Detection of Residual Leukemia After Bone Marrow Transplant for Chronic Myeloid Leukemia: Role of Polymerase Chain Reaction in Predicting Relapse

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We used the polymerase chain reaction (PCR) to detect residual leukemia-specific mRNA in blood and marrow from 37 patients in complete hematologic and cytogenetic remission after allogeneic bone marrow transplant (BMT) for chronic myeloid leukemia (CML). Our two-step PCR method involved the use of "nested primers" in the second step and could detect one K562 cell diluted into 10^5 normal cells. Elaborate measures were taken to exclude false-positive and false-negative results. In nine patients whose blood and marrow were studied simultaneously the results were concordant (two positive and seven negative). Twenty-three patients transplanted in chronic phase (CP) with unmanipulated donor marrow were studied. Blood cells from nine of these patients were studied 3 to 6 months post-BMT and six were PCR positive; three were negative on subsequent studies. Blood cells from 18 patients studied between 8 months and 8 years post-BMT were all PCR negative. Nine patients transplanted in CP with T-cell–depleted marrow cells were studied. Blood from five was positive 3 to 24 months post-BMT; blood from five was negative 3 to 6 years post-BMT. Four patients no longer in first CP were studied after BMT with unmanipulated donor marrow. Blood from all four was positive 5 to 19 months post-BMT. Based on the known clinical results of transplant in these three cohorts we conclude that PCR may be positive within 6 months of BMT in patients who can expect long-lasting remission, whereas PCR positivity later after BMT may indicate that the probability of cure is reduced. Thus, the technique may prove useful for early assessment of new transplant protocols that might inadvertently increase the risk of relapse.

THE PROBABILITY OF RELAPSE at 4 years for patients with chronic myeloid leukemia (CML) in chronic phase (CP) who receive unmanipulated marrow cells from HLA-identical sibling donors after high-dose chemoradiotherapy is 10% to 20%.[1,2] In contrast, the probability of relapse at 4 years for patients who receive marrow that has been T-cell depleted in vitro to prevent graft-versus-host disease (GVHD) is 40% to 60%.[1] Thus, new regimens designed to improve survival must control GVHD without significantly increasing relapse. It would be valuable if one could monitor patients posttransplant in a manner that permitted early recognition of protocols that inadvertently increased the risk of relapse.

In Philadelphia (Ph) chromosome-positive CML the unique chimeric gene formed by fusion of the BCR gene on chromosome 22 with the ABL gene from chromosome 9 represents a leukemia-specific sequence that can be used to identify residual leukemia after bone marrow transplantation (BMT). The polymerase chain reaction (PCR) could theoretically be used to amplify DNA across the fusion point on the chimeric BCR/ABL gene, but for practical reasons it is easier to amplify (by PCR) the BCR/ABL mRNA.[3-5] This mRNA includes the B2 or the B3 exon from the M-BCR region of BCR and the A2 exon from ABL to form the so-called B2A2 or B3A2 fusion mRNAs, respectively. This provides a highly sensitive technique for detection of residual leukemia but has been associated often with false positive and false negative results.[6,7]

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We have previously shown that long-term survivors of BMT with unmanipulated marrow usually lack the fusion mRNA (PCR negative).[8] On the other hand, the fusion mRNA is often detectable (PCR positive) in patients studied early posttransplant. Therefore, we designed a study to determine the patterns of PCR positivity posttransplant in patients treated by different BMT regimens.

MATERIALS AND METHODS

Patients. Three groups of patients were studied: 23 patients with CP CML who received unmanipulated donor marrow and GVHD prophylaxis of cyclosporin alone (nine patients) or cyclosporin and methotrexate (14 patients); nine patients with CP CML who received donor marrow T-cell depleted in vitro using Campath 1M; and four patients who were transplanted in accelerated phase (AP) or second CP (see Table 3 for details). The conditioning regimen in all groups included cyclophosphamide 120 mg/kg and fractionated total body irradiation (TBI) to a total dose of 10 or 12 Gy at a dose rate of 15 cGy/minute. Details of the conditioning regimens have been reported previously.[9] All patients were in complete cytogenetic and clinical remission at the time of the PCR study.

Patients were studied at various intervals posttransplant. Where possible, both blood and bone marrow were studied. Cytogenetic studies were performed on marrow cells from all patients posttransplant at 3, 6, 9, 12, 18, and 24 months posttransplant and annually thereafter. Where possible, at least 20 metaphases were studied.

Statistical methods. Fisher's exact *t*-test was used to correlate relapse with PCR results.

Date of analysis. Results were analyzed as of September 30, 1990.

