Comparison of Genomic DNA and cDNA for Detection of Residual Disease After Treatment of Chronic Myeloid Leukemia With Allogeneic Bone Marrow Transplantation

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To test whether patients in remission after allogeneic bone marrow transplantation (BMT) possess a pool of chronic myeloid leukemia (CML) cells that do not express BCR-ABL mRNA, we have compared the results and sensitivity of amplification of BCR-ABL from genomic DNA with conventional reverse transcription-polymerase chain reaction (RT-PCR). Bubble PCR was used to amplify the genomic BCR-ABL translocation breakpoints from chronic-phase DNA of 10 patients with CML who subsequently underwent BMT. After cloning and sequencing of the amplification products, patient-specific ABL primers were synthesized and tested for both specificity and sensitivity in nested or heminested combinations with a variety of primers derived from the major breakpoint cluster region of the BCR gene. In all cases, combinations of primers were selected that enabled the detection of chronic-phase DNA from a specific patient at up to a 10^6 dilution into DNA from a normal individual. Patterns of residual disease obtained by serial RT-PCR and DNA-PCR analyses of blood and bone marrow samples obtained after BMT were similar for most patients, including one treated for relapse by infusion of donor leukocytes. Of the 24 samples for direct comparison of RT-PCR and DNA-PCR, results were concordant in 19 (79%) cases. Five results were discordant. In two instances, RT-PCR was positive, while PCR from genomic DNA was negative; this discrepancy might have arisen due to the slightly greater sensitivity of RT-PCR compared with DNA-PCR. In three samples from three patients, two of whom had been transplanted in the accelerated phase, PCR from genomic DNA was positive while RT-PCR was negative; this could mean that some CML cells in these samples had a reduced or absent capacity to express BCR-ABL mRNA post-transplant. Of these three patients, one subsequently relapsed; and two are in remission at 21 and 24 months after the discordant result. Thus, the finding of a single DNA-PCR-positive, RT-PCR-negative result does not necessarily predict relapse. Because the great majority of samples (79%) gave concordant results with the two assays, we believe that patients in remission do not generally harbor a substantial pool of CML cells that do not express BCR-ABL mRNA.

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Materials and Methods

Patients. Ten patients with CML who had undergone allogeneic BMT were studied. At the time of transplant, eight patients were in chronic phase, and two were in accelerated phase. Four patients received grafts from HLA-identical sibling donors and six from unrelated volunteer donors. The conditioning regimen in all groups included cyclophosphamide 120 mg/kg and fractionated total body irradiation to a total dose of 10, 12, or 13.2 Gy at a dose rate of 15 Gy/min. All patients received cyclosporin A (CSA) plus methotrexate (MTX) as graft-versus-host disease prophylaxis, and those with unrelated donors also underwent in vivo T-cell depletion using Campath 1G. One patient relapsed and was treated by donor leukocyte transfusion (DLT).

Cytogenetic analysis. Cytogenetic analysis of marrow cells was performed using standard methods.
performed routinely on all patients at 3, 6, 9, and 12 months post-transplant and subsequently at 6-month intervals whenever possible. At least 30 consecutive metaphases were studied. All patients were Ph-positive before BMT.

RT-PCR for BCR-ABL. Peripheral blood or bone marrow cells were studied prospectively at various intervals posttransplant. Informed consent was obtained as required by the Declaration of Helsinki. After extraction of leukocyte RNA, samples were tested for the presence of BCR-ABL mRNA by reverse transcription followed by nested PCR. BCR-ABL transcripts were quantified in 15 of the 36 RT-PCR-positive specimens by competitive PCR. Results were standardized by deriving the number of BCR-ABL transcripts per microgram RNA. PCR-positive samples were scored as having less than 10 BCR-ABL transcripts per microgram RNA if the competitor band predominated at 10 molecules of competitor added. High BCR-ABL levels were defined as 50 transcripts per microgram RNA or greater; detectable but low BCR-ABL levels had less than 50 transcripts per microgram RNA. Using competitive PCR, we have previously demonstrated concordance in levels of residual disease between blood and bone marrow.

