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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1629

THE PHILADELPHIA (Ph) chromosome, the result of a translocation between the abl proto-oncogene on chromosome 9 and the bcr gene on chromosome 22, is found in more than 95% of chronic myelogenous leukemias (CMLs), and also between 10% and 25% of acute lymphoblastic leukemias (ALLs). In virtually all Ph-positive CMLs, and about half of the Ph-positive ALLs, the leukemic cells express bcr-abl fusion messenger RNAs (mRNAs) in which either exons “2” (b2a2 junction) or “3” (b3a2 junction) from the major breakpoint cluster region of the bcr gene are spliced to exon 2 of the abl gene. The b3a2 and the b2a2 fusion mRNAs both encode 210-Kd chimeric bcr-abl proteins with oncogenic activity. In the remaining cases of Ph-positive ALLs, the first exon of the bcr gene is spliced to exon 2 of abl, leading to the production of a smaller 185-Kd fusion protein.

Bone marrow transplantation (BMT) is presently the most effective curative treatment of CML. In many cases, the mechanism of cure does not appear to derive solely from disease eradication during the initial marrow-ablative treatment, but from an additional progressive antileukemic effect of the donor marrow, termed the graft-versus-leukemia (GVL) effect. This finding is supported by the results from studies using the conventional polymerase chain reaction (PCR) amplification technique to detect the presence of bcr-abl fusion message, which have shown that Ph-positive cells often persist in CML patients after BMT. The GVL effect may be more prevalent in allogeneic BMTs, which exhibit significantly higher cure rates than either syngeneic BMTs, or BMTs performed with T-cell-depleted marrow as a prophylaxis for graft-versus-host disease (GVHD).

The prognostic value of detecting bcr-abl fusion message by conventional PCR amplification after BMT remains a central clinical question because not all patients that are PCR-positive after treatment progress to clinical relapse. A recent study has suggested that patients in which expression of bcr-abl mRNA is detected more than 6 months after BMT may have a reduced probability for cure. Based on this report, residual leukemic activity should decline sometime during the first 6 months after BMT in most cured patients. Nevertheless, a detailed study of disease activity after BMT has not been possible previously due to the lack of an appropriate assay to quantitatively analyze low numbers of leukemic cells.

Cytogenetic and Southern blotting techniques routinely used to diagnose CML may not detect leukemic cells comprising less than 1% of the total cell population, and therefore lack the sensitivity necessary to quantitatively study minimal residual disease. On the other hand, conventional PCR amplification of bcr-abl fusion message can detect one Ph-positive cell in a pool of up to 10^9 to 10^10 normal cells in dilution experiments, but it is not quantitative when unknown patient samples are tested.

To effectively monitor residual leukemic activity after BMT, we used a competitive PCR titration technique to quantify expression of bcr-abl fusion message in peripheral blood (PB) and BM of CML patients after BMT. Our data suggest that this technique can provide a means to either detect relapse early after treatment, or to monitor the progressive disappearance of disease in CML, while the patients are in complete clinical remission.

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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.27 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 primer bcr1A: AGTTCACAGCTTCCTGATCTC and abl2C: TTATCTCCACTGGCCACAAA (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.46 They included primers bcr1B: TCTGACTATGAGCGTGCAGA and abl2D: AGTGCTGCTCAGGTTCCAGG.

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One group of patients was used to establish the titration curve. For each PCR amplification, the PCR amplification product was visualized by electrophoresis and bands visualized with ethidium bromide staining. We also included the product of a known positive control (K562 cell line) and a known negative control (Ph-negative lymphocyte). We also included an internal control of a known bcr-abl fusion reaction product (K562 cell line) and a known negative control (Ph-negative lymphocyte).

RESULTS

We synthesized an exogenous competitor RNA, termed ECR (Fig 1), capable of specifically competing during PCR amplification with the b3a2 type bcr-abl fusion message prevalent in CML. The ECR used was identical to the junction 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 Xho I 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 288-bp product from the b2a2 bcr-abl fusion message.
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 \(\mu\)g of RNA from the Ph-negative Daudi B-cell line. Such dilutions correspond to approximately 100, 10, or 1 K562 cells in \(10^6\) 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).

A comparative cytogenetic, Southern blotting, and \(bcr-abl\) fusion mRNA expression analysis was performed on consecutive PB or BM samples from two high-risk CML patients after BMT. The marrow ablative regimen used high expression of the b3a2 type of \(bcr-abl\) fusion message. In reconstitution experiments, b3a2 mRNA was quantified in as little as 1 cell equivalent (10 pg) of K562 RNA in an admixture with 1 \(\mu\)g of RNA (equivalent to approximately \(10^6\) cells) from a Ph-negative cell line. With this ECR titration preparation, an average of approximately 250 molecules of the ECR was required to titrate \(bcr-abl\) mRNA present in one cell equivalent of K562 RNA (Fig 2). Although the precision of the competition strategy used is limited by the magnitude of the dilutions used in the ECR titrations, a reasonably good accuracy was achieved in the present study using a 10:7:5:2:1 dilution series spanning two orders of magnitude (Fig 2). This is further illustrated in Fig 4, in which similar results were obtained from titration experiments of two separate RNA preparations, extracted 6 months apart from a frozen PB lysate of a CML patient 6 months post-BMT (Table 1, case 1).

A comparative cytogenetic, Southern blotting, and \(bcr-abl\) fusion mRNA expression analysis was performed on consecutive PB or BM samples from two high-risk CML patients after BMT. The marrow ablative regimen used
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/ Total</th>
<th>bcr Southern Blot</th>
<th>bcr-abl mRNA (% relative to K562 control)*</th>
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<tr>
<td>1</td>
<td>55/F</td>
<td>b3a2</td>
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<td>ND</td>
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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.
Fig 5. bcr-abl expression in consecutive BM samples 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 obtained 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).

Fig 6. Southern blot bcr gene rearrangement analysis of PB and BM samples from case 1 collected before 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 bcr -3 probe that hybridizes to the major breakpoint cluster region of the bcr gene. Rearranged bands are indicated by arrows. For details, see text and Table 1.

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 conventional 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 granulocytes 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. 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 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/IgH 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.

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REFERENCES

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