Preparation of material. A buffy coat was prepared from 20 to 40 mL of peripheral blood by dextran sedimentation. Mononuclear cells (less than 1.077 g/mL) were prepared from marrow by density gradient separation using Lymphoprep (Nycomed, Norway). RNA was prepared by the acid guanidium/phenol/chloroform method.[10] Between 2 to 5 μg of total RNA suspended in 18 μL of annealing buffer (250 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.3, 1 mmol/L EDTA) was mixed with 1 μg of primer A (Fig 1) and heated to 85°C for 3 minutes, incubated at 70°C for 25 minutes, and gradually allowed to cool to 45°C over 25 minutes. The mixture was then incubated at 40°C for 60 minutes after addition of 200 IU of Moloney murine leukemia virus (MMLV) reverse transcriptase

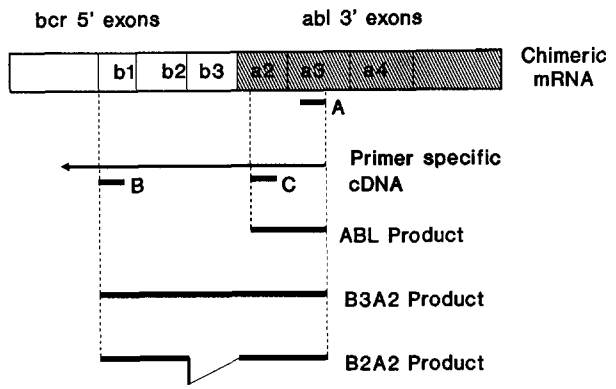

Fig 1. Model showing the chimeric mRNA formed by BCR and ABL genes that join together on chromosome 22. The chimeric mRNA resulting from the B3A2 junction is shown. This chimeric mRNA is used to produce a primer-directed cDNA using antisense primer A (5'-TGTGATTATAGCCTAAGACCCGGAGCTTTT-3'), which is complementary to a sequence on the A3 exon. The cDNA can be amplified either with sense primer B (5'-GAGCGTGCAGAGTGGAGGGAGAACATCCGG-3'), which is complementary to a sequence on the B1 exon, or with sense primer C (5'-TTCAGCGGCCAGTAGCATCTGACTT-3'), which is complementary to a sequence of the A2 exon. Primers A and B will amplify the B2A2 or B3A2 products (these two products differ by 75 base pairs (bp), the size of the B3 exon). Primers A and C will amplify the internal control ABL product (180 bp).

(GIBCO/BRL, Grand Island, NY) and 30 μL of cDNA buffer (24 mmol/L Tris-HCl, pH 8.3, 16 mmol/L $MgCl_2$, 8 mmol/L dithiothreitol [DTT], 0.4 mmol/L each of dATP, dCTP, dGTP, and dTTP). The cDNA was then divided between two tubes each containing 55 μL of PCR buffer (100 mmol/L Tris-HCl, pH 8.8, 30 mmol/L $(NH_4)_2SO_4$, 20 μL of 2.5 mmol/L dNTP solution, and 2.5 U of Taq polymerase (Amplitaq; Cetus, USA). To one half of the cDNA primer C was added, which will result in a positive band from all human RNA and will therefore control for the quality of RNA and successful PCR amplification especially in the absence of any detectable fusion-product amplification. Primer B was added to the other half of the cDNA mix, which will amplify the cDNA derived from the B2A2 and B3A2 transcripts if present. Of this PCR product, 2.5% was added to a second PCR reaction that used nested primers D and E in addition to 10 μL annealing buffer, 15 μL cDNA buffer, 20 μL 2.5 mmol/L dNTP, 55 μL PCR buffer and 2.5 IU of Taq polymerase. These primers will specifically amplify an internal segment of the B2A2 or the B3A2 PCR products derived from the first PCR (Fig 2).

A programmable heating block (MJ Research, MA) was used for the PCR. This block was set to give 35 cycles, the first cycle being 10 minutes at 94°C, 5 minutes at 55°C, and 2 minutes at 72°C, with the remaining cycles being 45 seconds at 94°C, 45 seconds at 55°C, and 60 seconds at 72°C, and the last step being held at 72°C for 10 minutes. PCR samples were then run on an ethidium bromide-stained 1.5% agarose gel.

Using this approach we could detect a single control-positive (K562) cell in 10^5 normal cells. We did not attempt to enhance sensitivity further, as others have done, by Southern blotting and hybridisation to radiolabeled probes.

