Detection of Residual Lymphoma Cells by Polymerase Chain Reaction in Peripheral Blood Is Significantly Less Predictive for Relapse Than Detection in Bone Marrow

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Polymerase chain reaction (PCR) amplification of the t(14;18) has been shown to be a highly sensitive method to detect minimal residual disease in patients with non-Hodgkin’s lymphoma (NHL) whose tumors bear this translocation. The ideal tissue source to detect residual lymphoma would be from a previously involved lymph node. However, lymphoid tissue is rarely available once patients achieve complete remission. Although PCR amplification has been used to detect residual lymphoma cells in both bone marrow (BM) and peripheral blood (PB) of patients in complete remission, it is presently unknown whether BM and PB are equivalent tissue sources to detect residual disease. In the present study, we compared the clinical utility of the detection of residual lymphoma in both the BM and the PB of patients with advanced-stage non-Hodgkin’s lymphoma before, at the time of, and after high-dose therapy and autologous BM transplantation (ABMT). The detection of residual lymphoma in either the BM or PB was associated with decreased disease-free survival. However, in the present study, 44% of patients who relapsed had no evidence of circulating lymphoma cells in their PB. At the time of BM harvest, PCR-detectable residual lymphoma cells were detected in 211 of 212 patients; although, in a subset of these patients analyzed, lymphoma cells were detected in the peripheral blood of only 49% of patients. When residual lymphoma cells within the autologous BM are infused into the patient these cells are rapidly detectable circulating in the PB in the patient. These cells continue to circulate during the immediate posttransplant period and be detectable in the PB in the majority of patients who are infused with marrow containing residual lymphoma. We conclude that BM is a more informative tissue source than PB in detecting minimal residual disease at the time of and after ABMT, and that contamination of PB early after ABMT appears to be the consequence of reinfection of lymphoma cells within autologous marrow.

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Submitted December 16, 1993; accepted February 14, 1994.

Supported by Grants No. CA-34183 (L.M.N) and CA-55207 (A.S.F.) from the National Cancer Institute, F05-TWO44 (JGG) from the Fogarty International Center, and CA 06516-28 (D.N.) from the National Institutes of Health.

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the patients studied. In addition, PCR amplification is capable of detecting circulating lymphoma cells within 2 hours of infusion of residual lymphoma in the autologous harvested BM. We conclude that the clinical significance of the assessment of minimal residual lymphoma is clearly influenced by the tissue source used to detect such disease and that BM is a more informative source of tissue to assess minimal residual lymphomatous infiltration than PB.

MATERIALS AND METHODS

Patients. From 1982 to December 1992, 386 patients with advanced stage B-cell NHL have undergone ABMT at a single institution. In all cases, immunophenotyping confirmed that the lymphoma cells expressed CD20. All patients had chemosensitive disease at the time of ABMT as assessed by the achievement of protocol-eligible minimal disease after induction or salvage therapy. Minimal disease criteria were defined as either a clinical CR or a partial remission (PR) to maximum tumor masses of 2 cm or less, and BM infiltration of less than 20% of the inter trabecular space. A PCR-amplifiable BCL2/IGH translocation was identified in BM or lymph node samples obtained before ABMT from 212 of these patients. Samples analyzed from these 212 patients are included for subsequent analysis. All patients were treated with an identical high-dose therapy regimen consisting of high-dose cyclophosphamide and total body irradiation (TBI) before ABMT. Immunologic purging was performed using a cocktail of three monoclonal antibodies (MoAbs) as previously described.11 All patients were monitored for palpable disease, radiologic progression or evidence of morphologic infiltration in the BM at six monthly intervals for the first 2 years after ABMT and at yearly intervals thereafter.

