Acute Lymphoblastic Leukemia Using a Cocktail of Three Monoclonal Antibodies (BA-1/CD24, BA-2/CD9, and BA-3/CD10) Plus Complement and 4-Hydroperoxycyclophosphamide for Ex Vivo Bone Marrow Purging

By Fatih M. Uckun, John H. Kersey, Robert Haake, Daniel Weisdorf, and Norma K.C. Ramsay

Fourteen patients with high-risk B-lineage acute lymphoblastic leukemia (ALL) in complete remission underwent autologous bone marrow transplantation (BMT) using a combined immunochemopurging protocol. A monoclonal antibody (MoAb) cocktail of BA-1, BA-2, and BA-3 plus rabbit complement (C') plus 4-hydroperoxycyclophosphamide (4-HC) was used to eliminate residual occult leukemia cells from autografts. All patients were conditioned with single-dose total body irradiation (TBI) followed by high-dose Ara-C. All 14 patients engrafted at a median of 24 days (range, 12 to 36 days). Three patients are alive and disease free at 3.5 years, 3.9 years, and 4.1 years post-BMT. The Kaplan-Meier estimates of BMT provided disease-free survival (DFS) at 3.5 years was 21% ± 11%, with a mean DFS time of 1.3 ± 0.4 years. A novel and quantitative minimal residual disease (MRD) detection assay, which combines fluorescence-activated multiparameter flow cytometry and cell sorting with leukemic progenitor cell (LPC) colony assays, was used to analyze remission BM samples from B-lineage ALL patients for residual LPC, and to evaluate the efficacy of ex vivo BM purging. Notably, the minimal residual leukemia burden before BMT, as measured by the percentage of B-lineage LPC in the pre-BMT remission BM samples, indicated the outcome of the BMT. The median value for the minimal residual leukemia burden before BMT was 0.0035% (35 LPC/10^6 mononuclear cells). The Kaplan-Meier estimates and standard errors of the probability of remaining in remission after BMT were 43% ± 19% for patients whose BM samples contained ≤0.0035% LPC and 0% ± 0% for patients whose BM samples contained greater than 0.0035% B-lineage LPC (P < .05). In contrast to the minimal residual leukemia burden measured by the described MRD assay system, the percentage of blasts or TdT' cells in the remission BM samples did not correlate with the probability of relapse. The applied purging protocol showed variable success in destroying target B-lineage LPC populations contaminating the autografts. While in some cases purging was highly effective, eliminating up to 24 logs of residual B-lineage LPC, in other cases only 0.1 to 0.2 logs of B-lineage LPC were purged. Because of this variation in efficacy, the estimated numbers of B-lineage LPC remaining in autografts, as well as the estimated numbers of reinfluenced LPC, showed a marked interpatient variation. The investigators conclude that improvements in both pretransplant conditioning and ex vivo BM purging may be needed to significantly increase the percentage of long-term survivors among high-risk B-lineage ALL patients undergoing autologous BMT.

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Despite pretransplant total body irradiation (TBI) combined with high-dose chemotherapy, posttransplant recurrence of leukemia remains a major obstacle in autologous bone marrow transplantation (BMT) for high-risk remission acute lymphoblastic leukemia (ALL). In a recent study at our institution, which used autologous BMT for treatment of refractory residual leukemia in high-risk remission ALL patients, 91% of primary failures were caused by leukemic relapses. The factors contributing to the high incidence of relapse include the inability of available pretransplant conditioning regimens to effectively eradicate the residual leukemia burden in vivo, lack of graft-versus-leukemia effects, and, possibly, inefficient purging of autografts ex vivo. To minimize the reinfection of significant numbers of leukemic blasts, most BMT centers harvest BM in complete remission, when the residual leukemia burden is believed to be minimal, and purge the autografts ex vivo by either immunologic or pharmacologic methods to eliminate occult leukemic cells. Recent evaluation of the antileukemic efficacy of various purging protocols at the level of primary clonogenic blasts (also referred to as leukemic progenitor cells [LPC]) showed that some protocols, including our standard monoclonal antibody (MoAb) cocktail of BA-1/anti-CD24, BA-2/anti-CD9, and BA-3/anti-CD10 plus rabbit complement (C') for B-lineage ALL, may be suboptimal. The addition of 4-hydroperoxycyclophosphamide (4-HC) or mafosfamide (ASTA-Z) enhanced the antileukemic activity of this purging protocol against clonogenic blasts from B-lineage ALL cell lines and primary clonogenic blasts freshly obtained from B-lineage ALL patients. These results prompted us to conduct a clinical study using the BA-1,2,3 MoAb cocktail plus rabbit C' in combination with 4-HC for purging BM from high-risk remission B-lineage ALL patients who did not have a matched sibling donor for allogeneic BMT. To evaluate the efficacy of ex vivo BM purging and to examine whether the relative quantities of residual LPC influence the probability of relapse, pre purge...
and postpurge samples of clinical autografts were analyzed for the presence of B-lineage LPC. This report details our laboratory and clinical findings using the minimal residual disease (MRD) assay system with 14 high-risk B-lineage ALL patients who underwent autologous BMT during complete remission.

