Eradication of Polymerase Chain Reaction-Detectable Chronic Lymphocytic Leukemia Cells Is Associated With Improved Outcome After Bone Marrow Transplantation

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In chronic lymphocytic leukemia (CLL), clonal rearrangement of the immunoglobulin heavy chain locus (IgH) provides a useful marker for the detection of minimal residual disease (MRD) after treatment. At the time of initial presentation, DNA from patients with CLL was polymerase chain reaction (PCR)-amplified using consensus Variable (V\text{H}) and Joining (J\text{H}) region primers using complementarity determining region III consensus region primers or a panel of V\text{H} family-specific framework region 1 (FR1) primers. The clonal product was directly sequenced and patient-specific probes constructed using N region nucleotide sequences. We amplified and sequenced the CDRIII region and designed patient-specific oligonucleotide probes for the detection of MRD in 55 of 66 patients (84%, 90% Confidence Intervals [CI]: 74% to 90%) with poor prognosis CLL referred for autologous and allogeneic bone marrow transplantation (BMT). To determine the clinical utility of this technique, PCR amplification was performed on patient samples at the time of and following autologous (21 patients) and allogeneic (10 patients) BMT in whom serial bone marrow samples obtained after BMT were available for analysis. We show that the persistence of MRD after BMT is associated with increased probability of relapse. In all cases that have relapsed to date, the IgH CDRIII region was identical at the time of initial presentation and at relapse suggesting that clonal evolution of the IgH locus is unusual in this disease. The finding that a significant number of patients remain disease free and with no evidence of PCR-detectable MRD after BMT suggests that high-dose therapy may contribute to improved outcome in selected patients with CLL.

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Despite increases in response rates to therapy using purine analogs, chronic lymphocytic leukemia (CLL) remains incurable. In 1989, we initiated a pilot study to investigate the feasibility and toxicity of high-dose myeloablative therapy with bone marrow transplantation (BMT) in the management of young patients with advanced stage disease. Preliminary results demonstrated that this approach is capable of inducing very high response rates with durable complete remissions in many of these patients. However, in view of the chronic nature of the disease, long-term follow-up is clearly required to determine whether any of these patients are cured of their disease.

CLL, in common with other human lymphoid malignancies, is characterized by proliferation of cells that have undergone malignant transformation with subsequent clonal expansion. During lymphocyte development, somatic recombination of immunoglobulin loci takes place in both normal and malignant B cells. Clonal rearrangement of the immunoglobulin heavy chain locus (IgH) provides a useful marker both to confirm the diagnosis and to detect minimal residual disease (MRD). We demonstrated previously that, when assessed by restriction fragment analysis and Southern blotting, BMT is capable of eradicating clonal Ig gene rearrangements. However, this technique is capable of detecting the malignant clone only when it comprises more than 1% of the total cellular component. The sensitivity of detection of MRD has been enhanced greatly by the use of polymerase chain reaction (PCR) amplification of specific DNA sequences or chromosomal translocations. PCR amplification of the complementarity determining region (CDR) III region of the rearranged IgH is possible due to the presence of highly conserved sequences within the V and J regions. The product is specific to the rearranged allele and serves as a clonal marker for MRD detection. Following PCR amplification of the CDRIII region, PCR products may be sequenced and from the data obtained patient-specific oligonucleotides constructed as probes or for allele-specific PCR for sensitive detection of MRD following treatment of B-cell malignancies.

In the present report, we demonstrate that PCR amplification and direct sequence analysis allow the design of a patient specific oligonucleotide probe in 84% of patients with advanced stage CLL. In these advanced stage patients referred for consideration of BMT, standard induction or salvage therapy never eradicated PCR detectable disease. Following BMT, we demonstrate that persistence of PCR-detectable CLL after bone marrow transplantation is associated with increased risk of relapse. However, the majority of patients remain disease free and free of PCR-detectable MRD, suggesting that this treatment modality may be capable of curing selected patients with advanced stage CLL.