BM and PB samples. BM aspirates and biopsies were obtained from all 212 patients at the time of initial evaluation and at the time of BM harvest before ABMT. These samples were used to document that the lymphoma expressed the BCL2/IGH translocation. PB and BM samples were obtained at the time of follow up clinic visits at 6, 12, 18, and 24 months after ABMT, and yearly thereafter until the time of documented clinical relapse of disease. All samples obtained before January 1990 were cryopreserved. Previous analysis has shown that identical results are obtained by PCR analysis of fresh and cryopreserved BM samples.13 Normal BM and PB were obtained from normal volunteers. In addition, samples were collected from all patients who had NHL that did not contain a PCR detectable BCL2/IGH translocation and these samples were used throughout the study to ensure that false positive results did not occur. Samples of the harvested BM after immunologic purging were available for analysis in 202 of these 212 patients. Although the investigative protocol included the collection and cryopreservation of BM samples from these patients, PB samples were not routinely collected. Therefore, at the time of BM harvest, only 153 PB samples, collected from 45 patients, were available for analysis. To address particular questions regarding the utility of PCR to detect infused lymphoma cells after ABMT, PB samples were collected prospectively from 60 consecutive patients undergoing ABMT who had a documented BCL2/IGH translocation. Samples from these patients were collected before and at 2 hours after infusion of autologous BM, on days 1 and 7 after ABMT, and weekly thereafter until discharge from hospital. All samples were obtained with the patients' informed consent and with the approval of the human protection committee of our institution. Genomic DNA was isolated by cell lysis using nonionic detergents and digestion with proteinase K (Sigma Chemical Co, St Louis MO).14 DNA was extracted from the cell lines DHL-6 and RL (which contain a PCR-amplifiable translocation at the major breakpoint region [MBR]) and from DHL-16 (which contains a PCR-amplifiable translocation at the minor cluster region [mcr]) and were used as positive controls. RL was a gift from Dr W. Urba, National Institutes of Health Biologic Response Modifiers Branch, Frederick, MD. DHL-6 and DHL-16 were gifts from Dr A. Epstein, University of Southern California, Los Angeles, CA.

PCR analysis. PCR amplification of the major11 and minor23 breakpoint regions of the BCL2/IGH translocation were performed using nested oligonucleotides in a modification of the method previously described.19 Samples containing 1 μg of genomic DNA were amplified for 25 cycles in a thermal cycler (Cetus, Emoryville, CA) in a 50-μL final volume containing 50 mM Tris hydrochloride (pH 8.3), 2.25 mM magnesium chloride, 0.001% gelatin, 200 μM of each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate and deoxycytosine triphosphate, 1.5 U Taq polymerase (Cetus), and 20 mM of oligonucleotide primers, 5′ ACC TGA GAC GAC GGT GAC C 3′ for the consensus JH region, with 5′ CAG CCT TGA AAC ATT GAT GG 3′ for the MBR or 5′ CGT GCT GGT GAC ACC ACT CCT G 3′ for the mcr. Amplification was performed by denaturation for 1 minute at 94°C, annealing for 1 minute at 55°C for the MBR and 58°C for the mcr and extension at 72°C for 1 minute with a final extension period of 10 minutes. Reamplification for 30 cycles of a 5-μL aliquot of the initially amplified mixture was reamplified in a final volume of 50 μL using oligonucleotides internal to the original primers, 5′ ACC AGG GTC CCT TGG CCC CA 3′; for the consensus JH region, with 5′ TAT GGT GTC TTT TTT AG 3′ for the MBR or 5′ CCT GGC TTC TCC TCT G 3′ for the mcr, using the identical cycle parameters used in the initial amplification step. Aliquots of the final reaction product were analyzed by electrophoresis in 4% agarose gels containing ethidium bromide and visualized under ultraviolet light. Standard precautions against cross-contamination of amplified material were taken.20 With each amplification a weak-positive control consisting of DNA from a 10-4 dilution of the appropriate cell line in normal BM cells and a negative control consisting of DNA from a cell line that did not contain the appropriate BCL2/IGH translocation were performed. In samples with no detectable PCR product, PCR amplification was repeated using oligonucleotides for the V region of the human B-cell activation antigen B7 and confirmed that PCR-amplifiable DNA was isolated from each sample that was included in the present analysis.

Statistical analysis. Disease-free survival was estimated by the method of Kaplan and Meier.22 Differences in disease-free survival were assessed using the log rank test.22 Associations between the results of PCR analysis of BM samples after immunologic purging and of PB samples, both at the time of marrow harvest and within 1 month after ABMT, were assessed using the Fisher exact test.22 The results of PCR analysis of PB samples obtained before and after infusion of autologous BM were examined using McNemar's test.22

RESULTS

Identification of patients with a BCL2 translocation. At the time of analysis, 386 patients with B-cell NHL had been treated at our institution with an identical protocol that included treatment to a protocol-eligible minimal disease state followed by TBI, high-dose cyclophosphamide and immunologic purging of autologous marrow before transplantation. A PCR-amplifiable BCL2/IGH translocation was identified in diagnostic tissue obtained from 212 patients. The patient characteristics are shown in Table 1.

