Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment.

Short title: BCR/ABL+ progenitors in imatinib mesylate treated patients

Scientific Section: Clinical Observations

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ABSTRACT

The BCR/ABL tyrosine kinase inhibitor imatinib mesylate (Gleevec, STI571) has shown remarkable efficacy in the treatment of chronic myelogenous leukemia (CML), with a high proportion of patients achieving complete cytogenetic responses (CCR). However, it is not clear whether remissions will be durable and whether imatinib mesylate can eliminate the malignant primitive progenitors in which the disease arises. We investigated whether residual BCR/ABL+ hematopoietic progenitors were present in patients who achieved complete cytogenetic responses (CCR) with imatinib mesylate treatment. CD34+ progenitor cells were selected from bone marrow mononuclear cells (MNC) and analyzed for the presence of the BCR/ABL fusion gene by fluorescence in situ hybridization (FISH). CD34+ cells were also plated in committed progenitor (colony forming cells or CFC) and primitive progenitor (long-term bone marrow culture initiating cell or LTCIC) cultures and resulting colonies analyzed for the presence of BCR/ABL+ cells by FISH. Using these assays, residual BCR/ABL+ progenitors were detected in all patients studied. Quantitative RT-PCR analysis demonstrated increased levels of BCR/ABL mRNA in CD34+ cells compared with total MNC. Evaluation of samples collected at different time points demonstrated persistence of BCR/ABL+ progenitors despite continued treatment with imatinib mesylate. Our results indicate that inhibition of BCR/ABL tyrosine kinase activity by imatinib mesylate does not eliminate malignant primitive progenitors in CML patients. Patients in CCR with imatinib mesylate treatment need to be followed carefully to assess for risk of relapse.
INTRODUCTION

Chronic myelogenous leukemia (CML) is a lethal hematological disorder, which results from malignant transformation of a very primitive hematopoietic cell. The disease usually has a triphasic course, presenting in an initial chronic phase (CP) but invariably progressing over time to an intermediate accelerated phase (AP) and a terminal blast crisis (BC). Cells of malignant clonal origin in CML are characterized by a balanced translocation between chromosomes 9 and 22, leading to the fusion of a portion of the ABL gene from chromosome 9 with the BCR gene on chromosome 22\textsuperscript{1,2}. The resulting BCR/ABL gene has been shown to play a critical role in the pathogenesis of CML\textsuperscript{3-5}. The c-ABL gene product is a tyrosine kinase with tightly regulated activity\textsuperscript{6,7}. The BCR/ABL gene product, p210\textsuperscript{BCR/ABL}, demonstrates constitutive activation of tyrosine kinase activity with enhanced substrate phosphorylation compared with c-ABL\textsuperscript{8-10}. Abnormal kinase activity appears to be critical for BCR/ABL induced transformation\textsuperscript{8,11}.

Imatinib mesylate (STI571, Imatinib mesylate) is a 2-phenylaminopyramidine compound which has been demonstrated to be a potent inhibitor of all ABL tyrosine kinases (c-ABL/BCR/ABL and Tel/ABL), as well as the PDGF receptor tyrosine kinase and c-kit tyrosine kinases\textsuperscript{12}. Other tyrosine kinases are not significantly inhibited. In preclinical studies, imatinib mesylate demonstrated high levels of activity against BCR/ABL containing cells in vitro and in vivo\textsuperscript{13,14}. Subsequently phase 1 and phase 2 clinical trials have shown remarkable activity of imatinib mesylate in the treatment of CML\textsuperscript{15-17}. Almost all patients in CP achieve hematological response and a high proportion of patients achieve major and complete cytogenetic responses\textsuperscript{17}. The drug
is also effective in patients with AP and BC CML though with a considerably lower rate of response\textsuperscript{18-20}.

Although the success of this molecular therapeutic approach represents an exciting advance in targeted cancer therapy, it still needs to be determined whether responses to imatinib mesylate in CML patients will be durable. Recent reports indicate that BCR/ABL transcripts, although significantly reduced, can still be detected in patients in CCR on Imatinib, and that molecular remissions are rare \textsuperscript{21,22}. In patients with CML in AP and BC, relapses are observed even after induction of complete cytogenetic response\textsuperscript{18,19}. Relapses have been infrequent in patients with CP disease, but follow up is limited\textsuperscript{17}. It is not clear whether imatinib mesylate effectively targets and eliminates malignant primitive progenitors in which the disease originates. Persistent malignant progenitors could be a source of relapse in patients in CCR.