Cloning and characterization of BCR-ABL breakpoints. Characterization of genomic DNA breakpoints for patients M.L., D.M., A.H., M.T., A.J., A.L., and A.E. by bubble PCR has been described in detail elsewhere. Breakpoints for patients B.Y., B.M., and R.K. were determined in exactly the same way. Briefly, DNA was extracted from peripheral blood samples collected before BMT. Fragments containing BCR-ABL breakpoints were amplified using a panel of oligonucleotide primers directed against the major breakpoint cluster region (M-BCR) in conjunction with primers complementary to the bubble sequence. Products were cloned into M13mp19 and sequenced. The position of the breakpoint was determined by comparison with the published M-BCR sequence, and either one or two 22-bp to 25-bp primers were synthesized to match the ABL sequence for each patient.

DNA extraction. DNA was extracted from cryopreserved chronic-phase leukocytes as described and from fresh peripheral blood (n = 29) or bone marrow specimens (n = 13) at various times after BMT. In some cases, DNA was extracted from stained marrow slides (n = 33). First, coverslips were removed after soaking the slide overnight in xylene. Using a razor blade, bone marrow material was scraped into 200 μL of 10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 0.5% sodium dodecyl sulfate (SDS). Proteinase K (40 μg) was added, and the sample was incubated at 65°C overnight. After extraction with phenol/chloroform, DNA was precipitated and resuspended in 10 μL to 50 μL water. DNA was quantified using a spectrophotometer, and the concentration and integrity were confirmed by agarose gel electrophoresis. Quality was confirmed by amplification of a 318-bp BCR exon 1 fragment from 500 ng genomic DNA with primers BCR-B (5′-CCCCGGAGGTCTGGGTGATCTG 3′) and BCR-D (5′-ATGGAAAGGCCCGGTGGCATC 3′) for 30 cycles of 96°C for 30 seconds, 64°C for 50 seconds, and 72°C for 1 minute. All DNA samples resulted in the amplification of a clear product of the expected size after a single-step PCR and were, therefore, considered to be of sufficient quality for analysis of residual disease.

Genomic DNA-PCR for BCR-ABL. Optimal PCR conditions and combinations of ABL and M-BCR primers were determined to yield specific amplification of BCR-ABL from chronic-phase DNA of each patient. For nested or heminested PCRs, an initial amplification reaction was performed with a pair of patient-specific primers. One microliter of product was reamplified in a second PCR using either a pair of patient-specific primers internal to the first set or the same ABL primer plus an internal M-BCR primer. Chronic-phase DNA was serially diluted with DNA of a normal individual to test the sensitivity of the assay for each individual. For analysis or residual disease, the first-step PCR was seeded with 1 to 2 μg genomic DNA. After reamplification with nested or heminested primers, products were fractionated on ethidium bromide-stained agarose gels, and results were scored as positive or negative. For both RT-PCR and DNA-PCR, rigorous precautions were taken to prevent contamination of samples and reaction components by PCR products. At least two negative controls were performed along with each sample, none of which resulted in an amplification product.

RESULTS

Combinations of ABL and M-BCR primers enabled the BCR-ABL breakpoints to be detected in chronic-phase DNA from each of the 10 patients studied. As expected, primer pairs were patient-specific; ie, a particular combination only resulted in an amplified product from a single patient (Fig 1).

Chronic-phase DNA from each patient was diluted into normal DNA and subjected to two-step PCR using either nested or heminested combinations of patient-specific primers. Using 100 ng genomic DNA as a template, the 10⁻¹ dilution usually resulted in a specific amplification product, but at the 10⁻³ dilution, BCR-ABL was not detectable. By increasing the amount of template DNA to either 2 or 5 μg, an amplification product was obtained from the 10⁻³ dilution (Fig 2). A specific product was obtained using a 10⁻⁴ dilution for all patients when 500 ng of genomic DNA was amplified.

For the 10 patients after BMT, a total of 75 DNA speci-
mens from 61 timepoints were analyzed by two-step PCR for BCR-ABL. Twenty seven timepoints were DNA-PCR–positive, and 34 were DNA-PCR–negative. At 49 timepoints, a single sample was analyzed, whereas for 12 timepoints, either two or three independent samples (total = 26) were analyzed. Complete concordance was found between results of contemporaneous DNAs extracted from fresh peripheral blood, fresh bone marrow, and stained bone marrow slides.