MATERIALS AND METHODS

Patients. Patients with B-CLL who were less than 60 years old with advanced stage disease (stage C or stage B with poor prognostic features) who had chemosensitive disease were considered eligible for this study. Chemosensitivity was determined by response to induction of salvage therapy such that at the completion of therapy the largest nodal mass was less than 2 cm and the bone marrow was infiltrated with less than 20% of the intertrabecular space. Patients with HLA identical sibling donors were offered CD6-depleted allogeneic bone marrow transplantation (BMT) in whom serial bone marrow samples obtained after BMT were analyzed for the presence of PCR detectable disease. We show that the persistence of MRD after BMT is associated with increased probability of relapse. In all cases that have relapsed to date, the IgH CDRIII region was identical at the time of initial presentation and at relapse suggesting that clonal evolution of the IgH locus is unusual in this disease. The finding that a significant number of patients remain disease free and with no evidence of PCR-detectable MRD after BMT suggests that high-dose therapy may contribute to improved outcome in selected patients with CLL.
necic BMT. All others underwent anti-B-cell purged autologous BMT. All patients received cyclophosphamide 60 mg/kg intravenously (IV) on each of 2 consecutive days followed by total body irradiation (TBI) to 14 Gy hyper-fractionated in 200 cGy fractions. Institutional Review Board approval was obtained and informed consent obtained in all cases.

**DNA preparation.** Peripheral blood and bone marrow mononuclear cells were isolated by density gradient centrifugation on a Ficoll/Hypaque gradient (Pharmacia, Uppsala, Sweden). Cells were centrifuged, the cell pellet lysed, and DNA extracted as previously described.

**PCR amplification.** DNA extracted from patients at the time of diagnosis was heated to 96°C for 10 minutes to denature proteinase K activity. Target DNA (1 µg) was amplified in a PCR reaction with a degenerate consensus V (ACACCGGCTCTAGTGGCTTACTGTG) and J region (ACCTGGAGACGGGTGACC) oligomers in 100 µL final volume comprising PCR Buffer II (Perkin Elmer-Cetus, Norwalk, CT) 100 mM Tris-HCl pH 8.3; 500 mMol/L KCl, 2.5 mMol/L MgCl2, 200 µMol/L of each of deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and deoxythymidine triphosphate (dTTP) (Perkin Elmer) and 2.5 U Amplitaq DNA polymerase, (Perkin Elmer-Cetus). Samples were amplified “hot start” using Ampli wax 100 beads (Perkin Elmer-Cetus) with initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds with a final single extension step at 72°C for 7 minutes. All PCR reactions were performed in triplicate using the GeneAmp PCR 9600 (Cetus, Emeryville, CA). A 20-µL aliquot of PCR product was analyzed on 3% agarose DNA polymerase, (Perkin Elmer-Cetus). Samples were amplified “hot start” using Ampli wax 100 beads (Perkin Elmer-Cetus) with initial denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds with a final single extension step at 72°C for 7 minutes. All PCR reactions were performed in triplicate using the GeneAmp PCR 9600 (Cetus, Emeryville, CA). A 20-µL aliquot of PCR product was analyzed on 3% agarose gels. Amplification of a clonal product resulted in a sharp band between 80 to 120 bp whereas amplification of normal blood or marrow resulted in a polyclonal broad smear at approximately 120 bp. Where a clonal band could be seen in the presence of a polyclonal background, polyacrylamide gel electrophoresis was performed and allowed better resolution of the clonal product.

Samples in which no clonal PCR product was obtained using the consensus primers were amplified using V region family-specific primers for the framework region 1 (FR1). Reaction mixtures for FR1 amplification were identical to those used for CDRIII amplification with the exception of the 5’ oligomers. All samples were amplified in seven separate reactions using each of the different 5’ primers (VH 1, 2, 3, 4a, 4b, 5, and 6) and a JH consensus primer. Samples were amplified using a single denaturation step at 94°C for 4 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 10 minutes. PCR product was assessed by electrophoresis of a 20-µL aliquot on a 2% agarose gel and visualized by transillumination. Amplification of a clonal product resulted in a band of approximately 120 bp. Where a clonal band could be seen in the presence of a polyclonal background, polyacrylamide gel electrophoresis was performed and allowed better resolution of the clonal product.