MATERIALS AND METHODS

Patients. Fourteen patients with high-risk B-lineage ALL in complete remission were enrolled in a clinical study of autologous BMT. The study protocol was approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota, and written informed consent was obtained from all patients according to Department of Health and Human Services guidelines. To be eligible for this protocol, patients had to have leukemic blasts that were immunophenotyped at diagnosis or relapse and found positive for CD9, CD10, or CD24 B-lineage–associated differentiation antigens. Fourteen patients were enrolled in this study. There were two females and 12 males with a median age of 13 years (mean ± SD = 14 ± 3 years; range, 4 to 36 years). The initial white blood cell (WBC) count ranged from 1,200 to 1,290,000/μL (median, 12,650; mean ± SD = 113,343 ± 90,802). The surface antigen profile of leukemic blasts was determined in all patients at the time of diagnosis and/or relapse before BMT. Six of seven cases (100%) expressed CD9, 14 of 14 cases (100%) expressed CD10, and 8 of 8 cases (100%) expressed CD24. One patient was in first remission (UPN 815, an adult male with Philadelphia chromosome [Ph]-positive ALL), seven were in second remission, five were in third remission, and one patient was in fourth remission. Except for UPN 815, all patients had prior BM relapses and five patients had prior extramedullary relapses as well. Of these five patients, three had central nervous system (CNS) relapses and two had testicular relapses. In the 13 patients who were in their second, third, or fourth remission, the duration of their first remissions had ranged from 6 months to 62 months (mean ± SD = 30 ± 5 months; median, 31 months).

BM harvest. BM was harvested under general anesthesia by multiple aspirations from the iliac crests in volumes to yield a minimum of 4.5 × 10^6 nucleated cells/kg. The BM was processed and treated ex vivo with a combination of three MoAb + C' and 4-HC, as described below. Nucleated cells (0.5 to 1.0 × 10^7) were stored unfrozen as backup BM to be used in case of graft failure.

Ex vivo purging of autologous BM grafts. The treatment of BM mononuclear cells (MNC) from remission autologous BM grafts with BA-1, BA-2, BA-3 MoAb plus C' in combination with 4-HC for elimination of residual leukemic blasts has been described in detail elsewhere.12-14 4-HC was used at 10 μg/mL, a concentration that was found to augment the antileukemic activity of the MoAb + C' purging protocol without significant additional BM progenitor cell toxicity.14

Detection of residual leukemic blasts in autologous BM grafts before and after purging. Ficoll-Hypaque–separated BM MNC from purpure and postpurge samples of autologous BM grafts were analyzed by two-color immunofluorescence for the “early” B-lineage cell surface differentiation antigen CD19 using phycocyanin (PE)-labeled B43 (anti-CD19) MoAb, and the “late” B-lineage cell surface differentiation antigen sIgM using fluorescein isothiocyanate (FITC)-conjugated 2C3 (anti-IgM) MoAb, as described.15,16 Sterile cell sorting at 1,500 cells/s was performed to isolate viable CD19(red) sIgM (green) B-cell precursors (BCP) in the “lymphoid” and “blast” light scattering windows, as described.15,16 Fluorescence-activated cell sorting (FACS) was performed and sorted BCP were assayed for B-lineage ALL blast colony formation in the presence of 10% vol/vol low molecular weight B-cell growth factor (L-BCGF; Cellular Products, Buffalo, NY) in a LPC assay system, as detailed in previous publications.15,16 After 7 days of culture, whole petri dishes containing the cultures were harvested for morphologic and immunologic characterization of colony cells, as previously described.15,16 In addition to this MRD detection assay, we also used multicolor multiparameter flow cytometry (FCM) without cell sorting to monitor the lysis of target B-lineage lymphoid cell populations by the MoAb + C' component of the purging protocol. To this end, BM MNC from purpure and postpurge samples of autologous BM grafts were analyzed in a comparative fashion by two-color immunofluorescence for the presence of CD10(J5-PE') CD19(B43-FITC'), CD10(J5-PE') CD22(G28-7-FITC'), and CD19(B43-PE') sIgM(2C3-FITC') BCP within the lymphoid light scattering window, as described.13,18