Presence of residual lymphoma cells in BM and in PB is predictive for outcome after ABMT. Serial BM and PB samples were obtained after ABMT and PCR analysis performed to determine whether the presence of residual lymphoma cells in the BM and PB predicted for subsequent relapse. PCR analysis at the MBR and mcr were performed
and investigators were blinded to clinical outcome. For inclusion in this analysis, patients had a documented history of lymphoma containing a BCL2/IgH translocation that could be detected by PCR, and samples were available for analysis that were obtained while the patient was documented to be in clinical CR after ABMT. Remission samples were not available from all of the 212 patients with a documented PCR-detectable BCL2/IgH translocation. A total of 863 remission BM samples were available after ABMT from 146 patients, 47 of whom have relapsed to date. A total of 1478 remission PB samples were available after ABMT from 168 patients, 36 of whom have relapsed to date. A further 108 PB and BM samples were obtained at the time of documented relapse and were excluded from subsequent analysis because it was felt that these samples had no predictive value.

No detectable lymphoma cells were found at any time after ABMT in any of the BM samples analyzed from 49 patients (34%). Two of these patients (4%) relapsed at 10 and 16 months after ABMT. BM samples from these two patients showed no evidence of lymphoma on PCR analysis at 3 or 6 months after ABMT and no further BM samples were available for analysis before their subsequent relapse. PCR analysis showed lymphoma in the BM at some time after ABMT in 97 patients, 43 of whom (44%) have relapsed. The median disease-free survival for this group was 27 months after ABMT. All 43 of these patients had lymphoma detected by PCR analysis in the BM samples obtained at the previous clinic visit before their subsequent relapse. Therefore, PCR analysis detected lymphoma cells in the BM of 43 of 45 patients (96%) who have relapsed to date. The estimated disease-free survival of these patient groups is shown in Fig 1B. The presence of PCR-detectable lymphoma cells in the PB after ABMT was associated with an increased likelihood of subsequent relapse ($P = .01$ by log rank test). Although the detection of lymphoma cells in both the BM and PB was associated with decreased disease-free survival after ABMT, a detection of lymphoma in PB was less predictive than the detection of lymphoma in BM (logrank $P = .001$). As a predictive assay for subsequent relapse, the detection of PCR-detectable lymphoma cells in the BM at any time after ABMT was associated with a 96% sensitivity and a 44% specificity. Further follow-up will be required to determine whether this low specificity is a result of insufficient follow-up of the patients who currently have minimal detectable disease or if indeed the detection of minimal disease is not always a harbinger of subsequent relapse in these patients. In contrast, the detection of residual lymphoma in PB was associated with only a 55% sensitivity and a 67% specificity. Therefore, the BM is a more informative tissue source in which to detect residual disease.

**Detection of residual lymphoma cells in the BM and PB at the time of marrow harvest.** Because we found that BM was a more informative tissue source to detect minimal residual disease after ABMT than PB, we sought to determine whether this was also the case at the time of BM harvest. We previously reported that PCR analysis of BM samples at the time of BM harvest detected residual disease in all patients analyzed. Although 96 patients (45%) had morphologic evidence of BM infiltration at the time of marrow harvest, PCR analysis of the BM obtained showed residual BM infiltration in 211 of 212 patients (99.5%) in the present series. Only one patient had no evidence of BM infiltration by PCR analysis in any of four BM samples obtained at the time of harvest.

Unfortunately, PB samples at the time of their marrow harvest had not been cryopreserved because collection of PB samples was not included in the investigational protocol. Therefore, we obtained multiple PB samples at the time of marrow harvest from 45 patients. The patient characteristics of these 45 patients are shown in Table 2. This subgroup of patients is representative of the whole group of 212 patients in all aspects except the results obtained after purging. After purging, residual lymphoma cells were detected after immunologic purging in 67% of patients in this group, but in 52% of the remaining 157 patients for whom post purging samples were available ($P = .09$).

A total of 153 PB samples and 144 BM samples were obtained from these 45 patients at the time of marrow harvest. Lymphoma cells were detected in the BM by PCR analysis in 44 of these patients (98%). In contrast, PCR analysis detected circulating lymphoma cells in the PB in only 22 of these patients (49%). Of note, the single patient who had no residual lymphoma cells detected in any of the four BM samples obtained at the time of marrow harvest had lymphoma cells detectable in 1 of 6 PB samples obtained at that time. This suggests that this patient had residual lymphoma present only at the very limit of detection by PCR analysis.