DNA Sequencer using the deoxy sequencing method and Taq polymerase (Perkin-Elmer Cetus).

**Cloning and sequencing PCR products.** In six patients, PCR products were cloned into the vector pCRII using the TA Cloning Kit (Invitrogen, San Diego, CA). Briefly, PCR product was ligated into 50 ng pCRII vector and competent Escherichia coli were transformed and plated onto L-broth medium containing ampicillin (50 µg/mL) and x-gal (4 µg/mL) and incubated overnight at 37°C. For each patient studied, 10 white colonies were selected for plasmid isolation, restriction endonuclease digestion, and sequencing. An inoculum from each colony was grown by shaking overnight at 37°C in L-broth containing ampicillin (50 µg/mL) and 2 µL centrifuged at 12,000g to pellet the cells. DNA was extracted and the plasmid preparation purified using Wizard Minipreps (Promega Corp). The vector pCRII contains EcoRI sites adjacent to the site of PCR product insertion and restriction digests were performed on each plasmid miniprep. The digestion products were run on 3% agarose gels to confirm the presence of a 3.9-kbp band representing the pCRII vector and 120-µL PCR product insert. Both strands of the PCR products were sequenced as outlined above.

**Design of patient-specific oligonucleotide probes.** Sense and antisense DNA sequences were aligned and contiguous sequences generated using Sequencher software (Gene Codes Corp., Ann Arbor, MI). Identification of germline V, D, and J regions was performed using Blast network service (National Center for Biotechnology Information, Bethesda, MD) and FASTA (NIH GenBank database, Bethesda, MD). Comparisons were also made using Sequencher software comparing patient-specific FR1 and CDRIII sequences with previously published V, D, and J sequences (National Center for Biotechnology Information). Patient-specific oligonucleotide probes were designed for each patient corresponding to the V, D, and J regions.

**3’ End-labeling of patient-specific oligonucleotide probes.** Patient-specific oligonucleotide probes were labeled for MRD detection using a 3’ end-labeled digoxigenin method (Genius Systems, Boehringer Mannheim, Indianapolis, IN). Briefly, 2.0 µL T4 tailing buffer, 4.0 µL CoCl2 (25 mMol/L), 1.0 µL patient-specific oligonucleotide (100 pmol/L), 1.0 µL Dig-dideoxyuridine triphosphate (ddUTP) and 1.0 µL terminal transferase were added in a volume of 20 µL and incubated at 37°C for 15 minutes and then placed on ice. Reactions were stopped by the addition of 2 µL of a glycerogen: 200 mmol/L EDTA mix and the labeled oligonucleotides precipitated in 2.5 µL of 4 mol/L LiCl and 75 µL 100% ethanol at −20°C. Samples were placed at −70°C for 30 minutes, after which they were centrifuged at 12,000g for 15 minutes, washed with 70% ethanol at −20°C, and centrifuged for 5 minutes at 12,000g. After aspirating the ethanol, the oligonucleotides were dried, resuspended in 20 µL of sterile water, and stored at −20°C.

**Southern transfer and hybridization using patient-specific oligonucleotide probes.** Follow-up patient samples were PCR-amplified as described above. Following amplification, a 20-µL aliquot was analyzed by electrophoresis on 3% agarose gels. Following electrophoresis, gels were denatured in 3 mol/L NaCl, 0.4 mol/L NaOH for 15 minutes (twice). Vertical transfer of PCR products onto positively charged nylon membranes (Boehringer Mannheim) was performed with transfer buffer (3 mol/L NaCl; 8 mmol/L NaOH; 2 mmol/L N-lauroylsarcosine) using the TurboBlotter system (Schleicher & Schuell, Keene, NH) with transfer times ranging from 2 hours to overnight. The nylon membranes were neutralized using neutralizing blocking buffer (0.2 mol/L sodium phosphate, pH 6.8) for 5 minutes. air dried, and fixed using a UV Stratalinker (Stratagene, La Jolla, CA) before hybridization.

