Autologous Bone Marrow Transplantation in High-Risk Remission T-Lineage Acute Lymphoblastic Leukemia Using Immunotoxins Plus 4-Hydroperoxycyclop phosphamide for Marrow Purging

By Fatih M. Uckun, John H. Kersey, Daniel A. Vallera, Jeffrey A. Ledbetter, Daniel Weisdorf, Dorothea E. Myers, Robert Haake, and Norma K.C. Ramsay

Fourteen patients with high-risk T-lineage acute lymphoblastic leukemia (ALL) in complete remission underwent autologous bone marrow transplantation (BMT) in an attempt to eradicate their residual disease burden. A combined immunochemotherapy protocol using a cocktail of two immunotoxins directed against CD5/TP67 and CD7/TP41 T-lineage differentiation antigens in combination with the in vitro active cyclophosphamide congener 4-hydroperoxycyclophosphamide (4-HC) was used to purge autografts. Despite high dose pretransplant radiochemotherapy and effective purging of autografts, 9 of 14 patients relapsed at a median of 2.5 months (range, 1.2 to 16.8 months) post BMT. Two patients remain alive and disease free at 26 and 28 months post BMT. We used a novel assay to detect minimal residual disease (MRD) at diagnosis and to predict disease-free survival post BMT. These results indicate that the primary reason for the recurrence of leukemia was inefficient pretransplant radiochemotherapy rather than inefficient purging of autografts.

Material and Methods

Patient selection. Between October 1986 and May 1989, 14 consecutive patients with T-lineage ALL in complete remission were treated with autologous BMT using an immunotoxin protocol. Notably, high numbers of residual Lac detected in remission BM before BMT constituted a poor prognostic indicator. Providing the first evidence for the biologic significance and clinical value of in vitro T-lineage ALL LPC assays. The median value for the residual leukemia burden before BMT was \( -8.6 \times 10^3 \text{LPC} \times 10^8 \text{MNC} (\text{MNC}) \). Patients with a residual leukemia burden less than this median value appeared to have a better outcome for remaining free of relapse after autologous BMT than patients with a greater leukemia burden (53 ± 25% \( \pm_0 \pm_1 \% \text{LPC} \)). By comparison, the log kill efficacy of purging, the remaining numbers of LPC in purged autografts, or the estimated numbers of reinfused LPC, did not correlate with the probability of disease-free survival (DFS). These results indicate that the primary reason for the recurrence of leukemia was inefficient pretransplant radiochemotherapy rather than inefficient purging of autografts.

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Submitted March 12, 1990; accepted July 2, 1990.

Supported in part by US Public Health Service Grants No. P01-CA-21737, R01-CA-42633, R01-CA-31618, R01-CA-35725, R01-CA-25097, R29-CA-42111, Bristol Myers Research Grant No. 220, and special grants awarded by the Minnesota Medical Foundation, Children's Cancer Research Fund, Bone Marrow Transplant Research Fund, and the Coleman Leukemia Research Fund, University of Minnesota.

F.M.U. is a Scholar of the Leukemia Society of America. J.H.K. is Recipient of an Outstanding Investigator Grant (CA-41729) from the National Cancer Institute. This is publication no. 40 from the Tumor Immunology Laboratory.

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enrolled in a clinical study of autologous BMT for high-risk T-lineage ALL. Follow-up was complete through May 1990. 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 before entering the study according to Department of Health and Human Services (DHHS) guidelines. To be eligible for this protocol, patients had to have leukemic blasts that were immunophenotyped at diagnosis or relapse and found positive for CD5(T,p67) and/or CD7(T,p41) T-lineage differentiation antigens. Eligible patients included: (1) all patients less than 45 years of age and in second or third BM remission who lacked a histocompatible matched donor; (2) high-risk adolescents, defined as patients 16 to 20 years of age whose (a) WBC at the time of diagnosis was greater than 10,000/μL, or (b) leukemic blasts displayed structural chromosomal abnormalities (eg, translocations, deletions) in their first or subsequent remission; (3) children less than 16 years of age whose leukemic blasts had either a 4;11 translocation or a Philadelphia chromosome in their first or subsequent remission; and (4) adults 21 to 45 years of age in their first or subsequent remission. Patients who had a chemotherapy-induced remission after experiencing an isolated extramedullary relapse while receiving maintenance chemotherapy were eligible. Patients relapsing more than 1 year after cessation of maintenance chemotherapy were not eligible.

**Immunologic surface marker analyses.** Immunologic marker analyses of ALL BM mononuclear cell suspensions by indirect immunofluorescence and flow cytometry were performed as previously described in detail.12-16 Samples were scored positive when more than 20% of cells bound the antibody used.

