Molecular Quantification of Residual Disease in Chronic Myelogenous Leukemia After Bone Marrow Transplantation

By James D. Thompson, Isadore Brodsky, and Jorge J. Yunis

Residual disease remains a major problem in the treatment of human neoplasia. To effectively monitor minimal leukemic activity after bone marrow transplantation (BMT), we used a competitive polymerase chain reaction (PCR) amplification technique to quantify expression of the characteristic bcr-abl fusion message in patients with chronic myelogenous leukemia (CML). Quantitative results were obtained between the 0.001% and 0.1% level in control experiments. This represents a significant advantage over cytogenetic and Southern blotting techniques routinely used to diagnose CML, which may not be sensitive below the 1% level. To illustrate the potential clinical usefulness of the quantitative PCR strategy, we compared results of bcr-abl messenger RNA expression with those obtained using cytogenetic and Southern blotting techniques, in a study of consecutive BM and peripheral blood (PB) samples from two CML patients at high risk for relapse after BMT. One patient received a syngeneic transplant during the chronic phase of the disease and relapse was apparent at the molecular level 4.5 months after BMT, while the patient was in complete clinical remission. The second patient was treated with an allogeneic BMT during the accelerated phase of the disease. A slow, but progressive decrease in bcr-abl expression was observed during the first 12 months after BMT, and expression was undetectable thereafter. Our results indicate that the competitive PCR technique can be used to monitor disease activity in patients at high risk of relapse, while the patients are in complete clinical remission, which should facilitate the early detection of relapse or the identification of progressive disappearance of leukemic activity. The approach used may serve as a model for the study of residual disease in an increasing number of other hematologic malignancies that express cancer-specific RNAs.

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Submitted March 1, 1991; accepted November 6, 1991.

Supported in part by National Cancer Institute Grant No. CA33314 and Grant No. 04269-06-J from the Mary L. Smith Charitable Lead Trust.

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0006-4971/92/7906-0018$3.00/0


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MATERIALS AND METHODS

Sample preparation. PB or BM samples were diluted into 5 vol of a freshly made red blood cell lysis buffer (38.8 mmol/L NH₄Cl, 2.5 mmol/L KHCO₃, 0.1 mmol/L EDTA, pH 8.0), incubated at room temperature for 10 minutes, and nucleated cells pelleted and washed in the same buffer. RNA was extracted from total PB or BM cells using the method of Chomczynski and Sacchi, and DNA was extracted using the Elu-Quik DNA extraction kit (Schleicher and Schuell, Keene, NH).

Cytogenetic and DNA studies. Cytogenetic studies were performed using a methotrexate cell synchronization technique, as previously described by our laboratory. bcr gene rearrangement by Southern blot analysis was performed using the bcr-3 probe (Oncogene Science, Manhasset, NY) essentially as described by the manufacturer.

Reagents for PCR amplification. A two-step amplification protocol with two sets of primers was used to accomplish PCR amplification. The first primer set included primers bcrA: AGTATACAGG-TCTCTGATC and abl2C: TTAATCCACCTGGCCACAA (Genosys Biotechnologies, Inc, The Woodlands, TX). A second or “nested” set of primers was used to enhance the sensitivity and specificity of the PCR amplification. They included primers bcrB: TCTGACTATGACGCTGCA and ablB2D: AGTGCACGAAAAAGTTGGG (Fig 1). Solutions for PCR amplification were as follows: reverse-transcriptase mix consisting of 2X Taq buffer (as described by Boehringer Mannheim Biochemicals [BMB], Indianapolis, IN), 0.4 mmol/L each of dATP, dCTP, dGTP, and dTTP, 0.5 mmol/L primer abl2C, 2 ÌM pancreatic RNase inhibitor (BMB), and 20 ÌL/L of M-MLV reverse-transcriptase (GIBCO BRL, Gaithersburg, MD) Taq1 mix consisting of 1X Taq buffer, 1 mmol/L primer bcr1A, and 0.25 ÌM Taq polymerase (BMB); Taq2 mix containing 1X Taq buffer, 0.2 mmol/L dNTPs, 0.2 mmol/L/L each of primers bcrB1 and ablB2D, and 0.05 ÌM Taq polymerase; Xho1 mix containing 3X Taq buffer and 2 ÌM/L Xho1 restriction enzyme (BMB). All mixes were made in advance without enzyme and stored in aliquots; enzyme was added just before use.