**Prehybridization/hybridization of 3’ end-labeled Dig-ddUTP probes.** Membranes were prehybridized by rotation at 68°C in 20 mL hybridization buffer consisting of 5X SSC, 1% (w/vol) blocking
reagent, 0.1% (wt/vol) N-lauroylsarcosine, 0.02% (wt/vol) sodium dodecyl sulfate (SDS), for 60 minutes following which 100 pmol of 3' labeled oligonucleotide was added. The melting temperature of the oligonucleotide (Tm) was calculated and membranes were hybridized at (Tm-5)°C for 6 hours, rinsed twice in 2X SSC/0.1% SDS for 5 minutes at room temperature followed by two rinses at 50°C (15 minutes each time) in 0.1X SSC 0.1% SDS. Membranes were blotted lightly and air-dried before the detection stage.

**Detection of bound Dig-ddUTP labeled patient-specific probes.**  For the detection of 3' end-labeled, Dig-ddUTP-labeled patient-specific probes, membranes were equilibrated in Buffer 1 (0.1 mol/L Tris, pH 7.5; 0.15 mol/L NaCl) for 1 minute then placed in Buffer 2 (0.1 mol/L Tris, pH 7.5; 0.15 mol/L NaCl, 1% blocking reagent (w/vol)) for 60 minutes, after which anti-digoxigenin antibody (3 μL in 30 mL Buffer 2) was added and membranes agitated for 30 minutes. Membranes were then rinsed twice for 15 minutes in Buffer 1 and then transferred to acetate film. Lumiphos (Boehringer Mannheim; 500 μL per membrane) was added and the membrane covered with a second acetate sheet. After incubation at room temperature for 1 minute, excess Lumiphos was removed from the membranes that were then exposed to autoradiograph film (DuPont Inc, Boston, MA). Exposures were performed for 40 to 90 minutes.

**Statistical methods.** Descriptive statistical methods have been employed in this paper. Exact binomial confidence intervals have been provided for all estimates of proportions.

**RESULTS**

**Construction of patient-specific oligonucleotide probes for detection of MRD.** We first determined which patient samples could be PCR-amplified and sequenced using the strategy outlined in Fig 1. Samples from 66 patients with CLL who were referred to the Dana-Farber Cancer Institute for consideration for bone marrow transplantation were analyzed. Using the CDRIII consensus primers, a clonal PCR product could be obtained and sequenced in 42 patients (64%). In six additional patients, although there appeared to be a clonal product on gel electrophoresis, sequencing was repeatedly unsuccessful. In the remaining 18 patients, there was either no visible band or a polyclonal smear was evident. Samples from these 24 patients that could not be sequenced after amplification using the CDRIII consensus primers were then PCR amplified using the V_H family specific FR1 region primers. A clonal product was obtained and sequenced in 13 of these patients (54%). Sequencing of what appeared to be a clonal product on electrophoresis was unsuccessful in eight additional patients. This was presumably because normal B-cell rearrangements were coamplified with the malignant clone making it impossible to read the sequence of the CDRIII region. Confirmation that the sequence obtained represented that of the malignant clone was obtained in two ways. First, sequencing of PCR product from each patient was performed on at least two occasions on the diagnostic sample and on at least two samples at different time points. The identical sequence was obtained on serial samples obtained from each of the patients. In addition, we cloned and sequenced 8 to 10 recombinant colonies from six patients in whom a sequence could be obtained by direct sequencing. Demonstration of clonality was confirmed by finding the identical sequence in at least three isolates. In each case, the sequence obtained after cloning was identical to that obtained by direct sequencing, further demonstrating that the sequence obtained by direct sequencing was that of the leukemic clone. Therefore, using this combined strategy, a clonal product could be obtained and the IgH rearrangement of the malignant clone sequenced successfully in 55 of the 66 patients (84%).