We have previously shown that \textit{in vitro} exposure to imatinib mesylate results in inhibition of primitive progenitor growth. Suppression of primitive progenitor growth was partial and occurred mainly through inhibition of their abnormally increased proliferation rather than through a selective increase in progenitor apoptosis\textsuperscript{23}. These observations suggest that BCR/ABL tyrosine kinase inhibition by imatinib mesylate may suppress but not eliminate all malignant progenitors in CML patients. On the other hand, it is possible that prolonged exposure to imatinib mesylate in vivo could have an increased suppressive effect on malignant primitive progenitors. In this study we investigated whether malignant primitive hematopoietic progenitors could be detected in bone marrow samples obtained from CML patients in complete cytogenetic remission following imatinib mesylate treatment. CD34+ cells selected from bone marrow
mononuclear cells, and colonies derived from in vitro assays of committed and primitive progenitors, were analyzed for the presence of the BCR/ABL gene.
METHODS

Samples. Bone marrow samples were obtained from 15 CML patients who were identified to be in complete cytogenetic remission (CCR) after imatinib mesylate treatment using guidelines approved by the Institutional Review Board of the City of Hope National Medical Center. CCR was defined as the complete absence of t (9; 22) on karyotypic analysis or 6% or less BCR/ABL positive cells (normal background limits) on FISH analysis. For some patients two or more samples were obtained at different time points.24

Bone marrow mononuclear cells (MNC) were isolated by Ficoll-Hypaque (Sigma Diagnostics, St. Louis, MO) density gradient separation (specific gravity 1.077) for 30 minutes at 400g. CD34+ cell enriched populations were selected from MNC using immunomagnetic column separation (Miltenyi Biotech Inc., Auburn, CA).

Progenitor assays:

Colony forming cells. CD34+ cells were plated in semisolid methylcellulose progenitor culture for 14-18 days and assessed for the presence of CFU-GM and BFU-E colonies as previously described25.

Long-term culture initiating cells. Cells were plated in LTBMC medium on M2-10B4 murine fibroblast feeders previously subcultured in 24 well plates. Cultures were maintained at 37°C in a humidified atmosphere with 5% CO2 and fed at weekly intervals by removal of half the medium from the wells and replacement with fresh medium. After 6 weeks all non-adherent and adherent cells were harvested, pooled and plated in methylcellulose progenitor culture and the number of CFC evaluated after 14 days26.
**Fluorescent in situ hybridization (FISH) analysis.** FISH analysis was performed on freshly isolated total nucleated cells or selected CD34+ cells or on cells derived from colonies resulting from CFC and LTCIC assays, which were harvested and pooled. Cells were resuspended in hypotonic KCl solution at 37°C for 20 minutes, centrifuged and fixed using Carnoy’s fixative (1 acetic acid: 3 methanol) on ice for one hour. Just prior to slide preparation, fixed cells were resuspended in 5-10 µl of Carnoy’s fixative. Hybridization using the LSI dual labeled BCR/ABL DNA probe followed manufacturer’s instruction (Vysis, Inc., Downers Grove, IL). Lymphocytes from a normal individual were used as a BCR/ABL negative control; SD1 cell lines, derived from an ALL patient, served as BCR/ABL positive control. Two hundred nuclei were scored for each sample. A percentage less than or equal to 6% of cells with a BCR/ABL fusion signal is considered within background limits based on laboratory validation studies (sensitivity, 95.2%, with 95% confidence limits of 76.2%-99.9%, and specificity, 100.0%, with 95% confidence limits of 82.4% -100.0%).