Competitive PCR titration analyses. For use in quantitative PCR, we produced a DNA template for synthesis of an Exogenous Competitor RNA (ECR) (Fig 1) using the “megaprimer” method of site directed mutagenesis. The ECR was synthesized using T7 RNA polymerase (GIBCO-BRL) as described by the manufacturer. Template was removed with 20 U DNase I (RNase-free; BMB) at 37°C for 60 minutes. RNA was purified by three extractions with phenol/chloroform followed by Sephadex G50 chromatography. If needed, the DNase I step was repeated until 30 cycles of PCR amplification of 10⁶ molecules (2.74 ng) of the ECR failed to generate a detectable amplification product. For quantitative PCR analysis, the RNA to be tested (usually 0.3 Ìg dissolved in 5 ÌL water) was aliquoted to 0.5 mL reaction tubes (Perkin-Elmer, Norwalk, CT), and 5 ÌL of the ECR dilutions and 10 ÌL of the reverse-transcriptase mix stated above were added and then incubated for 1 hour at 42°C. Five microliters of Taq1 mix was then added, and the reactions were overlaid with 20 ÌL of mineral oil and subjected to 30 cycles of amplification in a Cetus thermocycler, using cycles of 95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 60 seconds. One microliter was removed and added to 25 ÌL of Taq2 mix and then subjected to an additional 30 cycles of amplification as above.

Analysis of PCR amplification product. Four microliters of the final amplification product was combined with 16 ÌL of fresh Taq2 mix, heated to 95°C for 45 seconds, 55°C for 30 seconds, and then 72°C for 10 minutes. This step was performed to convert residual single-stranded PCR amplification product to the double-stranded form, and to resolve heterodimers formed between ECR and bcr-abl fusion message amplification products. Ten microliters of product from this reaction was then combined with 5 ÌL of Xho1 mix, heated to 37°C for at least 1 hour, and subjected to agarose gel electrophoresis.

Precautions used and data analysis. A known complication associated with the PCR technique is contamination of sample RNA and amplification reagents with amplification product. Consequently, separate pipets were used for RNA extraction, PCR amplification, and analysis of amplification product. Ph-negative samples were coextracted with patient samples to monitor cross-contamination, and blank reactions lacking sample RNA were used to monitor PCR reagent contamination. As a quality control measure, we tested several different preparations of ECR with common lots of Taq polymerase and frozen aliquots of K562 RNA. Possibly due to extensive ECR serial dilutions, it was found that different ECR dilutions gave twofold to fourfold differences in titration results. To minimize this, ECR was diluted in a solution containing Escherichia coli (RNA (100 Ìg/mL) as carrier, and aliquots of a single ECR dilution series were used for comparative analysis of samples from a given patient. Titration results were also standardized against a common K562 RNA preparation to directly compare results from different patients. Confidence in competitive PCR amplification results was increased by analyzing simultaneous PB and BM samples, and at least two independent titration experiments were performed on each sample.

RESULTS

We synthesized an exogenous competitor RNA, termed ECR (Fig 1), capable of specifically competing during PCR amplification with the b3a2 (Fig 2) and b2a2 (Fig 3) type bcr-abl fusion messages prevalent in CML. The ECR used was identical to the junction region of the b3a2 type bcr-abl fusion mRNA except for three mutated bases at the b2a2 junction, creating a novel Xho1 restriction endonuclease recognition sequence used to distinguish, by agarose gel electrophoresis, the otherwise similar ECR and b3a2 amplification products. We quantified expression of bcr-abl fusion message during the amplification of the ECR, and a 289-bp product from the b2a2 bcr-abl fusion message.

