bound UIC-2, MAF = 0.3). Since the data indicated the increase of Rh123+ cases during the development of imatinib resistance as well during prolonged use of this drug for therapy, we compared the mode of Rh123 efflux by peripheral blood cells from CML patients ("Rh123+" and "Rh123–" indicate efflux and lack of efflux, respectively) and clinical parameters of these patients treated with imatinib for 6-24 months (Table 3). In the "Rh123+" group, five of eight patients died, whereas in the "Rh123–" group all patients were alive. CCR was observed in five of seven patients from "Rh123–" and just one patient from "Rh123+". Although the numbers of patients in both groups were rather small, the differences in survival of "Rh123+"and "Rh123–" patients were highly significant (Fig. 7).

Table 3. Lethality and cytogenetic response of AP-CML patients to imatinib therapy depending on Rh123 efflux by their peripheral blood cells after imatinib therapy for 6-24 months

Pgp activity and outcome of treatment	Rh123+ *	Rh123– **
Died	5	0
CCR***	1	5
CR****	2	2
Total number of patients	8	7

* Peripheral blood cells from patients exhibited Rh123 efflux sensitive to inhibition by verapamil (MAF > 0.1).
** Peripheral blood cells from patients did not exhibit any Rh123 efflux (MAF ≤ 0.1).
*** Complete cytogenetic response to imatinib therapy (100% disappearance of bone marrow cells carrying Ph chromosome).
**** Lack of (or) partial cytogenetic response to imatinib therapy (presence of more than 1% of bone marrow cells carrying Ph chromosome).

DISCUSSION

Use of a BCR-ABL tyrosine kinase inhibitor, imatinib, for treatment of CML is an important event for both therapy of this particular disease and therapy of malignant tumors in general. However, in spite of effectiveness of this drug in some patients CML recurrence is observed after imatinib therapy (i.e. reproduction of cells carrying *BCR-ABL*). For understanding of these processes, it is important to realize how the cell population treated with imatinib evolves.

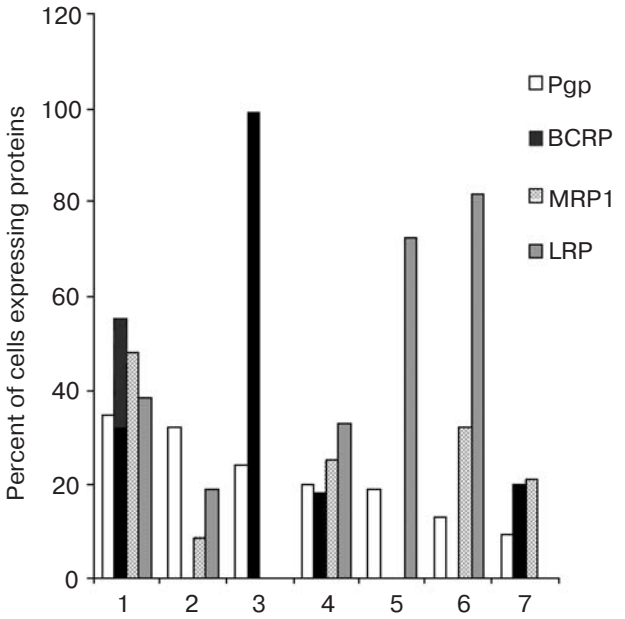

Fig. 5. Expression of MDR proteins in peripheral blood cells of AP-CML patients resistant to imatinib. Data represent percent of cells with detected MDR proteins determined by cytofluorimetry after staining with corresponding antibodies.

Since proteins of the ABC family can provide resistance of cells to imatinib, their treatment with this preparation can cause selection of cells expressing ABC transporters. Besides involvement into mechanisms underlying drug resistance of malignant tumors, the proteins of the ABC family may play important physiological functions including some yet unclear functions in stem cells, for example in bone marrow stem cells (the early hemopoietic precursors) [26, 27]. Study of evolution of cells expressing these proteins would give valuable information about reproduction of those cell clones that determine the fate of this disease.