**Conventional cytogenetic analysis.** Chromosome studies were performed according to standard methods on metaphase cells derived from 24-and 48-hour unstimulated and at least one culture supplemented with supernatant derived from a human bladder carcinoma cell line (ATCC #5637) known to contain hematopoietic growth factors. Chromosome abnormalities were described according to the International System for Human Cytogenetic Nomenclature. When available, a minimum of 20 metaphase cells per sample was analyzed.
**Reverse transcriptase-polymerase chain reaction (RT-PCR) for BCR/ABL mRNA.** A nested PCR approach was used to detect BCR/ABL mRNA in total MNC and CD34+ cells. Total cellular RNA was extracted using Triazol reagent, and cDNA generated by reverse transcription using oligo dT primers. PCR amplification was carried out using primers designed to specifically detect the transcripts from the breakpoint region of the p210 bcr-abl fusion gene. Initially 30 cycles of PCR amplification were carried out using the BCR68 and ABL3 primers (Table 1). Subsequently a fraction of the first stage product was subjected to an additional 30 cycles of PCR amplification using internal nested primers, BCRfwd and ABLrev. Amplified samples were size separated by gel-electrophoresis, ethidium bromide stained and photographed. In preliminary studies it was established that this assay could reliably detect 1 in $10^6$ primary CML CD34+ cells diluted in normal MNC. Cell lines K562 and Meg01 were used as positive controls and HL-60 as a negative control. In addition negative (reagent only) controls from each stage of the assay were included in each run to exclude contamination. Each sample was also analyzed by nested PCR for the presence of the normal BCR gene as an internal control to confirm RNA integrity and loading (first stage primers BCR68 and BCR15; second stage primers BCRfwd and BCR20). Samples were considered to be negative for BCR/ABL if no BCR/ABL product was detected in 4 separate PCR assays.

**Real time quantitative RT-PCR (Q-PCR).** Q-PCR using TaqMan technology was used to quantitate BCR/ABL mRNA levels in MNC and CD34+ cells. Total cellular RNA was converted to cDNA by reverse transcription and used as the template for the PCR procedure using primers and probes designed to detect the p210 transcripts of the BCR/ABL fusion gene (Table 1). The PCR reaction exploits the 5’ nuclease activity of AmpliTaq Gold DNA polymerase to cleave a
TaqMan probe containing a reporter dye at the 5’ end and a quencher dye at the 3’ end. The separation of the reporter dye from the quencher dye results in an increase of fluorescence of the reporter allowing real-time detection of target amplification during PCR. Forty-five amplification cycles were run using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with a standardized protocol. Cell lines K562 and SD-1 were used as positive controls with serial cell line dilutions and a known positive patient sample included in each run to confirm the linearity and reproducibility of the test. Internal control RNA amplification for GAPDH was also performed on each sample. A quantitative result was calculated using a formula based on the ratio of amplification of BCR/ABL to the internal control gene GAPDH. The procedure was initially tested on cell line dilutions to confirm the ability to accurately quantitate the result, and could detect 1 in $10^4$-$10^5$ BCR/ABL+ cells.

**Statistical analysis.** Differences between groups were evaluated using the Students-t test. Relationships between two sets of variables were evaluated using linear regression analysis.
RESULTS

Fifteen patients who achieved complete cytogenetic remission (CCR) following imatinib mesylate treatment were studied. CCR was defined as 100% Ph-negative metaphases by conventional cytogenetics, and/or BCR/ABL negative by FISH analysis of bone marrow nucleated cells. Multiple samples were collected at different time points from some patients while in continued CCR. A total of 23 samples were studied. Table 2 shows characteristics of the patients studied and the samples collected. Twelve patients were in CP and 3 patients in AP at time of initiation of imatinib mesylate treatment. Eleven patients had received prior treatment with interferon or a combination of interferon and cytosine arabinoside whereas 4 patients received imatinib mesylate as initial therapy (excluding Hydroxyurea). The time from diagnosis of CML to start of imatinib mesylate treatment ranged from 1-61 months (median 17 months). The time from initiation of imatinib mesylate treatment to the time when the first sample was obtained for the purpose of this study ranged from 3-18 months (median 9 months). MNC from 12 of 13 evaluable patients were BCR/ABL+ by a nested RT-PCR assay (18 of 19 samples).