![Fig 1. Diagram of the bcr-abl-specific ECR template. The ECR template is identical to a 520 nucleotide region of the b3a2 type bcr-abl fusion message except for three mutated bases (indicated by asterisks) at the bcr-abl junction, which generate an Xho1 recognition sequence (CTCGAG). T7, T7 RNA polymerase promoter. bcr 1B and abl 2D indicate positions of the second primer set used during PCR amplification. These primers yield a 385-bp amplification product from both the b3a2 bcr-abl fusion message and the ECR, and a 289-bp product from the b2a2 bcr-abl fusion message.](www.bloodjournal.org)
Fig 2. Quantification of bcr-abl fusion message in the Ph-positive cell line K562. Varying amounts of ECR molecules were coamplified with 1, 0.1, or 0.01 ng of total K562 RNA, mixed with 1 µg of RNA from the Ph-negative Daudi B-cell line. Such dilutions correspond to approximately 100, 10, or 1 K562 cells in 10⁶ Ph-negative cells, respectively. A 10:7:5:2:1 dilution series of ECR molecules was used in the titrations and the product of each of the PCR amplifications was treated with Xho I restriction endonuclease, which cleaves ECR but not b3a2. After Xho I digestion, the products were electrophoresed in separate lanes on 1.8% agarose gel, stained with ethidium bromide, and photographed. The b3a2 amplification product (363 bp) and the two Xho I fragments derived from cleavage of the ECR-amplified product (235 bp and 128 bp) are marked in the figure. The equivalence point of ECR titrations was considered as the point at which the ethidium bromide fluorescence intensities of the b3a2 fusion message and the two ECR Xho I restriction fragments were roughly equivalent after agarose gel electrophoresis. Arrows indicate the equivalence points in the ECR titration. Lane M = 123-bp DNA ladder (GIBCO BRL).

Fig 3. Quantification of b2a2 type bcr-abl fusion message. Lanes 1 and 2, ECR and b2a2 control amplification products, respectively. Lanes 3 through 11, reactions containing 0.3 µg of PB RNA from case 2 (Table 1), +162 days post-BMT, and 10⁶, 3 x 10⁷, 10⁸, 3 x 10⁸, 10⁹, 3 x 10⁹, 10¹⁰, 3 x 10¹⁰, and 10¹ⁱ molecules of ECR, respectively. Because the 75 nucleotide bcr exon 3 is absent in the b2a2 type fusion message, the smaller b2a2 amplification product migrated faster than the ECR product in agarose gels (Fig 5), eliminating the need for the Xho I digestion step that was used to distinguish ECR and b3a2 amplification products. The equivalence point of titration is shown in lane 7. The equivalence point obtained from a K562 control titration performed in parallel was 1,000 molecules/0.01 ng K562 RNA (not shown).
before BMT consisted of busulfan (16 mg/kg × 4 days) and cyclophosphamide (60 mg/kg × 2 days).  

Case 1 is a 55-year-old white female, transplanted with unmanipulated (non-T-cell depleted) marrow from her identical twin sister during the initial chronic phase of the disease. Such syngeneic BMTs have been shown to have a higher incidence of relapse.  

Time of sample collection ranged from 23 days to 10 months after BMT. The patient expressed a b3a2 type bcr-abl fusion message and such transcript increased more than 10-fold between 1 and 4.5 months, with further increases 6 and 10 months after BMT (Table 1, Fig 5). While bcr rearrangement was not detected by Southern blotting in PB or BM at 4.5 months post-BMT (Table 1, Fig 6), the increase in expression of bcr-abl mRNA detected by competitive PCR analysis was supported by the finding of 2 Ph-positive cells among 64 metaphases in BM at 4.5 months post-BMT, using methotrexate-synchronized cells (Table 1). These results, in a syngeneic BMT, strongly suggested that the patient was in the early stages of relapse at 4.5 months BMT, even though she was in complete clinical remission.