Preparative regimens and supportive care. All 14 patients were prepared with single-dose TBI (850 cGy) total dose delivered using 10-MeV X-rays at a dose rate of 26 cGy/min on day −8) followed by 12 doses of Ara-C on days −7 through −2 at a dose of 3 g/m² administered as a 1-hour intravenous infusion twice daily (total dose of Ara-C, 36 g/m²). After 1 day of rest (day −1), autologous BM grafts were thawed and administered on day 0. The details of this preparative regimen were previously published.10,11 Supportive care during the hospital stay was provided as previously detailed.13

Data analysis. The clinical and laboratory data were retrieved from the University of Minnesota BMT Data Base, which systematically and prospectively collects data on all of our BMT transplant recipients. The data were analyzed by standard statistical methods with the assistance of the BMDP-90 software program (University of California Press, Berkley, CA). Posttransplant event times were measured from the day of BMT to the day of relapse, death, or last follow-up. Disease-free survival (DFS) was defined as the time from date of BMT to date of relapse or death, whichever occurred first. The probabilities for remaining in remission and DFS were estimated and relapse-free interval curves were generated using the Kaplan-Meier product limit method. We used the Cox regression method19 to examine the relationship between the five potential predictor variables (%TdT⁺ cells, % lymphoblasts, % B-lineage LPC in the preparative BM samples, LPC/kg reinfused in the autografts, and the log kill of LPC by the applied purging protocol) and relapse post-BMT. Given the wide ranges in values for the percent LPC in the preparative BM samples and for LPC per kilogram, log transforms were applied to the above listed potential predictors. Due to the number of statistical tests performed, Bonferroni adjustments were made to the resulting P values. Statistical significance is quoted only for those predictors that remain significant (P < .05) after adjustment for multiple comparisons.

RESULTS

Quantitative analysis of BM remission status by multiparameter FCM, cell sorting, and LPC colony assays. The percentage of lymphoblasts in the pretransplant BM specimens ranged from 0% to 2.7% (mean ± SD = 0.9% ± 0.2%; median, 0.6%) and the percentage of TdT⁺ cells ranged from 0% to 4.5% (mean ± SD = 1.3% ± 0.4%; median, 1.0%) (Table 1). In an attempt to more accurately examine the BM remission status of patients, we performed a quantitative analysis of residual leukemia in remission BM specimens. Specifically, we analyzed the CD19⁺ sIgM⁺ BCP fraction in the “lymphoid” plus “blast” light scattering windows for the presence of B-lineage LPC. The percentage of CD19⁺ sIgM⁺ BCP among the BM MNC showed a marked interpatient variation ranging from 0.2% (UPN

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Table 1. Residual Leukemia in High-Risk Remission B-Lineage ALL BM Samples

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<th>%CD19&lt;sup&gt;+&lt;/sup&gt;IgM&lt;sup&gt;-&lt;/sup&gt; BCP&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Mean No. of Blast Colonies per 10&lt;sup&gt;6&lt;/sup&gt; FACS-Sorted BCP&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Immunophenotypic Features of Colony Blasts in L-BCGF-Stimulated Cultures of FACS-Sorted CD19&lt;sup&gt;+&lt;/sup&gt;IgM&lt;sup&gt;-&lt;/sup&gt; BCP&lt;sup&gt;+&lt;/sup&gt;</th>
<th>B-Lineage LMC per 10&lt;sup&gt;6&lt;/sup&gt; MNC</th>
<th>B-Lineage LMC&lt;sup&gt;+&lt;/sup&gt; %</th>
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Normal BM controls

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B-lineage ALL BM controls

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<td>930</td>
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Abbreviation: ND, not determined.

Fresh BM samples obtained at the time of BM harvest in complete remission were examined for the presence of residual LPC, as described in Materials and Methods. Colony assays were set up in duplicate. The variation in the number of TdT<sup>+</sup>CD19<sup>+</sup> blast colonies from individual dishes did not exceed 30% of the mean values. The immunophenotypic features of colony blasts were determined as described in Materials and Methods, and the phenotype data are shown as the percentage of colony blasts expressing each marker. The numbers of B-lineage LMC per 10<sup>6</sup> MNC were calculated from the percentage of FACS-sorted BCP among MNC and the numbers of TdT<sup>+</sup>CD19<sup>+</sup> blast colonies using the formula: B-lineage LMC/10<sup>6</sup> MNC = [CD19<sup>+</sup>IgM<sup>-</sup> MNC (%)/100] × [mean no. of blast colonies/10<sup>6</sup> FACS-sorted cells] × 10<sup>9</sup>/. Controls included normal BM specimens from four healthy volunteers and leukemic BM specimens from five newly diagnosed B-lineage ALL patients.