One of the purposes of this study was to investigate whether CML cells expressing Pgp always exhibit imatinib resistance. There are contradicting data on this problem [12-18]. Results of our study on K562/i-S9 cells confirm that these cells exhibit Pgp mediated MDR: they contain increased level of *MDR1* mRNA and express increased levels of Pgp. The latter is involved in active efflux of Rh123, and this suggests functional activity of this protein. Our data also suggest that Pgp expressed by K562/i-S9 cells recognizes imatinib along with other Pgp substrates, and imatinib competes with these substrates (verapamil and vinblastine) for Pgp binding (Fig. 3). Thus, imatinib is a substrate for Pgp expressed by K562/i-S9 cells. However, our experiments also demonstrated

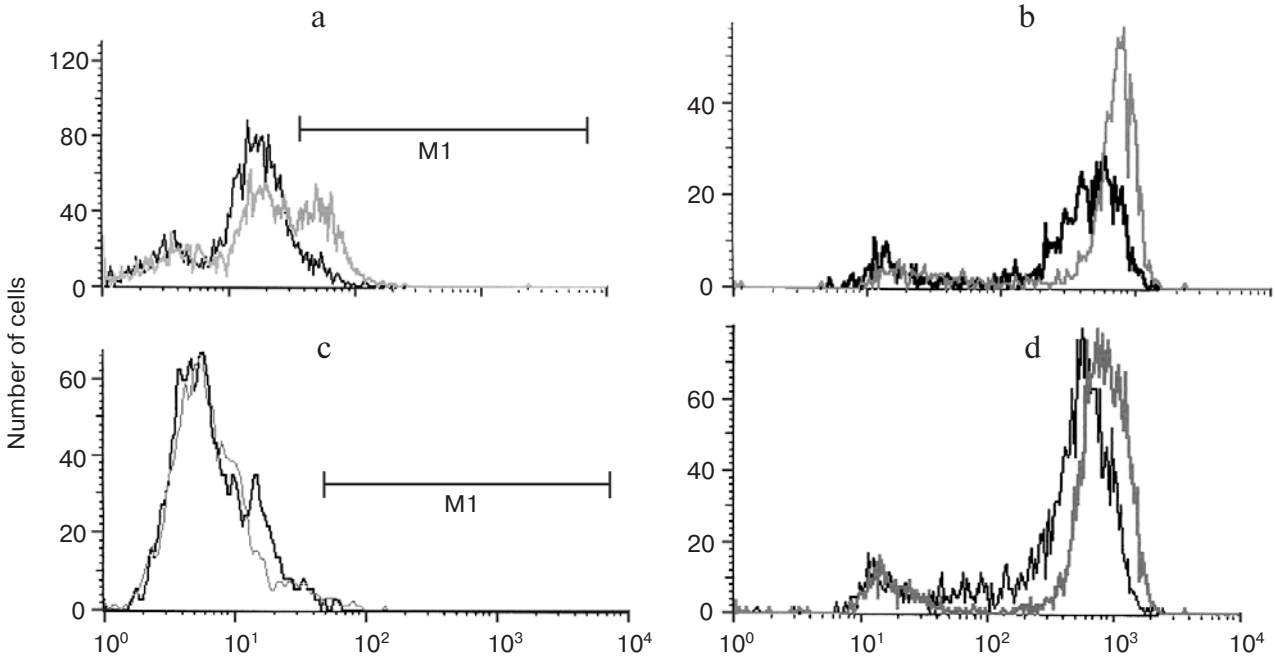

Fig. 6. Examples of UIC-2 antibody binding by peripheral blood cells and Rh123 efflux by cells from two AP-CML patients (patient 1, a and b; patient 2, c and d) resistant to imatinib. a, c) UIC-2 antibody binding by peripheral blood cells. The black line shows binding of secondary antibodies to the cells (control). The gray line shows UIC-2 antibody binding to Pgp. Values of M1 are given in the text. b, d) Rh123 efflux by cells from a patient (black line) and the effect of verapamil of Rh123 efflux (gray line). The abscissa shows fluorescence intensity of cells, the ordinate shows number of cells with certain level of fluorescence.