CD34+ cells were selected from MNC of patients in CCR by immunomagnetic column separation and analyzed for the presence of BCR/ABL+ cells by FISH. Results are summarized in Table 3. Residual BCR/ABL+ cells were detected in the CD34+ cell fraction from the first sample studied from 11 of the 15 patients (Figure 1) including all 3 patients in AP and 8 of 12 patients in CP. Considerable variability in numbers of residual BCR/ABL+ CD34+ cells was observed between patients. There was a trend towards a higher percentage of BCR/ABL+CD34+ cells in patients in AP (12.7±1.8) compared with CP 7.8±1 (p=0.09). There was no significant
correlation between the percentage of BCR/ABL+ CD34+ cells and time from diagnosis to start of imatinib mesylate treatment or the duration of imatinib mesylate treatment. The percentage of BCR/ABL+ CD34+ cells was also not significantly different between patients who had received prior interferon-based treatment or had received imatinib mesylate as initial therapy.

To determine whether BCR/ABL+ CD34+ cells had functional primitive and committed progenitor capacity, CD34+ cells were plated in CFC (committed progenitor) and LTCIC (primitive progenitor) assays and the resulting colonies were harvested and analyzed for BCR/ABL+ cells by FISH. BCR/ABL+ CFC were present in the first sample studied from 11 of 15 patients and BCR/ABL+ LTCIC in 10 of 13 patients for whom results are available (Figure 2). Of the four patients in whom CD34+ cells were BCR/ABL- by FISH on initial examination, BCR/ABL+ CFC were seen in one patient and BCR/ABL+ LTCIC in 2 patients. One of the patients with BCR/ABL- CD34+ cells but BCR/ABL+ LTCIC on first examination (#222) had detectable BCR/ABL+ CD34+ cells on analysis of a subsequent evaluation 6 months later. One patient (#255) did not have detectable BCR/ABL+ cells on evaluation of CD34+ cells, CFC or LTCIC on initial examination. However, BCR/ABL+ LTCIC were present in a sample obtained from this patient 3 months later. When the results for CD34+ cells, CFC and LTCIC are taken together, BCR/ABL+ progenitor cells were present in all patients included in this study.

Using the nested PCR assay, BCR/ABL mRNA was detected in CD34+ cells from 9 of 13 evaluable patients (15 of 19 samples) including all patients with BCR/ABL- CD34+ cells on FISH analysis. Availability of only limited amounts of cells and low RNA yield may have compromised the sensitivity of the PCR assay for samples in which BCR/ABL message was not
detectable. A real time quantitative RT-PCR (Q-PCR) assay was used to compare BCR/ABL mRNA levels in MNC and CD34+ cells. This assay is less sensitive than the nested PCR assay, but has the advantage of allowing quantitative comparison of results obtained from MNC and CD34+ cells. Results are expressed as a ratio of BCR/ABL to the internal control gene GAPDH. Q-PCR results comparing BCR/ABL expression (BCR/ABL: GAPDH ratio) in CD34+ cells and MNC are available for 8 patients (Figure 3). Increased levels of BCR/ABL mRNA were detected in CD34+ cells when compared with MNC in 6 patients, consistent with the presence of an increased proportion of BCR/ABL+ cells in the CD34+ fraction. Two patients did not have detectable BCR/ABL message in either total nucleated cells or CD34+ cells using this assay, likely reflecting the lower sensitivity of the Q-PCR assay.

Four patients were evaluated on more than one occasion. BM samples obtained at 4 different time points from one patient showed no significant trend towards an increase or decrease in BCR/ABL+ CD34+ cells by FISH over the period from 9 to 26 months after initiation of imatinib mesylate treatment (Figure 4). BCR/ABL mRNA could be detected by nested PCR in CD34+ cells on all occasions. Q-PCR measurements indicated variable levels of BCR/ABL mRNA in CD34+ cells over time. Two other patients also showed persistent BCR/ABL+ progenitors without a definite trend towards an increase or decrease in the percentage of BCR/ABL+ progenitor cells. Finally, in one patient, also described earlier, CD34+ cells, CFC and LTCIC were all BCR/ABL- 6 months after start of treatment, whereas BCR/ABL+ LTCIC were detected at 9 months.
Thirteen of the 15 patients included in this study were in CCR at last follow up, whereas two (#144, #261) have developed evidence of cytogenetic relapse. These two patients were both in AP when imatinib mesylate was started. For patient #144, results shown are based on analysis of samples obtained when the patient was in CCR (BCR/ABL FISH negative), 16 and 21 months after initiation of imatinib mesylate. A subsequent bone marrow sample, obtained 27 months after initiation of imatinib mesylate, was BCR/ABL positive by FISH. For patient #261, results shown are for a sample obtained 18 months after initiation of imatinib mesylate, when the patient was Ph- and BCR/ABL FISH negative. An unrelated clone with trisomy 8 but without t (9; 22) was noted at that time. A subsequent bone marrow examination, performed 27 months after initiation of imatinib mesylate, showed evidence of cytogenetic relapse with 2 of 22 cells having the t (9; 22) but without trisomy 8 on karyotypic analysis. Cytogenetic relapse was confirmed by BCR/ABL FISH. Two additional cells had trisomy 8 but did not have t (9;22), indicating persistence of the unrelated clone.
DISCUSSION