**Bone marrow harvest.** BM was harvested under general anesthesia by multiple aspirations from the iliac crests in volumes to yield a minimum of 4.5 × 10^9 nucleated cells/kg. The BM was processed and treated ex vivo with a combination of two immunotoxins and 4-HC, as described below; 0.5 to 1.0 × 10^9 nucleated cells were stored untreated as backup BM to be used in case of graft failure.

**Ex vivo purging of autologous bone marrow grafts.** Anti-CD5 monoclonal antibody (MoAb) T101 (lgG2a) and anti-CD7 MoAb G3.7 (lgG1) were linked to intact ricin, as previously described.12,16,17,18 BM MNCs were transferred to 5-L Belco bottles with a stir bar containing a prefrozen solution of RPMI 1640 supplemented with 300 mmol/L lactose plus 2% human serum albumin (HSA) plus 2% penicillin/streptomycin (PS) plus an appropriate amount of the immunotoxin cocktail to yield a final cell concentration of 10 × 10^6 cells/mL, a final immunotoxin concentration of 500 ng/mL for T101 (anti-CD5) Ricin and 500 ng/mL for G3.7 (anti-CD7) Ricin, and a final lactose concentration of 200 mmol/L. Cells were incubated at 37°C and 5% CO₂, under stirring at a moderate speed. After 90 minutes of incubation, an appropriate amount of a freshly prepared buffered 4-HC stock solution was added to each bottle to yield a final concentration of 10 μg/mL and a pH of 7.4 without significantly changing the final cell or immunotoxin concentrations. Following another 30 minutes of incubation at 37°C and 5% CO₂, the reaction was stopped with cold medium and cells were washed in cold medium twice to remove remaining 4-HC and unbound immunotoxin. After this purging procedure, cells were resuspended at a concentration of 50 to 100 × 10^6 nucleated cells/mL, and autologous irradiated or microporefiltered plasma and cold dimethylsulfoxide as cryoprotectant (Cryoserv; Research Industries Corp, Salt Lake City, UT) were rapidly added to give a final volume proportion of 10% for each in freezing bags (Delmed, Canton, MA). Bags were immediately transferred to a controlled-rate freezer equipped with a model 1010 microcomputer programmable freezer controller for precision cooling rates (Cryomed Freezing System; Cryomed, Mt Clemens, MI), brought to 0°C and cooled to −60°C at −1°C/min, and then to −100°C at −3°C/min. Frozen bags were stored in liquid nitrogen until reinfusion.

**Detection of residual leukemic blasts in autologous marrow grafts before and after purging.** Ficol-Hypaque separated BM MNCs from prepurge and postpurge samples of autologous BM grafts were analyzed by two-color immunofluorescence for the "early" T-lineage cell surface differentiation antigens CD5 and CD7 using a mixture of FITC labeled 10.2 (anti-CD5) and G3.7 (anti-CD7) MoAbs and the "late" T-lineage cell surface differentiation antigen CD3 using the phycoerythrin (PE) conjugate of G19.4 (anti-CD3) MoAb, as described elsewhere.15,16 Sterile cell sorting at 1,500 cells/s was performed to isolate viable CD5⁺(green) CD3⁻(red) T-cell precursors as well as CD5⁺ CD3⁻ T-lineage cells, as described previously.15,16 CD5⁺ CD3⁻ cell fractions were analyzed since some ALL cases had a CD3⁻ mature T-cell ALL immunophenotype. FACS sorted cells were assayed for T-lineage ALL blast colony formation in a LPC assay system, as reported in detail in previous publications.12,13 After 7 days of culture, whole petri dishes containing the cultures were harvested for morphologic and immunologic characterization of colony cells.

**Preparative regimens and supportive care.** Two different pretransplant preparative regimens were used in this study. The first nine consecutive 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 (IV) infusion twice daily (total dose of Ara-C, 36 g/m²) (regimen A). In an attempt to decrease the posttransplant relapse rate, the last five consecutive patients were prepared with hyperfractionated TBI (1,375 cGy total dose delivered in 11 × 125 cGy fractions on days −7, −6, −5, and −4) followed by 60 mg/kg/d cyclophosphamide on days −3 and −2, which was administered as a 1-hour IV infusion on each day (regimen B). After 1 day of rest (day −1), autologous BM grafts were thawed and administered on day 0. The details of these preparative regimens were published previously.8,10,20 Supportive care during the hospital stay was provided as previously detailed.8,10

**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 BM transplant recipients. The data were analyzed by standard statistical methods with the assistance of the BMDP-87 software program. Posttransplant event times were measured from the day of BMT to the day of relapse, death, or last follow-up. DFS was defined as the time from date of BMT to date of relapse or death, whichever occurred first. The probabilities for DFS and for remaining in remission were estimated and relapse free interval curves were generated using the Kaplan-Meier product limit method.