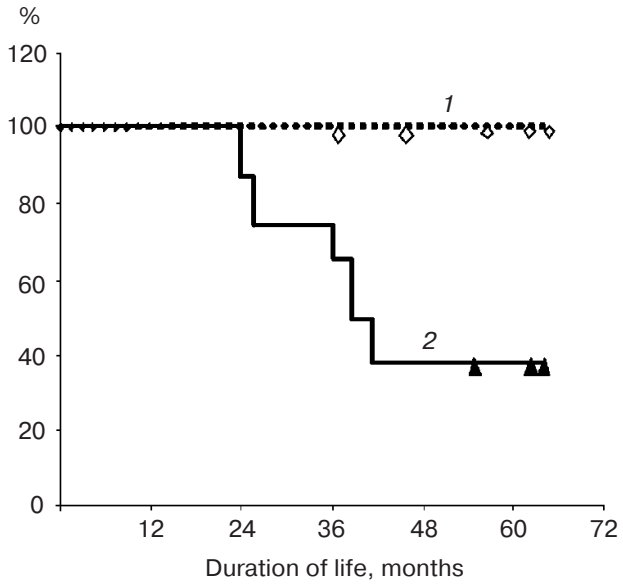

Fig. 7. Comparative survival of "Rh123–" (*1*) and "Rh123+" (*2*) patients, $p = 0.017$.

that these cells do not exhibit resistance to the cytotoxic effect of imatinib; moreover, these cells are approximately three times more sensitive to the cytotoxic effect of imatinib than the parent K562 cells. It should be noted that K562/i-S9 cells are resistant to vinblastine (Fig. 4). Thus, our results combined with the literature data suggest that CML cells with increased expression of Pgp may survive during treatment with imatinib (provided that Pgp determines resistance to imatinib) or die (when Pgp cannot determine resistance to this preparation).

The study of changes in the number of cells expressing Pgp during evolution of AP-CML in patients showed individual modality of this process. This was expected because processes of genetic variations in various patients and in various populations of cells should differ. However, repeated examinations of the same patients and also groups of patients at various time intervals after the beginning of imatinib therapy have shown time-dependent tendency to increase of Pgp+ cells in peripheral blood. Treatment with imatinib for 6-12 months increased the number of Pgp+ patients compared with previous periods of examinations, and it remained high at subsequent examinations. Evidently, in most patients these time intervals of imatinib therapy are required for formation of certain cell clones expressing Pgp (in some individuals formation of such clones may occur later).

Such clones of leukemic cells express not only Pgp but also other proteins conferring MDR. Our data demonstrate that peripheral blood cells from all patients undergoing imatinib therapy for more than six months express several MDR proteins: MRP1, BCRP, LRP (Fig. 5). K562/i-S9 cells selected for increased expression of Pgp [22] were also characterized by increased expression of these proteins (Fig. 1). Evidently, selection of Pgp-positive cells is accompanied by selection of cells with overexpression of other transporters of the ABC family and LRP protein.

Reproduction of leukemic stem cells or early precursors is critical for CML progression. Such cells are known to express not only Pgp, but also other transporters belonging to the ABC family [28]. Stem cells and early precursor cells are characterized by active efflux of Rh123 [26]. To evaluate whether populations of leukemic cells selected during imatinib treatment consist of such stem cells (at least in some cases), we separated patients treated with imatinib for 6-24 months into two groups designated as "Rh123+" and "Rh123–" and analyzed survival of these patients and appearance of complete cytotoxic response (i.e. death of all BCR-ABL positive cells). Using these parameters, we found highly significant differences between these two groups (Table 3 and Fig. 7). These results show that in some AP-CML patients treated with imatinib for 6-24 months cell populations with a significant number of "Rh123+" cells are formed. They evidently represent populations of stem or early precursor cells and their reproduction results in death of these patients.

Our data suggest that Pgp expression plays a role in the processes of selection of "Rh123+" or "Rh123–" by imatinib. However, not only the status of Pgp but also the status of other ABC-transporters may by important factors for selection of CML cells by drugs of the new generation. It has been demonstrated that transporters of the ABC-family expressed by stem cells include all proteins determining MDR [28]. At least a fraction of these transporters may determine evolution of the population of CML cells during treatment with imatinib.

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