For most patients, the patterns of serial DNA-PCR results were similar to those described for RT-PCR (Fig 3). Patients M.L., B.M., and A.J. were initially DNA-PCR–positive after BMT but were subsequently persistently DNA-PCR–negative. Patient B.M. was RT-PCR–negative during the period of DNA-PCR positivity, while patients M.L. and A.J. were not studied by RT-PCR when they were DNA-PCR–positive. Patients D.M. and A.H. were persistently DNA-PCR– and RT-PCR–positive. Patients B.Y., M.T., A.L., and A.E. were persistently DNA-PCR–negative; RT-PCR assays were also negative except at a single timepoint for three of these individuals. Patient R.K. was persistently DNA-PCR–positive for 2 years after BMT. RT-PCR was initially negative but subsequently converted to positive. This patient was in cytogenetic remission for the first year, but at 21 months, 17% Ph-positive metaphases were detected in the marrow, rising to 40% Ph-positive metaphases at 24 months. Treatment for relapse with DLT was initiated at 21 months post-BMT. At 4 months later, Ph-positive metaphases were no longer detectable, and no residual disease was detected by either DNA-PCR or RT-PCR. Subsequent PCR assays were also negative.

Quantitative RT-PCR results have been described in detail elsewhere. Results are shown in Fig 3 only for those RT-PCR–positive datapoints that had low levels of BCR-ABL mRNA (less than 50 transcripts/μg RNA). All other RT-PCR–positive datapoints had high levels of BCR-ABL (greater than 50 transcripts/μg RNA), except for one positive specimen that was not quantified. Twenty-four paired samples were taken on the same day for analysis by RT-PCR and DNA-PCR and were, therefore, available for direct comparison. Nineteen samples were concordant, of which nine were RT-PCR– and DNA-PCR–negative and 10 were RT-PCR– and DNA-PCR–positive. Of the 10 that were positive, six had high levels of BCR-ABL mRNA (greater than 50 transcripts/μg RNA), and four had low levels. Five samples were discordant. Of these, three were DNA-PCR–positive and RT-PCR–negative. For these samples, RT-PCR was repeated in triplicate: one was persistently negative, and two yielded a single positive result. Two samples were DNA-PCR–negative and RT-PCR–positive, of which one had a low level of BCR-ABL mRNA and one was not quantified (Fig 4).

Cytogenetic analysis was performed on 18 of the 34 samples that were DNA-PCR–negative and 15 of the 27 samples that were DNA-PCR–positive. No Ph-positive metaphases were detected in any of the DNA-PCR–negative samples or in any of the 11 samples that was RT-PCR–negative. Of the 15 samples that were DNA-PCR–positive, only two had detectable Ph-positive metaphases. Both samples were also RT-PCR–positive.

**DISCUSSION**

Theoretically, DNA-based PCR has some advantages over RT-PCR for analysis of residual disease. Extraction of DNA from patient samples is technically easier than extraction of RNA followed by synthesis of cDNA. Genomic DNA-PCR would result in amplification of BCR-ABL whether the fusion gene was actually expressed or not. Moreover, the use of patient-specific primers rather than a common set of primers for all patients may help to minimize the problem of PCR contamination. In CML, however, the t(9;22) breakpoints are dispersed over a very wide region of genomic DNA, particularly within the ABL gene. Thus, to amplify BCR-ABL from genomic DNA, it is necessary to clone and sequence the breakpoint from each patient and to design patient-specific oligonucleotide primers. Recently, we have described the use of bubble PCR to facilitate cloning of BCR-ABL breakpoints. While this technique is considerably easier than the traditional method of constructing a genomic DNA library from each patient, it does, nevertheless, involve an appreciable amount of time and effort. Furthermore, as shown elsewhere, we were only able to amplify
DNA VERSUS cDNA FOR DETECTION OF MRD IN CML

Fig 3. DNA-PCR, RT-PCR, and cytogenetic results of the 10 patients after BMT. Open ellipses indicate either PCR negativity or Ph-negativity. Solid ellipses indicate either PCR positivity or the presence of Ph-positive metaphases in the marrow. Stippled ellipses indicate the two cDNA samples that were repeated in triplicate and yielded a single positive result. Patient R.K. was treated for relapse with DLT at 21 months post-BMT. Competitive RT-PCR results are shown for those samples that were PCR-positive but had low levels of BCR-ABL mRNA (less than 50 transcripts/ug RNA). All other RT-PCR-positive datapoints had high levels of BCR-ABL mRNA (greater than 50 transcripts/ug RNA), except for one that was not quantified (nq).

Published reports have suggested that not all CML cells actively express the BCR-ABL fusion gene. Purified CD34-positive CML cells were shown to be RT-PCR-negative despite possessing the fusion gene. Similarly, some Ph-positive myeloid colonies were found to be BCR-ABL mRNA-negative. While results of the latter study have not been confirmed, and both studies can be criticized on the grounds of inadequate controls for cDNA synthesis, it is possible that CML cells that do not express BCR-ABL mRNA do, in fact, exist. Such cells could potentially lead to false-negative assessment of residual disease in patients after treatment.