**Association between lymphoma in PB and detection of**
lymphoma after immunologic purging. After immunologic purging, samples were available for analysis from 202 of the 212 patients. After purging, no lymphoma cells were detectable by PCR in the BM of 91 patients (45%) including the single patient who had no detectable lymphoma cells in the harvested BM before purging. Residual lymphoma cells were detected in the BM after purging in 111 patients (55%). Of the 45 patients in whom PB and BM samples were available for analysis at the time of marrow harvest, those patients who had residual lymphoma cells detected in both the BM and PB at the time of ABMT were more likely to contain residual lymphoma cells after immunologic purging than those patients who had residual lymphoma in the BM but not in the PB \( (P = .01) \). As can be seen in Table 2, of 30 patients in whom PCR-detectable lymphoma cells were found after purging, 19 patients (63%) had detectable lymphoma cells in the PB at the time of BM harvest. In contrast, of 15 patients who had no detectable lymphoma cells after purging, 3 patients (20%) had detectable lymphoma cells in PB at the time of BM harvest. Results obtained from two patient samples are shown in Fig 2. Patient A has detectable lymphoma cells in BM but not in PB and has no detectable lymphoma cells after purging. Patient B has detectable lymphoma cells in BM and PB, and has detectable lymphoma cells after purging.

Detection of lymphoma cells in the circulation after infusion of autologous BM. Because we had previously shown by PCR analysis that we were able to eradicate residual lymphoma cells by immunologic purging in only 50% of patients,\(^5\) we were interested to determine whether lymphoma cells could be detected in the patient after the infusion of autologous BM that contained PCR detectable lymphoma cells. For this reason we collected prospectively multiple PB samples from patients 2 hours before and at 2 hours after infusion of purged autologous BM. Samples were analyzed from 60 consecutive patients undergoing ABMT in whom it was documented that the lymphoma contained a PCR-detectable BCL2/IgH translocation. A total of 388 PB samples were obtained from these 60 patients, 195 samples obtained before infusion and 193 samples obtained 2 hours
after infusion of autologous marrow. These samples were not obtained from the lumen of the Hickman line that was used to infuse the BM. The results obtained by PCR analysis of PB samples obtained before and after infusion of marrow are shown in Fig 3. There is an obvious difference in the detection of lymphoma cells in the circulation after infusion of autologous BM (P < .0001 by McNemar’s test). After high-dose therapy, but before infusion of autologous BM, lymphoma cells were detected by PCR analysis in the PB samples of only 8 patients (13%). This suggests that high-dose therapy is relatively effective in clearing detectable lymphoma cells from the majority of patients. In contrast, 2 hours after infusion of autologous marrow, PCR-detected lymphoma cells were detected in the PB samples from 35 patients (58%). In 29 of 35 patients (83%), there was no evidence of circulating lymphoma cells before infusion of the autologous BM. Six patients had detectable lymphoma cells before and after infusion of BM. The reason 2 patients had detectable lymphoma cells in the PB before infusion of BM that were not detectable later that day is not clear, but these may represent patients in whom the lymphoma cells were in the process of being cleared from the circulation after high-dose therapy.

*Lymphoma cells infused with autologous marrow are de-
PCR ANALYSIS OF PERIPHERAL BLOOD AFTER ABMT

PCR Analysis of PB

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Fig 3. Results of PCR amplification at either the MBR or mcr of the BCL2/IgH translocation of PB samples obtained immediately before and at 2 hours after infusion of harvested autologous BM. (☐) PCR-negative; (■) PCR-positive.

of 637 samples were obtained from 60 patients from day 1 to day 26 after infusion of autologous BM. PCR-amplifiable DNA could be obtained from only 38 patients’ samples during days 1 to 6 because of the paucity of cells obtained from the PB during this period, from 57 patients during days 7 to 13, and from 53 patients during days 14 to 20. Samples were available for analysis from only 27 patients during days 21 to 27 as the remaining patients had already been discharged from hospital admission by this time. During each of these time periods, there was a statistically significant association between the detection of residual lymphoma cells after immunologic purging and the detection of circulating lymphoma cells in the PB (P < .0001 for each of the periods from days 1 to 20 and P = .0002 for days 21 to 27). Results obtained on representative samples are shown in Fig 5. Patients A and B have PCR-detectable lymphoma cells remaining after immunologic purging. Lymphoma cells were also detected in the PB at 2 hours after infusion of autologous BM. These lymphoma cells remained detectable in PB throughout the entire hospital admission for ABMT. In contrast, patient C has no PCR-detectable lymphoma after immunologic purging and no lymphoma cells were detected by PCR analysis of PB samples obtained during admission. PB samples contained either no PCR-detectable lymphoma cells in any sample or contained PCR-detectable lymphoma cells at all time periods in all but 12 of the patients. In these 12 patients, PCR analysis detected lymphoma cells at some, but not all time periods. No discernible pattern was apparent on detailed analysis of the samples analyzed from this small group of patients. These findings suggest that once lymphoma cells in the autologous marrow are infused into the patient, they appear in the circulation and continue to circulate for extended periods. This is in keeping with our previous observation that patients who were infused with marrow that contained residual lymphoma after purging had detectable lymphoma cells in the circulation at 6 months after ABMT.19