Abbreviation: ND, not determined.

0749) to 39.8% (UPN 0661) (mean ± SE = 7.2% ± 2.7%; median, 5.0%). When stimulated with L-BCGF (10% vol/vol), FACS-sorted virtually pure populations of CD19<sup>+</sup>IgM<sup>-</sup> cells from 14 of 14 patients formed 4 to 523 (mean ± SE = 164 ± 39; median, 145) blast colonies per 100,000 cells (Table 1). Colony cells displayed blast morphology with a high nuclear to cytoplasmic ratio, highly irregular nuclear membrane, and prominent nucleoli (Fig 1). In nine cases, sufficient numbers of colony cells were obtained for immunophenotypic analyses. As shown in Table 2, colony blasts were TdT<sup>+</sup> (7 of 7 cases; mean % positivity, 90% ± 3%), CD10/CALLA<sup>+</sup> (8 of 9 cases; mean % positivity, 68% ± 9%), CD19<sup>+</sup> (9 of 9 cases; mean % positivity, 93% ± 3%), cytoplasmic CD22<sup>+</sup> (4 of 4 cases; mean % positivity, 99% ± 1%), CD24<sup>+</sup> (7 of 7 cases; mean % positivity, 76% ± 5%), and slg<sup>-</sup> (8 of 8 cases; mean % positivity, 1% ± 0.5%), consistent with B-lineage ALL. In two cases (UPN 0661 and UPN 0771) colony blasts expressed cytoplasmic μ heavy chains (C<sub>μ</sub>), consistent with pre-B ALL, while the remaining seven cases were C<sub>μ</sub> negative (mean % positivity, 2% ± 1%), consistent with pre--pre-B ALL. The number of B-lineage LMC per 10<sup>6</sup> MNC in the analyzed remission BM specimens from all 14 patients was calculated using the formula: B-lineage LMC/10<sup>6</sup> MNC = [CD19<sup>+</sup>IgM<sup>-</sup> MNC (%)/100] × [mean no. of blast colonies/10<sup>6</sup> FACS-sorted CD19<sup>+</sup>IgM<sup>-</sup> BCPs × 10<sup>9</sup>/10<sup>6</sup>]. A pronounced interpatient variation was observed, ranging from 0.2 to 346.0 (mean ± SE = 82.7 ± 27.9; median, 35). Thus, the remainder BM specimens of these high-risk B-lineage ALL patients contained 0.0002% to 0.0346% (median, 0.0035%) residual B-lineage LMC. We have previously shown that L-BCGF can stimulate in vitro

Fig 1. Detection of residual CD19<sup>+</sup> LPC in remission BM samples of high-risk B-lineage ALL patients. (A) In situ morphology of day 7 blast colonies in L-BCGF-stimulated cultures of FACS-sorted CD19<sup>+</sup>IgM<sup>-</sup> BCPs. (B) Wright-Giemsa-stained preparations of colony blasts from day 7 blast colonies shown in (A). Note the immature blast morphology with irregular nuclei, prominent nucleoli, and high nuclear/cytoplasmic ratio. (C) Nuclear TdT staining of day 7 colony blasts shown in (B). (D) Surface CD19 antigen expression of day 7 colony blasts shown in (B) and (C).
Fig 1.
period in the presence of L-BCGF. Although FACS-sorted proliferation and colony formation of normal BCP from samples shown in this table. The immunophenotypic features of colony blasts were determined as described in Materials and Methods, and the fetal hematopoietic organs. Notably, unlike normal BCP specimens did not differentiate during the 7-day culture specimens as negative controls. As evident in Table 1, important to include normal adult and pediatric BM B-lineage LPC were calculated as described in the legend of Table 1.

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<th>UPN</th>
<th>% CD19'sIgM- BCP</th>
<th>CD19'sIgM- BCP</th>
<th>Mean No. of Blast Colonies/10⁵ FACS-Sorted</th>
<th>CD19'sIgM- BCP (°) Cells (%) positive</th>
<th>CD19'sIgM- BCP</th>
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Samples from purged autografts were analyzed for the presence of residual B-lineage LPC as described in Materials and Methods. The numbers of B-lineage LPC were calculated as described in the legend of Table 1. Log kill values were obtained by comparing on a logarithmic scale the number of B-lineage LPC per 1⁰⁶ MNC in pre purge autograft samples shown in Table 1 with the number of B-lineage LPC per 1⁰⁶ MNC in post purge autograft samples shown in this table. The immunophenotypic features of colony blasts were determined as described in Materials and Methods, and the phenotype data are shown as the percentage of colony blasts expressing each marker.