**RESULTS**

**Pretransplant characteristics of patients.** Fourteen consecutive patients were enrolled in this study. The pretransplant characteristics of patients are detailed in Table 1. There were 2 females and 12 males with a median age of 14 years (range, 6 to 35 years). The initial white blood cell count (WBC) ranged from 3,600/μL to 800,000/μL (median, 164,000/μL). In 11 patients, WBC was greater than 50,000/μL and in 8 patients, WBC was greater than 100,000/μL. The surface antigen profile of leukemic blasts was determined in all patients at the time of diagnosis and/or relapse before BMT. Two of 11 cases (18%) expressed CD1, 8 of 12 cases (67%) expressed CD2, 5 of 10 cases (50%) expressed CD3, 10 of 12 cases (83%) expressed CD5, and 14
of 14 cases (100%) expressed CD7. The cytogenetic features of leukemic blasts were examined in eight patients. Of these eight patients, four (UPN 643, 836, 882, 1051) had a pseudodiploid karyotype with structural chromosomal abnormalities (data not shown). Four patients were in first remission, five were in second remission, and five were in third remission. Five patients had prior extramedullary relapses. Of these five patients, two had central nervous system (CNS) relapses, one had testicular relapse, one had testicular involvement and leukemia cutis at relapse, and one had tonsillar relapse.

Quantitative analysis of BM remission status by multiparameter fluorescence-activated flow cytometry, cell sorting, and leukemic progenitor cell assays. The remission status of the BM was confirmed in all patients within 10 days before the preparative regimen. As shown in Table 1, the percentage of lymphoblasts in the pretransplant BM biopsy specimens ranged from 0% to 3.1% (median, 0.4%) and percentage of TdT+ cells ranged from 0% to 6% (median, 0%). To further assess the BM remission status of patients, we performed a quantitative analysis of residual leukemia in the remission BM specimens by using an experimental detection assay system for occult leukemia, which combines multiparameter fluorescence activated flow cytometry, cell sorting, and in vitro LPC assays, as described in the Materials and Methods. Because leukemic blasts from some patients (UPN 643, 665, 735, 882, 1081) at the time of diagnosis or relapse before BMT were CD3+, we analyzed both the immature CD3-CD5,7+ as well as the mature CD3+CD5,7+ fractions of CD5/CD7 (green)+ T-lineage cells in the lymphoid light scattering window for the presence of T-lineage LPCs. The percentage of CD3+CD5,7+ immature T-lineage lymphoid cells showed a significant degree of interpatient variation ranging from 5.8% (UPN 767) to 19.9% (UPN 1051) (median, 13.3%) (Table 2). FACS sorted virtually pure populations of CD3+CD5,7+ cells from 11 of 13 patients formed 12 to 540 (median, 86) compact 20- to 50-cell colonies per 100,000 cells when cultured in the presence of rIL-2 and PHA-LCM in the LPC assay system (Table 2). Colony cells had blast morphology with a basophilic cytoplasm, high nuclear to cytoplasmic ratio, highly irregular nuclear membrane, and prominent nucleoli (Fig 1 and 2). Sufficient cells for immunophenotypic analyses were obtained in six cases. As will be detailed later in this section, colony blasts in these six cases were found to be strongly positive for nuclear TdT, surface CD2, CD5, and CD7, as well as for cytoplasmic CD3, but they were negative for surface CD3 or CD25, consistent with T-lineage ALL. The percentage of CD3+CD5,7+ T-lineage lymphoid cells among MNCs ranged from 2.8% (UPN 767) to 37.3% (UPN 882) (median, 13.2%). FACS sorted CD3+CD5,7+ cells from 12 of 13 patients formed 20- to 500-cell colonies in the LPC assay system. However, in 7 of these 12 cases (UPN 692, 701, 735, 767, 817, 836, 1051), colonies were loose rather than compact, had an irregular shape, and were formed by diffusely associated 200 to 500 small cells growing in “sheets.” Isolated colony cells from these cultures displayed small mature normal lymphocyte morphology and had the composite immunophenotype...
cytospin preparations of pooled colonies, it was assumed that none of the observed colonies in these cases was composed of TdT' T-lineage ALL blasts.

\[
\text{CD3}^+\text{CD5.7}^+ \text{ cells} \times 10^5/10^5 \]

FACS sorted CD3+CD5.7+ lymphoid cells from UPN 692, 701, 735, 767, 817, 836, and 1051 as well as from controls included EM specimens from patients, and one high-risk remission E-lineage ALL patient. The numbers of CD5,7+ T-lineage LPC per 10’ MNC were calculated from the percentage of FACS sorted lymphoid cells.