From each patient sequence, the V, D, and J segments were identified. In cases where the V gene usage could not be identified, the diagnostic sample was also amplified using the FR1 family specific primer. The V and J segment usage in these patients are shown in Fig 2. As has been previously observed, the V and J segment usage was nonrandom.
Germline segments \( V_{H3} \), \( V_{H4} \), and \( V_{H6} \) and \( J_{H3} \), \( J_{H4} \), and \( J_{H6} \) were overrepresented. No CLL samples were identified using \( J_{H1} \) or \( J_{H2} \). Patient-specific oligonucleotide probes were designed based on the unique N region sequences identified in each patient as demonstrated in Fig 3. The specificity of the oligonucleotide probe was demonstrated by the finding that the oligonucleotide probe bound specifically to the clonal PCR product from that patient, but never cross-reacted with the clonal product obtained in other patients (data not shown). To determine the sensitivity of PCR amplification and patient-specific oligonucleotide probing, serial dilutions of leukemic cells into normal bone marrow mononuclear cells were amplified and probed. The sensitivity of detection in an individual patient appears to be dependent on the length of the N insertion, as this increases the specificity of the oligonucleotide probe. These experiments suggest that PCR amplification with probing using a patient-specific oligonucleotide probe is capable of detection at a sensitivity of one leukemic cell in \( 10^4 \) normal cells (range, \( 10^3 \) to \( 10^5 \)).

Assessment of residual leukemia cells at the time of BMT. To be eligible for BMT, patients had to achieve a protocol-eligible minimal disease state following induction or salvage chemotherapy. Of the 66 patients studied at the time of diagnosis, 48 patients achieved protocol-eligible minimal disease status and have undergone BMT. Thirty-five of these patients have undergone autologous BMT, and 13 patients have un-
deregone allogeneic BMT from HLA-matched sibling donors. PCR amplification of the IgH region could be performed successfully on 26 of the 35 patients (74%, 90% CI: 59% to 86%) undergoing autologous BMT and in 11 of 13 patients (85%, 90% CI: 58% to 97%) undergoing allogeneic BMT. In each of the 37 patients in whom PCR was capable of amplifying their IgH rearrangement, residual leukemia cells could be identified in the BM at the time of BMT. In 35 of these 37 patients, residual leukemia cells could be identified by the presence of a clonal band on agarose gel electrophoresis, Southern blotting, and probed with digoxigenin labeled patient-specific oligonucleotide probe. In the remaining two patients, no clonal band could be identified on agarose gel electrophoresis, but residual leukemia cells were identified by oligonucleotide probing. Therefore, in this group of patients with advanced stage disease, standard dose induction or salvage chemotherapy never eradicated PCR detectable leukemia. This result was hardly surprising, as 46 of these 48 patients had morphologic evidence of persistence of leukemic infiltration at the time of BMT.

Detection of MRD after BMT for CLL. To assess the clinical utility of the detection of MRD after BMT, we analyzed the results of PCR amplification of IgH rearrangements on samples obtained after BMT. The results obtained of PCR analysis followed by probing with a patient-specific oligonucleotide probe of samples obtained at the time of and following autologous BMT are shown in Fig 4. In this patient, there was persistence of PCR-detectable leukemia in every sample obtained after BMT and this patient subsequently relapsed. We analyzed the results of PCR performed on serial BM samples obtained after BMT in patients in whom PCR amplification of the IgH could be performed successfully at the time of diagnosis, a patient-specific oligonucleotide probe had been constructed, and who had undergone BMT more than 12 months prior to the time of this analysis. Of the 48 patients who have undergone BMT in whom a patient-specific oligonucleotide probe could be constructed, serial samples were available for analysis from 31 patients following BMT; 10 of these patients received allogeneic BMT and 21 patients autologous BMT. Because the aim of the study was to assess the clinical significance of MRD, only BM samples obtained while the patients remained in complete clinical remission and in whom there was no histological evidence of leukemic infiltration were included in this analysis. All PCR reactions were performed on at least three occasions. Discordant results within a single sample were not seen in this study. If more than one sample was available at a single time point and discordant results were obtained between samples, the patient was scored positive at that time point.