Abbreviation: ND, not determined.

proliferation and colony formation of normal BCP from fetal hematopoietic organs. Notably, unlike normal BCP from fetal hematopoietic organs, FACS-sorted CD19'sIgM- BCP from B-lineage ALL remission BM specimens did not differentiate during the 7-day culture period in the presence of L-BCGF. Although FACS-sorted BCP from B-lineage ALL remission BM did not display any degree of differentiation and postnatal BM contain much fewer BCP than fetal BM or fetal liver specimens, it was still important to include normal adult and pediatric BM specimens as negative controls. As evident in Table 1, negative control BM specimens from four healthy donors contained no detectable B-lineage LPC under these assay conditions. By contrast, all of the positive control BM specimens from newly diagnosed B-lineage ALL patients contained significant numbers of B-lineage LPC, which ranged from 1,987.2 LPC/1⁰⁶ cells to 16,651.8 LPC/1⁰⁶ cells. Thus, although the MRD detection assay system displays no exquisite specificity for in vitro growth of leukemic versus normal BCP, it clearly reflects the leukemic clonogenic cell burden of BM specimens. In additional experiments, we determined the LPC numbers in remission BM from two patients (UPN 723 and UPN 749) after the addition of varying numbers (0.1% to 50%) of L-BCGF-responsive allogeneic fresh leukemic B-lineage ALL blasts. In both experiments, we found a strong correlation between the numbers of added allogeneic leukemic blasts and the total numbers of LPC (UPN 723: number of LPC = 1/1⁰⁶ cells for 0.1% contamination, 218/1⁰⁶ cells for 1% contamination, 3,007/1⁰⁶ cells for 10% contamination, 7,752/1⁰⁶ cells for 50% contamination, and 11,610/1⁰⁶ cells for 100% contamination; correlation coefficient = .980, P = .001; UPN 749: number of LPC = 12/1⁰⁶ cells for 0.1% contamination, 90/1⁰⁶ cells for 1% contamination, 849/1⁰⁶ cells for 10% contamination, 4,906/1⁰⁶ cells for 50% contamination, and 5,865/1⁰⁶ cells for 100% contamination; correlation coefficient = .959, P = .002). When allogeneic leukemic blasts were irradiated in vitro with 20 Gy gamma rays before their addition to the remission BM samples to yield a 50% contamination, a decrease rather than an increase in LPC numbers was found because the fraction of viable LPC isolated by FACS sorting decreased because of the higher number of nonviable LPC contamination (data not shown). Thus, results obtained using remission BM from B-lineage ALL patients were not obscured by normal autologous BCP populations capable of clonogenic growth in the presence of L-BCGF.

Efficacy of ex vivo BM purging. As illustrated by the representative cases shown in Fig 2, the purging protocol had variable success in destroying CD19'CD10', CD19'CD10', CD19 'sIgM', or CD10'CD22' BCP populations within the lymphoid light scattering window. Comparison of the pre purge and post purge autograft specimens indicated a prompt and nearly complete elimination of these BCP populations in some cases, but not in others. We next used our MRD detection assay system to compare the numbers of residual B-lineage LPC in the pre purge versus post purge autograft samples. When the residual CD19'sIgM- BCP from post purge autograft samples were isolated by multiparameter FACS and cultured in the presence of L-BCGF, no blast colonies were observed in 7 of 14 cases. In the remaining seven cases, there were 5 to 359 (mean ± SE = 169 ± 53) blast colonies per 1⁰⁶ cells (Table 2). In five of seven cases with blast colony formation by BCP from post purge samples, we were able to determine the immunophenotype of colony blasts. As shown in Table
Fig 2. Multiparameter FCM analysis of elimination of BCP populations from autologous BM grafts by immediate lytic effects of the combined immunochemopurging protocol. MNC from prepurge and postpurge autograft samples were stained with various MoAb combinations and analyzed for the presence of CD19+slgM-, CD10+CD19+, CD10-CD19+, CD10'CD22' BCP populations. Note in (A) the effective elimination of BCP from the autograft of UPN 0661 by purging and, in sharp contrast to it, in (B) the failure of the purging protocol to eliminate BCP from the autograft of UPN 0837.