DISCUSSION

Although residual lymphoma cells have been detected by PCR in both BM and PB of patients who are in complete clinical remission, it is presently unknown whether these BM and PB are equivalent tissue sources to detect residual lymphoma. The present study compared the sensitivity and clinical utility of the detection of residual lymphoma cells by PCR amplification of the BCL2/IgH translocation in both BM and PB of patients with advanced stage B-cell non-Hodgkin’s lymphoma (NHL) before, at the time of, and after ABMT. Those patients who had no residual lymphoma cells detectable in BM samples obtained after ABMT had excellent prognosis. The presence of residual lymphoma cells in the PB in the present study was also associated with a statistically significant decrease in disease-free survival after ABMT. However, 44% of patients who subsequently relapsed had no detectable lymphoma cells in their PB. We conclude that analysis of BM is a far more informative and
sensitive detector of minimal residual disease than analysis of PB, and, more importantly, is significantly more predictive of which patients will relapse after ABMT.

A major concern with the use of ABMT has always been the fear that malignant cells harbored within the BM will be infused to the patient and contribute to subsequent relapse. Recently, three independent lines of evidence have suggested that the infusion of malignant cells within the autologous BM may contribute to subsequent relapse. First, Sharp and his colleagues2 at the University of Nebraska have shown that the detection of clonogenic lymphoma cells within autologous BM may contribute to subsequent relapse. Second, the infusion of PCR-detectable lymphoma cells after immunologic purging was also associated with an increased probability of relapse after ABMT.3 Most recently, gene-marker studies have shown the presence of a marker gene at the time of relapse, suggesting that these infused marrow cells contributed to subsequent relapse.31 In the present report, we show that when residual lymphoma cells are infused into the patient, these cells are rapidly detectable circulating in the PB in the patient. These cells continue to circulate during the immediate posttransplant period and are detectable in the PB in the majority of patients who are infused with marrow containing residual lymphoma. If lymphoma cells infused within the autologous marrow do indeed contribute to subsequent relapse, then it would seem likely that patients would relapse in a disseminated manner. However, after ABMT, patients relapse at the site of prior disease. A potential reason why patients subsequently relapse at the sites of prior disease might be that the infused lymphoma cells home back to previously involved lymph nodes.32

PCR analysis of BM at the time of harvest showed residual lymphoma cells in 211 of 212 patients studied (99%). However, analysis of PB samples obtained at the time of BM harvest from 45 of these patients showed circulating lymphoma cells in only 22 patients (49%). The finding that the PB is less likely than BM to have residual detectable lymphoma cells at the time of BM harvest might well suggest that PB is a less contaminated source of autologous hematopoietic stem cells than BM. However, because 49% of patients in the present study had residual lymphoma cells in PB samples, this suggests that PB stem cell (PBSC) collections from a considerable number of patients with NHL with the BCL2/IgH translocation are likely to be contaminated with lymphoma. These results are in keeping with a previously published study that showed that PB stem cell harvests were contaminated with lymphoma in up to 50% of the patients studied.33 This number is comparable with the results obtained using immunologic purging of autologous BM.15 In addition, it is presently unclear whether the use of chemotherapy and growth factors that are currently used to mobilize PBSC might also mobilize tumor cells. If the present results are confirmed in larger studies, then it will not be clear whether a patient would benefit more from the use of PBSC collection or purged autologous BM. To answer this question definitively, clinical trials comparing PBSC versus purged autologous BM would be required. Ongoing clinical studies in patients undergoing high-dose therapy with PBSC support can address whether the detection of residual lymphoma cells in PBSC collections will have the same adverse effect on subsequent outcome than the infusion of residual lymphoma cells in autologous BM. The results of such studies might help to determine the optimal tissue source as stem cell support for individual patients undergoing ABMT.

In conclusion, the results of concomitant assessment of BM and PB after ABMT show that serial BM samples are necessary to predict best for subsequent relapse. Moreover, these data are consistent with the notion that post-ABMT contamination of PB appears to be the consequence of reinfusion of lymphoma cells within autologous marrow and that to improve disease-free survival after ABMT, attention should be given to remove contaminating PCR-detectable lymphoma cells from either harvested autologous BM or PBSC collections. Furthermore, future studies will be required to address the issue of whether treatment attempting to deplete PCR-detectable lymphoma cells after ABMT will also result in improved survival after ABMT.

REFERENCES

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Detection of residual lymphoma cells by polymerase chain reaction in peripheral blood is significantly less predictive for relapse than detection in bone marrow

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