We demonstrate here that residual BCR/ABL+ progenitors persist in CML patients in complete cytogenetic remission on imatinib mesylate therapy. Analysis of colonies generated in methylcellulose progenitor culture and long-term bone marrow cultures confirmed that BCR/ABL+ CD34+ cells retain functional committed and primitive progenitor capacity. Since BCR/ABL+ cells were not detected by conventional cytogenetic or FISH analysis of total nucleated cells these results indicate selective persistence of malignant progenitor cells compared with more mature cells. Quantitative PCR assays also demonstrate that the level of BCR/ABL message was higher in the CD34+ cell population, consistent with enrichment of BCR/ABL+ cells in the progenitor fraction. BCR/ABL+ progenitor cells were not eliminated despite continued imatinib mesylate treatment for more than 2 years.

There was inter-individual variability in the frequency of BCR/ABL+ CD34+ cells. A trend towards an increased frequency of BCR/ABL+ CD34+ cells was seen in patients in whom treatment was initiated in AP compared to CP. No association between levels of BCR/ABL+ progenitors and risk factors such as time from diagnosis, prior therapy or length of time on imatinib mesylate treatment was apparent. Although 4 patients in CP did not have detectable CD34+ cells when first examined, residual BCR/ABL+ CFC or LTCIC were found in three patients. The fourth patient did not have BCR/ABL+ CD34+ cells, CFC or LTCIC on initial examination, but BCR/ABL+ LTCIC were detected when the patient was restudied 3 months later.
These observations indicate that imatinib mesylate treatment of CML may not eliminate primitive hematopoietic cells in which the disease arises. Evaluation of samples obtained from patients at different times after initiation of treatment, including serial evaluation of samples from individual patients, suggest that the residual BCR/ABL+ progenitor population persists despite continued treatment with imatinib mesylate. Persistence of residual BCR/ABL+ progenitors raises the possibility that patients in CCR on imatinib mesylate therapy may be at risk for disease relapse. Studies in patients receiving allogeneic BMT and IFN have shown that the level of residual malignant cells as determined by PCR is predictive of clinical outcome\textsuperscript{31,32}. Overall, BCR/ABL mRNA levels in marrow from patients in Imatinib-induced remissions were significantly lower than those observed in patients with IFN induced remissions\textsuperscript{21,22}. However, considerable variability in response was seen and BCR/ABL mRNA was still detectable in the majority of patients, consistent with our own results. Higher levels of CD34+ cells in patients treated in AP may be consistent with clinical observations of a higher rate of relapse compared to CP CML patients\textsuperscript{19}. Factors such as frequency and type of residual BCR/ABL+ progenitors, and their trend towards increase or decrease over time, may better correlate with clinical outcomes.

Interestingly, some previous reports have suggested that persistence of small populations of BCR/ABL+ progenitors may still be compatible with long-term remission from leukemia. Residual BCR/ABL positive progenitors were detected in marrow samples from CML patients who have achieved prolonged cytogenetic remission, and even complete molecular remission (RT-PCR-negative), following interferon-\(\alpha\) therapy. This suggests that the presence of a small population of Ph-positive CML progenitor cells for a very long period of time is compatible with a durable remission, and that interferon therapy may induce a state of tumor dormancy in CML.
Similarly, Miyamoto et al have reported that AML1/ETO expressing multipotent progenitors can be detected by RT-PCR in bone marrow of patients with AML with t(8;21) in remission after chemotherapy or autologous peripheral blood stem cell transplantation. However this finding did not necessarily indicate impending relapse since such progenitors could persist more than 10 years after achieving remission. Of the patients studied here, only two have developed evidence of clinical relapse on further follow up. Both patients had accelerated phase disease at initiation of Gleevec treatment. Longer follow up of a larger group of CML patients will be required to determine whether residual BCR/ABL+ progenitors are associated with risk of disease recurrence in a significant proportion on Imatinib-responsive patients, especially those in chronic phase. It will also be of interest to investigate in future whether BCR/ABL+ progenitors are eliminated in patients in long-term remission following allogeneic hematopoietic cell transplantation.