BCR/ABL negative cells were included in all RNA extraction procedures as negative controls to assess cross contamination between RNA samples. A blank control was added at the cDNA stage that included all reagents except RNA to control for contamination with PCR product in any of the buffers, enzymes, or primers, or cross contamination between tubes. A dilute positive control (RNA prepared in a separate procedure from a cell preparation containing one leukemic cell per 10^4 or 10^5 normal cells) was added at the cDNA stage.

Elaborate measures were taken to minimize contamination. The recommendations of Kwok and Higushi[11] were adopted. All samples were handled with disposable gloves, which were changed after any spillage and at frequent intervals. No samples known to be BCR/ABL positive (except 1 in 10^4 or 10^5 dilution of K562 cells) were handled with post-BMT specimens at any stage. Preparation of blood and marrow samples, RNA extraction, and cDNA and PCR preparation were performed in a laminar flow hood. Positive displacement pipettes were used for preparation and aliquoting of all buffers, primers, enzymes, and specimens. All primers and buffers were stored as aliquots sufficient for one to two experiments only. Eppendorfs containing PCR product were stored and opened in a separate room from the room where samples were handled, and separate pipettes and racks were used in the PCR-designated room. All pipette tips and eppendorfs were autoclaved before use.

Despite these measures, negative controls were positive in 10% to 20% of experiments. To minimise false positives, the criteria for accepting a result as positive were that all negative controls run simultaneously were negative, and that the same transcript was detected in a separate RNA sample taken at the same time but extracted separately from the first sample with a new set of negative controls that also proved negative. When separate experiments gave conflicting results, all data were discarded.

RESULTS

Peripheral blood cells were studied from 23 patients after they received unmanipulated donor marrow while in first CP (group A). Six out of nine studied at 3 or 6 months post-BMT were PCR positive (Table 1, Fig 3). All nine patients studied between 6 and 24 months were PCR negative, as were nine patients studied 3 to 8 years post-BMT. Eight patients were studied on more than one occasion; three who were positive 3 to 6 months post-BMT were negative on subsequent studies 6 to 12 months post-BMT and five who were negative on the first study remained negative on the second study. None of the 23 patients had any subsequent evidence of cytogenetic or clinical relapse with a median follow-up since PCR study of 11 months (range 6 to 15) in those studied within 2 years of

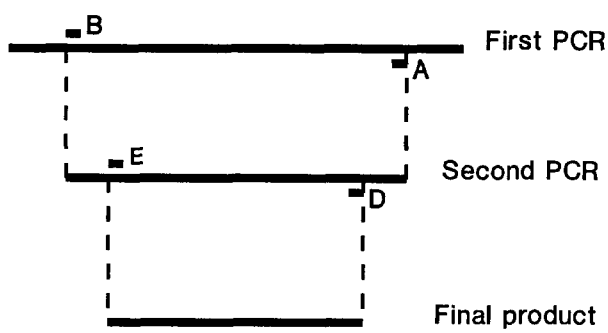

Fig 2. A small aliquot of the first PCR product produced by primers A and B is used for a second PCR that uses antisense primer D (5'-GACCCGGAGCTTTTCACCTTTAGTT-3'), which is complementary to a sequence spanning the junction between ABL exons 2 and 3, and sense primer E (5'-GAAGAAGTGTTTCAGAAGCTTCTCC-3'), which spans the junction between BCR exons 1 and 2. The primers are internal to primers A and B, respectively, to produce a final product of size 379 (B3A2) or 304 bp (B2A2), respectively.

Table 1. Clinical Details of Patients Who Received Unmanipulated Marrow Allografts

Patient No.	BMT Donor	Current Status and Duration of Follow-Up (mo post-BMT)	Results of PCR Studies (mo post-BMT)
1	UD	Rem(9)	B3A2(3)
2	ID SIB	Rem(9)	B3A2(3)
3	ID SIB	Rem(17)	B3A2(3), Neg(9)
4	ID SIB	Rem(16)	B3A2(3), Neg(6,9)
5	ID SIB	Rem(16)	Neg(3)*
6	ID SIB	Rem(18)	Neg(3)
7	ID SIB	Rem(12)	B3A2(6)
8	ID SIB	Rem(18)	B3A2(6), Neg(12)*
9	UD	Rem(17)	Neg(6), Neg(9)
10	ID SIB	Rem(19)	Neg(8), Neg(12)*
11	ID SIB	Rem(19)	Neg(8), Reg(12)
12	ID SIB	Rem(16)	Neg(9)
13	UD	Rem(22)	Neg(12)
14	ID SIB	Rem(23)	Neg(12)*, Neg(15)
15	ID SIB	Rem(43)	Neg(36)
16	ID SIB	Rem(86)	Neg(62)
17	ID SIB	Rem(96)	Neg(72,85)*
18	ID SIB	Rem(98)	Neg(74)
19	ID SIB	Rem(102)	Neg(78)
20	ID SIB	Rem(90)	Neg(78)
21	ID SIB	Rem(109)	Neg(85)
22	ID SIB	Rem(111)	Neg(87)
23	ID SIB	Rem(112)	Neg(88)

All results of PCR studies were from peripheral blood samples. An asterisk (*) indicates that bone marrow was tested simultaneously and gave the same result. Numbers in brackets represent months post-BMT when the test was performed.