Case 2 is a 24-year-old white female that was in the accelerated phase of CML before being transplanted with unmanipulated marrow from an HLA-matched sibling. The patient has remained in clinical remission for 1.5 years after BMT. Nevertheless, while bcr gene rearrangement could not be detected by Southern blotting, 1 in 60 and 1 in 84 Ph-positive cells were detected in cell-synchronized, non-stimulated BM at 4 and 5.5 months after BMT, respectively (Table 1). The patient showed a quantifiable expression of

Table 1. Residual Disease in Two CML Patients After BMT

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age/Sex</th>
<th>bcr-abl Junction Type</th>
<th>Time (d)</th>
<th>Disease Phase</th>
<th>Tissue</th>
<th>Ph+ Metaphases/</th>
<th>Southern Blot</th>
<th>bcr-abl mRNA (% relative to K562 control)*</th>
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<tbody>
<tr>
<td>1</td>
<td>55/F</td>
<td>b3a2</td>
<td>−47 BMT</td>
<td>Chronic</td>
<td>PB</td>
<td>20/20</td>
<td>+++</td>
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<td></td>
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<td>CR</td>
<td>PB</td>
<td></td>
<td></td>
<td>0.002</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>+132 BMT</td>
<td>CR</td>
<td>BM</td>
<td>0/20</td>
<td>−</td>
<td>0.008</td>
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<td></td>
<td></td>
<td></td>
<td>+180 BMT</td>
<td>Partial relapse</td>
<td>PB</td>
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<td>+</td>
<td>0.6</td>
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<td></td>
<td></td>
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<td>Relapse</td>
<td>BM</td>
<td>13/50</td>
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<td>1.2</td>
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<td>+++</td>
<td>5.0</td>
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<td>−45 BMT</td>
<td>Accelerated</td>
<td>BM</td>
<td>20/20</td>
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<td></td>
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<td>CR</td>
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<td>1/60</td>
<td>−</td>
<td></td>
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<td></td>
<td></td>
<td>+162 BMT</td>
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<td>1/84</td>
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<td>−</td>
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<td></td>
<td></td>
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<td>BM</td>
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<td>&lt;0.001†</td>
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<td>CR</td>
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<td></td>
<td></td>
<td>BM</td>
<td>0/100</td>
<td>−</td>
<td>ND</td>
</tr>
</tbody>
</table>

Case 1 treated with a syngeneic BMT; case 2 treated with an allogeneic BMT.

Abbreviations: CR, complete clinical remission; ND, none detected.

*Results were normalized against pretested frozen aliquots of K562 RNA run in parallel with patient sample RNA.

†Positive in one of three experiments.
QUANTIFICATION OF RESIDUAL DISEASE

Pre-BMT

+23

Flg

5.

ibcrJibl

expression In co" BM sam-

ples from

casa

Competitive PCR amplification was

performed as In Fig 2; 0.3 μg of patient sample RNA

was used in each lane, except for the ECR control lane. The arrows point out the equivalence points

obtalned from titrations of a PB sample collected

before BMT, and BM samples collected at indicated
days after BMT. The pre-BMT sample showed an

average of 22 molecules per nucleated cell, which

represents about 9% of that found in the K562 cell

line (Fig 2).

Days After BMT

Pre-BMT

+132

+180

+300

PB BM PB BM PB BM

Fig 5. bcr- abl expression in consecutive BM sam-

ples from case 1. Competitive PCR amplification was

performed as in Fig 2; 0.3 μg of patient sample RNA

was used in each lane, except for the ECR control lane. The arrows point out the equivalence points

obtalned from titrations of a PB sample collected

before BMT, and BM samples collected at indicated
days after BMT. The pre-BMT sample showed an

average of 22 molecules per nucleated cell, which

represents about 9% of that found in the K562 cell

line (Fig 2).