The mechanisms underlying persistence of malignant progenitors in patients in CCR are not known. Mechanisms such as BCR/ABL gene duplication, mutations in the ABL kinase domain, and acquisition of additional genetic abnormalities have been associated with primary or acquired resistance to imatinib mesylate in patients with advanced disease. Although the patients studied here were responsive to imatinib mesylate, it is possible that similar mechanisms could be active in a small subset of progenitors resulting in their persistence. However, refractoriness to imatinib mesylate would be expected to allow rapid emergence of the resistant clone, whereas several patients studied several months apart did not show evidence of increasing BCR/ABL+ cells, arguing against this possibility. We did not observe multiple copies of the BCR/ABL fusion gene in residual BCR/ABL+ CD34+ cells by FISH in any of the samples.
studied. Therefore other mechanisms may be responsible for persistence of malignant progenitors in patients responsive to imatinib mesylate. Normal hematopoietic stem cells demonstrate increased efflux pump activity, a characteristic that can be used to select primitive hematopoietic cell populations\textsuperscript{38,39}. It is possible that increased drug efflux in leukemic stem cells could lead to reduced intracellular levels of imatinib mesylate and be a mechanism of resistance to inhibition by imatinib mesylate.

Another potential mechanism for retention of BCR/ABL+ progenitors may be related to our previously reported observation that imatinib mesylate inhibits malignant primitive progenitor growth primarily through inhibition of their abnormally increased proliferation rather than selective induction of apoptosis\textsuperscript{23}. Consistent with this Graham et al have also reported that primitive, quiescent Ph+ progenitor cells from CML patients are insensitive to imatinib mesylate in vitro\textsuperscript{24}. These results suggest that BCR/ABL kinase activity may be required for abnormal proliferation and expansion of BCR/ABL+ cells in CML, but may not be essential for preservation of primitive malignant cells. BCR/ABL kinase inhibition by imatinib mesylate may therefore remove the proliferative advantage of BCR/ABL+ progenitors and their progeny cells and allow regrowth of coexisting BCR/ABL- cells, but not eliminate all BCR/ABL+ primitive progenitors.

In conclusion, we demonstrate that BCR/ABL+ hematopoietic progenitors persist in the marrow of patients in CCR indicating that malignant progenitors may be suppressed but not eliminated in the course of imatinib mesylate treatment. Future studies will examine potential mechanisms
underlying persistence of malignant progenitors and the significance of residual malignant progenitors with respect to clinical outcomes.
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REFERENCES


38. Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood. 2002;99:507-512

Table 1. Oligonucleotide primers used for RT-PCR

a. Nested PCR

First step

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Second step

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b. Real time Quantitative PCR

p210BCR/ABL

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TaqMan probe

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Table 2 Characteristics of patients and samples studied.

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<td>6m</td>
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<td>Pos</td>
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<td>-</td>
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<td>Pos</td>
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<td>Pos</td>
<td>1y</td>
<td>0/20</td>
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^1 Upper limit of normal \( \leq 6\% \) BCR/ABL+ cells
^2 7\% cells had trisomy 8, but were BCR/ABL-
^3 Variant BCR/ABL breakpoint, not amplified by the primers used
NE=Not evaluable
NA=Not available
Neg=negative
Pos=positive
MNC=mononuclear cells
Table 3. Results of progenitor analyses.