Abbreviations: ID SIB, HLA-identical sibling; UD, HLA-matched unrelated donor; Rem, clinical and cytogenetic remission; CyRel, Ph+ marrow metaphases detected post-BMT and remaining detectable on subsequent studies; HemRel, clinical evidence of leukemia recurrence.

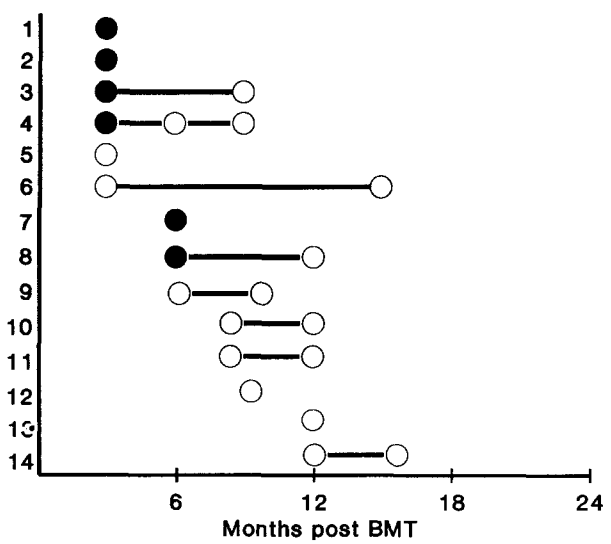

Fig 3. PCR results in the 14 patients receiving unmanipulated marrow while in first CP who received cyclosporin and methotrexate GVHD prophylaxis. The numbers on the vertical axis correspond to the patient number (given in Table 1). Each circle represents a PCR study from the specified patient. Serial studies are linked by a horizontal line. (●) Positive results. (○) Negative results.

BMT and 24 months (range 7 to 24) in those studied more than 2 years post-BMT.

In the group receiving T-cell–depleted donor marrow for CP disease (group B), two patients studied within 6 months of BMT were PCR positive, as were three patients studied between 6 to 24 months post-BMT (Table 2). Two of these three patients (patients 26 and 27), who were both PCR positive at 12 months, relapsed clinically at 18 months and 36 months, respectively. Five patients studied 3 to 5.5 years post-BMT were all PCR negative. None of these five has had subsequent evidence of relapse with a median follow-up of 7 months since PCR study (range 6 to 12 months).

In the group receiving unmanipulated donor marrow while in AP or second CP (group C) all four patients were positive 5 to 19 months post-BMT (Table 3). One patient who was PCR positive at 5 months relapsed cytogenetically at 6 months and hematologically at 9 months. The remaining three have not relapsed with follow-up of 6, 7, and 15 months after PCR study, respectively.

All these results were from blood cells; marrow cells studied simultaneously in nine cases (seven negative, two positive) gave identical results.

Fisher's exact t-test used to assess the value of PCR results to predict relapse in patients studied between 6 and 24 months post-BMT approaches but does not reach significance ($P = .07$, n = 13). However, the follow-up after PCR study in the positive group is longer (median 14 months) than in the negative group (median 7 months), limiting the validity of this analysis.

DISCUSSION

The technical problems encountered when PCR is used to amplify rare sequences are well documented.[7,11] In this study we adhered to a series of precautions to minimize contamination and used controls to detect false-positive and false-negative results. Furthermore, results were only accepted as true positives when they were concordant on at least two separate RNA samples. We have not attempted to increase sensitivity further, although this is technically possible, because the problem of false positives might be accentuated. We observed a high correlation between

Table 2. Clinical Details of Patients Who Received T-cell–Depleted Marrow Allografts

Patient No.	BMT Donor	Current Status and Duration of Follow-Up (mo post-BMT)	Results of PCR Studies (mo post-BMT)
24	UD	CyRel(16)	B3A2(3)
25	UD	Died(10)	B2A2(4)
26	ID SIB	HemRel(33)	B2A2(12)
27	ID SIB	HemRel(40)	B3A2(12)
28	UD	Rem(37)	B3A2(24)*, Neg(32)
29	ID SIB	Rem(43)	Neg(36)
30	UD	Rem(42)	Neg(36)
31	ID SIB	Rem(48)	Neg(42)
32	ID SIB	Rem(72)	Neg(60)
33	ID SIB	Rem(76)	Neg(66)*

See Table 1 for details of abbreviations and symbols.