2, colony blasts were TdT+ (4 of 4 cases, mean % positivity = 86% ± 6%), CD10+ (5 of 5 cases, mean % positivity = 81% ± 9%), CD19+ (5 of 5 cases, mean % positivity = 88% ± 2%), cytoplasmic CD22+ (2 of 2 cases), CD24+ (5 of 5 cases, mean % positivity = 79% ± 7%), and slg+ (4 of 4 cases, mean % positivity = 2% ± 2%), consistent with B-lineage ALL. In two cases (UPN 0661 and 0771), colony blasts were Cp+, consistent with pre-B ALL. Thus, the immunophenotypic features of colony blasts in the cultures of BCP from postpurge autograft samples were identical to those of colony blasts in the cultures of BCP from prepurge samples. In particular, blasts did not differ in expression of CD10 (prepurge, 68 ± 9% v postpurge 81% ± 9%, \( P = .4 \)) or CD24 (prepurge 76% ± 5% v postpurge 79% ± 7%, \( P = .8 \)), two of the three antigens targeted by the MoAb cocktail. This finding rules out lack of expression of these antigens as the underlying cause of inefficient purging. The estimated numbers of B-lineage LPC in the postpurge autografts ranged from less than 0.01 LPC/10⁶ MNC (ie, < 0.0000006% of MNC) in UPN 0749 to 208 LPC/10⁶ MNC (ie, 0.02% of MNC) in UPN 0755 (Table 2) (mean ± SE = 24.8 ± 16.2 LPC/10⁶ MNC; median, 0.250 LPC/10⁶ MNC) (ie, mean ± SE = 0.0025% ± 0.0016%; median, 0.000025%). The comparison of the B-lineage LPC numbers in the prepurge autograft samples (Table 1) versus postpurge samples (Table 2) showed a marked interpatient variation in the antileukemic efficacy of the applied BM purging protocol. In one case (UPN 0723), B-lineage LPC numbers in prepurge and postpurge samples were not sufficient to estimate the log kill efficacy of purging. In six cases, purging was highly effective, eliminating \( \geq 2.48 \) logs to \( \geq 4.28 \) logs of residual B-lineage LPC, and in five cases it was moderately effective, eliminating 1.12 logs to 2.13 logs of residual B-lineage LPC. The mean log kill efficacy in these 11 cases was 2.4 ± 0.3 logs. In the remaining two cases (UPN 0755 and UPN 0837), purging was not effective at all, and only 0.13 to 0.20 logs of B-lineage LPC were eliminated.

**BMT and posttransplant course.** The post-BMT events are detailed in Table 3. The cell dose of the BM grafts...
Table 3. BMT and Posttransplant Course

<table>
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<th>Time to Event (d)</th>
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The preparative regimen, supportive care, method of BM processing, and BMT were described in previous reports.2,10,11

Abbreviation: GI hemorrhage, gastrointestinal hemorrhage.

ranged from 0.32 × 10^9/kg to 0.99 × 10^9/kg (mean ± SE = 0.70 ± 0.05 × 10^9/kg; median, 0.73 × 10^9/kg). The estimated numbers of reinfused B-lineage LPC (LPC/kg = reinfused cell dose/kg × %LPC among MNC of post-purge autograft samples/100) ranged from less than 1 LPC/kg in UPN 0671, UPN 749, UPN 0770, and UPN 850, to 20,592 in UPN 0755 (mean ± SE = 2,214 ± 1,574; median, 13/kg). All 14 patients engrafted, as defined by a WBC greater than 1,000/μL for 3 consecutive days, at a median of 24 days (range, 12 to 36 days; mean ± SE = 24 ± 2 days). One patient died on day 72 of hemorrhage and respiratory failure due to adult type respiratory distress syndrome (ARDS). Ten patients relapsed at 73 to 731 days (mean ± SE = 233 ± 62 days; median, 182 days) post-BMT and eight have subsequently died of leukemia. Of the remaining two patients who relapsed, UPN 689 died of interstitial pneumonitis on day 544 and UPN 815 died of Aspergillus pneumonia on day 418. Three patients (UPN 0723, 0749, and 0814) are alive and disease free at 3.5 years, 3.9 years, and 4.1 years post-BMT. The Kaplan-Meier estimate and standard error of the probability of remaining in remission was 23% ± 12% at 3.5 years post-BMT with a mean relapse-free interval of 1.4 ± 0.4 years (median, 0.6 years) (Fig 3A). The overall DFS at 3.5 years was 21% ± 11% with a mean DFS time of 1.3 ± 0.4 years (median, 0.5 years).