The results obtained in the 10 patients who have received allogeneic BMT are shown in Fig 5. As described above, all 10 of these patients had evidence of PCR-detectable MRD at the time of BMT. Following allogeneic BMT, 44 follow-up samples were available for analysis ranging from 2 months to 4 years after BMT. Two patients (no. 9 and 28) had persistence of MRD detected in every sample analyzed after BMT. Both of these patients have relapsed. In both of these patients, sequence analysis confirmed that the sequence of the CDRIII region was identical at the time of presentation and at subsequent relapse after BMT. No PCR-detectable leukemia cells could be detected at any time after BMT in eight patients. None of these patients has relapsed to date. Two patients in whom no MRD was detected by PCR (nos. 2 and 16) died of treatment-related toxicity and had no clinical or histopathologic evidence of disease. The remaining six patients have received no additional therapy and remain in continuous complete remission from 1 year to 4 years after BMT.

The results obtained in the patients undergoing autologous BMT are shown in Fig 6. All 21 patients had evidence of PCR-detectable leukemia cells in their harvested autologous bone marrow before immunologic purging. After three rounds of immunologic purging of autologous BM using a panel of anti-B-cell monoclonal antibodies and rabbit complement, no PCR-detectable leukemia cells could be detected in 11 of these patients (52%). The sample sizes are too small to detect differences in relapse rates among autologous BMT patients whose BM remained PCR-positive after purging compared with those who were PCR-negative. We would have only a 32% power to detect the observed differences in relapse rates testing at the one-sided 5% level with only 10 and 11 patients per group.

A total of 126 follow-up samples were available for analysis ranging from 2 months to 5 years after autologous BMT. There was persistence of PCR-detectable leukemia early after BMT in samples obtained from eight patients. In three of these patients, PCR-detectable leukemia was found in every sample analyzed after BMT and all three patients subsequently relapsed. Again, in each of these patients, sequencing confirmed that the CDRIII region was identical at the time of initial presentation and at the time of relapse. However, in five patients there was no evidence of PCR-detectable leukemia in follow-up samples obtained at later time points. Although these patients received no further therapy, in none of these patients was there persistence of detectable leukemia more than 6 months after autologous BMT. In 12 patients there were no PCR-detectable leukemia cells found in any of the samples analyzed at any time after BMT. To date, none of these patients has relapsed. In patient no. 20, no PCR-detectable leukemia cells could be detected at 9
months after BMT, but MRD could be detected at 22, 32, and 38 months after BMT. This patient remains in clinical complete remission more than 3 years after autologous BMT with no morphologic evidence of leukemia in BM or any evidence of lymphadenopathy on computerized axial tomography (CAT) scan.

Following BMT, six patients had evidence of lymphoid nodules on their bone marrow biopsies and were considered to be in "Nodular CR." Three of these patients (nos. 9, 19, and 22) had evidence of PCR-detectable leukemia cells in their BM samples. All three of these patients progressed to a diffuse pattern of BM infiltration on later samples and relapsed. In contrast, three patients (nos. 10, 31, and 32) had no evidence of PCR-detectable leukemia despite the presence of these lymphoid nodules. All three of these patients have had stable lymphoid nodules reported on further follow-up and had no evidence of PCR-detectable leukemia in any of 14 serial BM aspirates analyzed. Although these patient numbers are small and further follow-up will clearly be required, assessment of clonality by PCR amplification of IgH may be a useful tool to discriminate between benign and malignant nodules in this patient population.

**DISCUSSION**

The aim of this study was to determine whether high-dose therapy was capable of eradicating PCR-detectable MRD in these patients and to determine whether detection of MRD might act as a surrogate endpoint for subsequent clinical relapse of disease in patients with B-cell CLL undergoing BMT. We demonstrate that PCR amplification and sequencing of the rearranged IgH from the malignant clone could be performed successfully in 55 of 66 (84%) CLL patients studied, allowing construction of a patient-specific oligonucleotide probe for the subsequent detection of MRD. In these patients with advanced stage disease, the use of conventional dose induction or salvage chemotherapy did not lead to the
eradication of MRD, and PCR-detectable leukemia cells could be found in the BM of all patients at the time of BMT. PCR-detectable leukemia cells could be found in every sample analyzed from five patients following BMT and all five patients have relapsed. In contrast, no PCR-detectable MRD could be detected in any of the serial samples obtained after BMT from 19 patients and none of these patients has relapsed. Of note, MRD was detected within the first 6 months after BMT in a number of patients in whom no PCR-detectable leukemia cells could be found on analysis of later samples, suggesting that the detection of MRD early after BMT will have limited predictive value to determine those patients who are at high-risk of subsequent relapse. The results of the PCR analysis are in keeping with our previous findings using restriction fragment analysis to assess MRD after ABMT.