<table>
<thead>
<tr>
<th>UPN</th>
<th>Phase (at start of imatinib mesylate treatment)</th>
<th>Duration of imatinib mesylate treatment (months)</th>
<th>PCR for BCR/ABL (MNC)</th>
<th>FISH (%BCR/ABL+ cells)(^1)</th>
<th>CD34+ cells</th>
<th>CFC</th>
<th>LTCIC</th>
<th>Length of additional follow up since last assessment (months)</th>
<th>Clinical status at last follow up</th>
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<tbody>
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<td>144</td>
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<td>NE</td>
<td>10 m</td>
<td>Cytogenetic relapse (FISH)</td>
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<td></td>
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<td>NE(^2)</td>
<td>13.2</td>
<td>6.9</td>
<td>NE</td>
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<td>6 m</td>
<td>6 m</td>
<td>CCR</td>
</tr>
</tbody>
</table>

\(^1\) Upper limit of normal ≤6% BCR/ABL+ cells

\(^2\) Variant BCR/ABL breakpoint, not amplified by the primers used

NE=Not evaluable

NA=Not available

Neg=negative

Pos=positive

CCR=Complete cytogenetic remission
LEGENDS FOR FIGURES

Figure 1. Persistence of BCR/ABL+ CD34+ cells in CML patients in complete cytogenetic remission (CCR) with imatinib mesylate treatment. CD34+ cells were selected from MNC of CML patients in CCR [CP (n=12) and AP (n=3)], by immunomagnetic column separation and analyzed for the presence of BCR/ABL+ cells by FISH. The percentage of BCR/ABL+ cells in the CD34+ cell fraction from the first sample studied is shown. A percentage less than or equal to 6% of cells with a BCR/ABL fusion signal is considered within background limits based on laboratory validation studies. CD34+ cells from 4 patients in CP were BCR/ABL negative by FISH.

Figure 2. Persistence of BCR/ABL+ cells with CFC and LTCIC capacity in CML patients in complete cytogenetic remission with imatinib mesylate treatment. CD34+ cells were plated in CFC (committed progenitor, n=15) and LTCIC (primitive progenitor, n=13) assays and the resulting colonies were harvested and analyzed for BCR/ABL+ cells by FISH. Results from the first sample studied are shown, and represent the percentage of BCR/ABL+ cells in colonies resulting from CFC and LTCIC assays. The percentage of CD34+ cells (n=15) which were BCR/ABL+ is shown for comparison. A percentage less than or equal to 6% of cells with a BCR/ABL fusion signal is considered within background limits. CD34+ cells from 4 patients, CFC from 4 patients and LTCIC from 3 patients were BCR/ABL negative by FISH.
Figure 3. Real time quantitative PCR (Q-PCR) analysis of mononuclear cells (MNC) and CD34+ cells from patients in complete cytogenetic remission with imatinib mesylate treatment. BCR/ABL mRNA levels in MNC and CD34+ cells were measured using a Q-PCR assay. Results were expressed as a ratio of BCR/ABL to the internal control gene GAPDH. Q-PCR results of BCR/ABL expression in CD34+ cells and MNC for 8 patients are shown. Two patients did not have detectable BCR/ABL message in either total nucleated cells or CD34+ cells using this assay.

Figure 4. Persistence of BCR/ABL+ progenitors in multiple samples obtained from a patient in continued CCR on imatinib mesylate treatment. Results for (A.) BCR/ABL:GAPDH mRNA levels measured by Q-PCR, and (B.) the percentage of BCR/ABL+ CD34+ cells detected by FISH are shown for BM samples obtained from one patient from 12 to 26 months after initiation of imatinib mesylate treatment. A percentage less than or equal to 6% of cells with a BCR/ABL fusion signal is considered within background limits for the FISH assay.
Figure 1.
Figure 2.

CFC assay for committed progenitors

- Analyze colonies by FISH

CD34+ cells

LTCIC assay for primitive progenitors

- Analyze colonies by FISH

CFC culture
14 days

LTBMC culture x 6 weeks
Figure 3.
Figure 4

A.  

B.  

BCR/ABL:GAPDH ratio

Not detected

Months on imatinib mesylate

% BCR/ABL+ cells

Months on imatinib mesylate
Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment

Ravi Bhatia, Melissa Holtz, Ning Niu, Rachel Gray, David S Snyder, Charles L Sawyers, Daniel A Arber, Marilyn L Slovak and Stephen J Forman