**Correlation between MRD and probability of relapse post-BMT.** Cox regression was used to determine the relationships between relapse-free interval and the five laboratory parameters of MRD. After making Bonferroni adjustments for multiple comparisons, the percent B-lineage LPC in the pre-BMT/prepurge remission BM samples was a significant predictor of relapse (P < .05). The product-limit estimates for the probability of sustained remission among patients with BM LPC percentages less than or equal to versus greater than the median value of 0.0035% are depicted in Fig 3B. The Kaplan-Meier estimates and standard errors of the probability of remaining in remission after BMT were...
43% ± 19% for patients whose BM samples contained ≤0.0035% B-lineage LPC and 0% ± 0% for patients whose BM samples contained greater than 0.0035% B-lineage LPC. LPC per kilogram was also a significant predictor of relapse post-BMT (P < .05). The Kaplan-Meier estimates and standard errors of the probability of remaining in remission after BMT were 33% ± 19% for patients who received less than the median estimated value of 13 LPC/kg in their reinfused autografts and 14% ± 13% for patients who received ≥13 LPC/kg in their reinfused autografts (data not shown). No significant relationships were found between the probability of remaining in remission and the percentage of TdT+ cells, percentage of lymphoblasts, or log kill of LPC contaminating the autografts by the purging protocol (all P > .05).

**DISCUSSION**

We used a new BM purging protocol that combines a cocktail of BA-1/anti-CD24, BA-2/anti-CD9, and BA-3/anti-CD10, MoAb, rabbit C', and 4-HC, in an attempt to minimize the chance of reinfusing significant numbers of leukemic blasts in purged autografts. All patients showed prompt engraftment at a median of 24 days, which provides evidence that this immunochemopurging protocol does not significantly impair the repopulating capacity of autografts. Three patients remain alive and disease free at 3.5 years, 3.9 years, and 4.1 years post-BMT, which shows that prolonged DFS can be achieved in some high-risk remission B-lineage ALL patients who do not have a matched sibling donor by autologous BMT during complete remission using ex vivo purged autografts.

In recent years, several laboratories have developed highly sensitive MRD detection methods to discern very small numbers of residual leukemic blasts in remission BM samples from acute and chronic leukemia patients. Proposed strategies include multiparameter FCM and immunophenotyping, cytogenetic, biophysical, and biochemical analyses, clonogenic assays, Southern blot hybridization analyses of Ig or T-cell receptor gene rearrangements, and polymerase chain reaction (PCR) technology for amplification of DNA sequences flanking the crossover sites of clonotypic chromosomal translocations or rearranged T-cell receptor γ- or δ-chain sequences. Hansen-Hagge et al reported that the PCR amplification of rearranged T-cell receptor δ-chain sequences using clonospecific probes permits the detection of as little as 0.0001% leukemic cell DNA. Similar levels of sensitivity in detecting residual ALL blasts were reported by d'Auriol et al, who used PCR amplification of T-cell receptor γ-chain DNA sequences, by Yamada et al, who used PCR amplification of complementary-determining region III DNA sequences, as well as by Kawasaki et al and Hunger et al, who used PCR amplification of leukemia-specific messenger RNA (mRNA) sequences. In more recent studies, several investigators have started to evaluate the clinical value of PCR-based MRD detection assays. Yamada et al presented molecular evidence for the persistence of residual disease during the first 18 months of treatment in eight of eight children with B-lineage ALL. Intriguing data published recently by Neale et al suggested that the detection of MRD in T-lineage ALL by PCR technology may predict impending relapse. Gehly et al provided elegant evidence that PCR-detectable chimeric BCR-ab1 mRNA can be used as a marker for MRD for prediction and/or early identification of relapse in Philadelphia chromosome-positive ALL patients undergoing BMT. Yokota et al reported that detection of MRD by PCR predicted clinical relapse in two patients with ALL. Recently, we developed a novel quantitative MRD detection assay system for T-lineage ALL, which combines multiparameter FCM and FACS sorting with LPC assays. Importantly, high numbers of residual LPC in remission BM of high-risk T-lineage ALL patients before BMT appeared to constitute a poor prognostic indicator, providing evidence for the clinical value of in vitro LPC colony assays in BMT.