Flg

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PB and

BM samples from cam 1

collected

More and at different times after

BMT. Approximately 4 μg of total DNA was subjected to Southern

blot hybridization as described in Materials and Methods, using the

6cr

probe that hybridizes to the major breakpoint cluster reglon

of

the

kr

gene. Rearranged bands

indicated by arrows. For details,

see text and Table 1.

Such expression decreased dramatically at 9 months

after BMT, falling below the accurate quantitative range of

the competitive assay (under 100 molecules/μg of total

RNA), but remaining detectable by standard PCR amplifi-

cation. At 12 months, only one of the triplicate experiments

showed the presence of bcr- abl fusion message by standard

PCR amplification, and expression of bcr- abl fusion mes-

sage could not be detected in two additional PB and BM

samples studied in triplicate at 14 and 18 months post-

BMT. Such suppression of bcr- abl mRNA expression was

concurrent with the loss of detectable Ph-positive cells by

extensive cytogenetic analysis (Table 1), and is probably

indicative of a gradual GVL effect.

DISCUSSION

Chemotherapy and radiotherapy, with or without BMT,

have achieved remarkable complete clinical remissions in

many hematologic malignancies. Nevertheless, despite the

absence of any abnormal clinical or laboratory markers, it is

not uncommon for patients to relapse, due to the presence

of minimal residual disease. Recently, using the very

sensitive PCR technique, it has been possible to detect such

residual disease. However, the usefulness of the conven-

tional PCR amplification strategy for detecting residual

disease activity after BMT has been controversial, because

it is unclear whether positive signals during the first 6

months after treatment derive from leukemogenic cells or

from nonproliferating Ph-positive lymphocytes or granulo-

cytes surviving the initial marrow-ablative regimen.

The competitive PCR titration strategy used in the

present study could help predict disease relapse, because

residual proliferating leukemic cells are expected to give

rise to increases in expression of bcr- abl fusion message

over time. Furthermore, although expression of bcr- abl

fusion message has been shown to vary among different

patients, the analysis of consecutive samples in a given
patient provides a built-in mechanism to determine relative decreases or increases in expression of the bcr-abl message (Table 1, Fig 5).

It is known that CML patients treated with syngeneic or autologous BMTs, as well as patients treated with BMT after the accelerated or acute blast phase of CML, all have a high risk of relapse.\(^2\) In these patients, there is a need to have available a sensitive and quantitative method to distinguish patients relapsing from those exhibiting a gradual reduction in leukemic activity beyond the initial 6 months after BMT. The two patients described in detail illustrate the usefulness of this approach, because in case 1 relapse was identifiable early after treatment, while the patient was in clinical remission after syngeneic BMT; while in case 2 a decrease in leukemic activity was measurable between 6 and 12 months post-BMT. There is now a need to study a large number of high-risk BMT patients to have available a sensitive and quantitative method to illustrate the usefulness of this approach, because in case 1 carefully evaluate the combined usefulness of the competitive PCR and refined cytogenetic methodologies used in this work, to objectively predict survival, and to tailor the use of new therapeutic modalities to achieve higher cure rates. The methodology used here should prove applicable to the study of residual disease in other hematologic malignancies that express distinctive mRNAs. Examples include most lymphoid malignancies, because they often express clonally rearranged antigen receptors, as well as t(14;18)-positive follicular lymphomas expressing a bcl-2/\(\Delta F\) fusion message, acute promyelocytic leukemias with the characteristic t(15;17) that express abnormal retinoic acid receptor mRNAs, T-cell leukemias expressing SIL-SCL fusion transcripts, and t(1;19) B-cell leukemias that express the E2a-PBX1 fusion message. In addition, the PCR technique can be used to amplify and possibly quantify DNA regions of the Ig or T-cell receptor genes that are rearranged in B- or T-cell leukemias.\(^6\)

ACKNOWLEDGMENT

We are grateful to Drs S. Bulowa, P. Crilley, and D. Topolsky for their procurement of patient samples.

REFERENCES


Molecular quantification of residual disease in chronic myelogenous leukemia after bone marrow transplantation

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