Table 3. Clinical Details of Four Patients Transplanted in Second CP or AP With Unmanipulated Donor Marrow

Patient No.	CML Status	BMT Donor	BMT Procedure	Current Status and Duration or Follow-Up (mo post-BMT)	Results of PCR Studies (mo post-BMT)
34	AP	ID SIB	Cyclophosphamide/TBI cyclosporin and methotrexate	HemRel(15)	B2A2(5)
35	AP	ID SIB	Cyclophosphamide/TBI cyclosporin only	Rem(34)	B3A2(19)
36	CP*	ID SIB	Cyclophosphamide/TBI cyslosporin and methotrexate	Rem(23)	B3A2(12)
37	CP†	ID SIB	Busulphan 16 mg/kg, cyclosporin and methotrexate	Rem(15)	B3A2(9)

See Table 1 for details of abbreviations and symbols.

*This patient presented in lymphoid blast-cell transformation and achieved CP after chemotherapy.

†This patient relapsed after a T-cell–depleted BMT and was in CP at the time of second BMT.

results of studying marrow and blood cells. This correlation suggests that it may be sufficient to study blood alone.

Previous studies have reported widely differing frequencies of PCR positivity after allogeneic BMT for CML in CP.[12-18] Some studies have found that the majority of patients studied were PCR positive regardless of the interval posttransplant or the T-cell content of the donor marrow.[12-14] Others have found PCR positivity generally confined to patients studied within 12 months of BMT[15] or patients who had received T-cell–depleted marrow transplants.[16] Two groups have suggested that PCR results might have prognostic significance.[17,18] Both groups found that PCR positivity persisting for more than 6 months posttransplant was commonly associated with subsequent cytogenetic relapse. In our study the pattern of PCR results in group A was very consistent: six of nine patients studied within 6 months of BMT were positive, whereas all studies beyond 6 months were negative. In contrast, all three patients in group B and all three in group C studied 9 to 24 months posttransplant were PCR positive.

The biologic implications of our results depend on the lineage of the cells from which the BCR/ABL mRNA responsible for the PCR positivity was derived. It is conceivable that a small number of long-living T or B lymphocytes or nonclonogenic myeloid cells that originated from the leukemic clone could survive the BMT procedure and be the only source of leukemic mRNA detected by PCR. If this were so, the BCR/ABL mRNA detected frequently in the first 6 months posttransplant might, in some cases, be derived exclusively from nonclonogenic leukemic cells incapable of proliferation or long-term survival. However, if PCR positivity is due to survival of clonogenic CML cells that must eventually be eradicated or suppressed indefinitely to prevent relapse, then the duration of PCR positivity would reflect the speed and effectiveness of the postulated graft-versus-leukemia (GVL) process. Prolonged PCR positivity in the T-cell–depleted group could then reflect impairment of GVL mechanisms, leading ultimately to relapse.

The relapse rate for patients in CP receiving unmanipulated donor marrow and cyclosporin or methotrexate alone for GVHD prophylaxis is low (10% to 20% at 4 years[1,2]). Although the use of cyclosporin and methotrexate in combination for GVHD prophylaxis is a more recent development and the risk of relapse is less well documented, we have observed no cytogenetic or clinical evidence of relapse in 23 patients receiving this protocol who have been followed a median of 17 months (range 6 to 28 months), suggesting the relapse rate will prove to be relatively low.

If the low risk of relapse with this protocol is confirmed, it would indicate that PCR positivity up to 6 months posttransplant has no adverse prognostic significance. On the other hand, PCR negativity post-BMT probably is predictive of a low risk of relapse. PCR positivity more than 6 months posttransplant might identify patients at a relatively high risk of relapse. Two of six patients PCR positive 9 to 24 months post-BMT have subsequently relapsed. This assumption has a number of possible practical implications. For example, we are currently assessing the use of high-dose busulphan alone with unmanipulated donor marrow to retransplant patients in relapse. The fact that one patient (no. 37) who received this protocol was PCR positive 9 months posttransplant might suggest that this regimen may be less effective at leukemia eradication than our current regimen for first transplants. Thus, serial PCR studies in other patient cohorts treated by experimental protocols may provide prognostic information at a relatively early stage.

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