Our published results on the use of multiparameter FCM and FACS sorting with LPC assays for detection of residual clonogenic blasts in remission BM from T-lineage ALL patients prompted us to design a similar MRD detection assay for B-lineage ALL. This new MRD assay for B-lineage ALL is based on FACS sorting of CD19/sIgM- BCP from B-lineage ALL remission marrow samples and their in vitro culture in the presence of L-BCGF using a B-lineage LPC colony assay system. Intriguingly, high numbers of residual B-lineage LPC in remission BM immediately before BMT predicted relapse, while the percentage of lymphoblasts and the percentage of TdT+ cells were not prognostic. We did not obtain sufficient numbers of cells from day 7 blast colonies to perform Southern blot hybridization or cytogenetic analyses to confirm the clonality and leukemic origin of colony blasts. Therefore, we cannot formally exclude the possibility that a small fraction of blast colonies counted as TdT+CD10-CD19+sIgM- BCP colonies were derived from normal CD10-CD19+sIgM- B-lineage lymphoid progenitor cells. However, (1) the correlation of LPC numbers with the probability of relapse; (2) the lack of differentiation of FACS-sorted CD19+sIgM- B-CP from B-lineage ALL remission BM samples, in sharp contrast to the rapid differentiation (as measured by acquisition of Cμ and sIgM) of normal BCP from fetal hematopoietic organs; (3) the absence of detectable B-lineage LPC in negative control BM specimens from healthy donors; (4) the presence of varying numbers of B-lineage LPC in all positive control BM specimens from B-lineage ALL patients in partial relapse or from newly diagnosed B-lineage ALL patients; and (5) the strong correlation between the number of viable leukemic blasts added to remission BM samples from B-lineage ALL patients and the numbers of LPC provide strong, albeit circumstantial, evidence that this MRD detection assay system measures the leukemic clonogenic cell burden of BM specimens, and that the results are not complicated by normal BCP populations capable of clonogenic growth in the presence of L-BCGF.

Our preliminary findings reported here indicate that the MRD detection assay system described in the present.
We therefore hypothesize that this assay system will help us to determine when the residual disease burden of high-risk ALL patients is sufficiently small to allow the eradication of occult leukemia cells by high-dose chemotherapy or radiochemotherapy before BMT. Separate long-term studies on a much larger patient population will be needed to determine whether the MRD data generated using this assay system will enhance the predictive strength of established prognostic parameters such as WBC at diagnosis. In comparison to cytogenetic or molecular genetic analyses, this MRD detection assay system does not require the presence of clonal chromosomal abnormalities or the availability of clonospecific probes, thereby providing an opportunity for routine analysis of MRD in remission ALL BM samples. Furthermore, a quantitative analysis of remission BM samples for the presence of residual clonogenic ALL blasts by this MRD assay system will likely yield more biologically relevant information about the quality of remission than qualitative analyses that do not discriminate between ALL blasts of different clonogenic or proliferative potential, such as PCR amplification of clonotypic DNA sequences. Perhaps the combined use of this MRD assay and a highly sensitive PCR method in future studies will yield the most reliable and biologically most significant information regarding the quality of remission in B-lineage ALL patients. Such “second generation” combined MRD assays would provide the opportunity (1) to compare the quality of remission achieved after different conditioning regimens in both allogeneic and autologous BMT; (2) to measure the effects of graft-versus-host disease or the regimens used to prevent graft-versus-host disease (such as cyclosporin A or methotrexate) on the residual leukemia burden; and, most importantly, (3) to compare the residual leukemia burden of long-term survivors of ALL who have undergone either multiagent chemotherapy or BMT.

Surprisingly, our BA-1,2,3 + C’ + 4-HC immunochemopurging protocol showed variable success in destroying target B-lineage LPC populations. While in some cases purging was highly effective, eliminating up to ≥4 logs of residual B-lineage LPC, in other cases purging eliminated only 0.1 to 0.2 logs of B-lineage LPC. Because of this inconsistency, the estimated numbers of remaining B-lineage LPC in postpurgate autografts as well as the estimated numbers of reinfused LPC showed a marked interpatient variation. While several factors (eg, variations in antigen expression, C’ sensitivity, 4-HC sensitivity, or shielding of leukemic blasts by normal cell populations with the same target B-lineage differentiation antigens) may contribute to the variable efficacy of this combined protocol, multiparameter FCM analysis of the immediate lytic effects of BM purging on target BCP populations provided strong evidence for a marked heterogeneity among patients’ sensitivity to C’. The immunophenotypic analyses of target BCP populations and LPC-derived colony blasts have shown that the interpatient variation in the efficacy of this immunochemopurging protocol was not caused by marked qualitative or quantitative differences in expression of target antigens. Notably, patients who received higher numbers of LPC in their autografts had earlier and more frequent relapses, as compared with patients whose autografts were contaminated with no or very few remaining LPC after purging. Because higher numbers of LPC in prepurge autograft samples were associated with higher numbers of remaining LPC after purging, the observed relationship between LPC per kilogram and relapse could be explained by the predictive value of the LPC numbers in the prepurge autograft samples. Whether reinfused LPC contributed to the higher relapse rate in patients who had more LPC in their autografts needs to be investigated in a larger series of patients. Improvements in both pretransplant conditioning and ex vivo BM purging should increase the percentage of long-term survivors among high-risk B-lineage ALL patients undergoing autologous BMT.

ACKNOWLEDGMENT

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FM Uckun, JH Kersey, R Haake, D Weisdorf and NK Ramsay