Because all of the patients studied had persistence of PCR-detectable disease at the time of BMT, harvested autologous BM always contained tumor contamination. Our attempts to eradicate leukemia cells by immunologic purging were successful in only half of the cases, as has been the case in our studies in non-Hodgkin’s lymphoma.22 Clearly, there are insufficient events to determine whether purging contributed to outcome in these patients, but, of note, all three patients who relapsed after autologous BMT were infused with marrow containing residual disease. However, a significant number of patients who were infused with autologous BM containing residual detectable leukemia cells have not relapsed, nor do they have PCR-detectable leukemia cells in follow-up samples obtained after BMT. It is interesting that those patients with PCR-detectable leukemia cells early after autologous BMT were those patients infused with BM containing detectable leukemia. This observation is also in keeping with our previous studies in non-Hodgkin’s lymphoma.23 However, the biggest argument against the contribution of infused cells to relapse is the finding that patients relapse at the same rate after allogeneic BMT where there can be no possibility that leukemia cells were infused to the patient.

The finding that so many of the patients had no PCR-detectable MRD after BMT was surprising and suggests one of three possibilities. First, high-dose therapy may be a highly effective therapy to eradicate MRD in patients with CLL. It has been demonstrated previously that induction therapy with fludarabine is capable of eradicating MRD in patients with earlier stage disease.24 Although all of the patients reported here had persistence of MRD at the time of BMT and most of the patients had received fludarabine, all of the patients in the present study had advanced stage, aggressive disease. However, all patients were also selected in that they had chemosensitive disease and may well have excellent response to high-dose therapy. Second, the patients may continue to harbor MRD below the limit of detection using the strategy outlined here. If this is the case, then on further follow-up, it would be expected that more patients will have evidence of PCR-detectable leukemia cells with time. A third possibility is that the technique is not capable of detecting MRD in these patients because there has been clonal evolution at the IgH locus. This may arise when subclones emerge that predominate over the original clone or there may be alteration such as V_{H\mu}-V_{H}\delta switching. Under these circumstances, the patient-specific oligonucleotide probe will no longer recognize the emerging leukemia clone. This has proved to be a significant problem in studies of MRD detection in acute lymphoblastic leukemia.25-29 CLL is regarded as a stable and indolent malignancy, with little or no intraclonal variation because of somatic mutations in the V_{H}, D, or J_{H} genes.30,31 Previous studies have shown that alterations in the genotype can occur with Richter’s transformation,32 but occur uncommonly in nontransformed CLL.33 Moreover, a number of factors argue against this possibility of intraclonal diversity in the CLL patients in the present study. Sequence analysis confirmed identity of the leukemia clone in all five of the patients who have relapsed to date. Only the CDRIII region was sequenced so that if mutations occurred in the other CDR regions or if there was V_{H}-V_{H}\delta switching, then this might not be detected in this analysis. In addition, even if the patient-specific probe no longer recognized the clonal product, we have not identified a clonal product on agarose gel electrophoresis after PCR amplification in any of the patients studied. In each of the patients who subsequently relapsed, although the patient-specific probe may have increased the sensitivity of detection of MRD, a clonal product was evident on agarose gel electrophoresis in each of these patient samples even before probing.

Therefore, the findings of the present study demonstrate that it is feasible to sequence the CDRIII region of the IgH in the majority of patients with CLL. Construction of a patient-specific oligonucleotide probe is then possible to assess MRD by PCR amplification using this approach in patients with CLL. The clinical utility of the technique is demonstrated by the finding that persistence of PCR-detectable leukemia is associated with increased likelihood of relapse and suggests that our therapeutic strategies should be aimed at the eradication of PCR detectable CLL cells for cure.

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