We have found that DNA-PCR can detect BCR-ABL when chronic-phase DNA from a particular individual is diluted into normal DNA up to a concentration of $10^{-3}$. RT-PCR is generally considered to be slightly more sensitive, capable of detecting a single CML cell in the background of $10^{5}$ to $10^{6}$ normal cells. The reason for the difference in maximum sensitivity is presumably that each CML cell carries a single copy of the fusion gene but, under steady-state conditions, may contain multiple copies of BCR-ABL mRNA. It might be expected, therefore, that at low levels of residual disease, RT-PCR could give a positive result, while DNA-PCR would be negative. If high levels of residual disease were found by RT-PCR, then DNA-PCR would also be expected to be positive, unless the positivity was due.
Fig 4. Concordance and discordance of DNA-PCR and RT-PCR on contemporaneous samples. Of the three samples that were DNA-PCR–positive, RT-PCR–negative on initial analysis, two yielded a single RT-PCR–positive result when repeated in triplicate, and one was persistently RT-PCR–negative. Pos, positive; Neg, negative.

respectively, after the discordant result. Thus, a single DNA-positive, RT-PCR–negative result is not necessarily an indicator of subsequent relapse. Interestingly, the two patients who did not relapse were transplanted while in the accelerated phase. This suggests that accelerated-phase CML cells, at least after BMT, might be more likely to harbor a transcriptionally silent BCR-ABL fusion gene than chronic-phase cells. As neither patient has relapsed, it is possible that the malignant cells were damaged by the transplant ablative regimen such that they did not express typical levels of BCR-ABL mRNA, but were still able to survive for an unusually long time posttransplant.

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12. Delage R, Soiffer RJ, Dear K, Ritz J: Clinical significance of to a very small number of CML cells expressing high levels of BCR-ABL mRNA. Conversely, if there were CML cells that did not express BCR-ABL mRNA, then DNA-PCR could be positive and RT-PCR negative.

Overall, we have found that for most patients the patterns of PCR results on serial analysis after BMT for CML were similar when tested with RT-PCR or DNA-PCR. On initial analysis of contemporaneous samples, we found that 19 of 24 (79%) gave concordant RT-PCR and DNA-PCR results. However, after repeating RT-PCR analysis for the three RT-PCR–negative, DNA-PCR–positive samples, 21 of 24 (88%) samples were concordant. This high degree of concordance suggests that patients in remission do not generally harbor a substantial pool of leukemia cells that do not express BCR-ABL mRNA. As concordance was found at both high and low levels of BCR-ABL mRNA, we believe it is unlikely that some CML cells express unusually high levels of fusion gene transcripts. Of the discordant results, two were RT-PCR–positive and DNA-PCR–negative. One of these RT-PCR samples was quantified and found to contain low levels of BCR-ABL mRNA; the other sample was not quantified. It is likely that these discrepancies result from the slightly superior detection level of RT-PCR over DNA-PCR.

Of the three samples that were DNA-PCR–positive, RT-PCR–negative, only one was persistently RT-PCR–negative when the analysis was repeated in triplicate. The cDNAs from all three samples appeared to be of good quality as judged by amplification of the normal ABL gene by single-step PCR, and, given the greater sensitivity of RT-PCR over DNA-PCR, it is possible that these specimens did harbor CML cells that were not actively expressing BCR-ABL mRNA. Alternatively, because the maximum sensitivity of the PCR assays cannot be absolutely guaranteed for every sample, the discordance could have arisen due to random variation. Of the three patients who had a DNA-positive, RT-PCR–negative sample on initial analysis, one subsequently relapsed and two are in remission at 21 and 24 months, respectively, after the discordant result. Thus, a single DNA-positive, RT-PCR–negative result is not necessarily an indicator of subsequent relapse. Interestingly, the two patients who did not relapse were transplanted while in the accelerated phase. This suggests that accelerated-phase CML cells, at least after BMT, might be more likely to harbor a transcriptionally silent BCR-ABL fusion gene than chronic-phase cells. As neither patient has relapsed, it is possible that the malignant cells were damaged by the transplant ablative regimen such that they did not express typical levels of BCR-ABL mRNA, but were still able to survive for an unusually long time posttransplant.


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