Spherical, compact, and were formed by 20 to 100 tightly associated round cells with a distinct cell border of high refractility. Isolated colony cells displayed blast morphology with a highly irregular nuclear membrane and prominent nucleoli, and, as detailed later in this section, they had the composite immunophenotype TdT+CD2+CD3+CD5+CD7+CD25+, consistent with T-lineage ALL (Fig 2). The number of CD5,7+ T-lineage LPC per 10^8 MNC in the remission BM, which was calculated from the percentages of CD3-CD5,7+ cells in these control BMs ranged from 0.5% to 2%. FACS sorted CD3-CD5,7+ lymphoid cells yielded 0 to 26 20- to 30-cell colonies per 10^5 MNCs in the remission.

<table>
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<tr>
<th>UPN</th>
<th>Lymphoid Cells ( % of MNC)</th>
<th>Lymphoid Cell Subpopulations ( % of MNC)</th>
<th>Mean No. TdT+CD5,7+ Blast Colonies/10^2 FACS Sorted Cells</th>
<th>CD5,7+ LPC/10^8 MNC</th>
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<td>66.6</td>
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<td>654</td>
<td>67.3</td>
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<td>CD5,7+ LPC/10^8 MNC</td>
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<td>CD3+CD5,7+ Fraction = 19.4</td>
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<td>882</td>
<td>69.8</td>
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<td>CD3+CD5,7+ Fraction = 37.3</td>
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<td>1051</td>
<td>60.3</td>
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<td>1081</td>
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<td>CD3-CD5,7+ Fraction = 15.1</td>
<td>CD3+CD5,7+ Fraction = 10.8</td>
<td>CD5,7+ LPC/10^8 MNC</td>
</tr>
</tbody>
</table>

Normal BM controls

| BM 1 | 45.0 | 2.0 | 2.7 | 9 | 0 | ND |
| BM 2 | 64.0 | 1.0 | 8.8 | 26 | 0 | ND |

B-lineage ALL BM controls

<table>
<thead>
<tr>
<th>UPN</th>
<th>Lymphoid Cells ( % of MNC)</th>
<th>Lymphoid Cell Subpopulations ( % of MNC)</th>
<th>Mean No. TdT+CD5,7+ Blast Colonies/10^2 FACS Sorted Cells</th>
<th>CD5,7+ LPC/10^8 MNC</th>
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<td>749</td>
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<td>CD3+CD5,7+ Fraction = 6.8</td>
<td>CD5,7+ LPC/10^8 MNC</td>
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<td>011</td>
<td>98.0</td>
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<td>CD3+CD5,7+ Fraction = 0.9</td>
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<td>98.0</td>
<td>CD3-CD5,7+ Fraction = 64.3</td>
<td>CD3+CD5,7+ Fraction = 27.8</td>
<td>CD5,7+ LPC/10^8 MNC</td>
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Fresh BM samples obtained at the time of BM harvest in complete remission were examined for the presence of residual LPC, as described in the Materials and Methods. Colony assays were set up in duplicate. The variation in the number of TdT' CD5,7+ blast colonies from individual dishes did not exceed 25% of the mean values. The numbers of CD5,7+ T-lineage LPC per 10^6 MNC were calculated from the percentage of FACS sorted lymphoid cell fractions among MNC and the numbers of TdT' CD5,7+ blast colonies in the cultures of 100,000 FACS sorted lymphoid cells using the formula: CD5,7+ LPC/10^6 MNC = CD3-CD5,7+ LPC/10^6 MNC + CD3+CD5,7+ LPC/10^6 MNC where CD3-CD5,7+ LPC = (CD3-CD5,7+ MNC [%]/100) x ([mean # TdT+ colonies per 10^6 CD3-CD5,7+ cells] x [10^6/10^9]) and CD3+CD5,7+ LPC = (CD3+CD5,7+ MNC [%]/100) x ([mean # TdT+ colonies per 10^6 CD3+CD5,7+ cells] x [10^6/10^9]). FACS sorted CD3+CD5,7+ lymphoid cells from UPN 692, 701, 735, 767, 817, 836, and 1051 as well as normal healthy volunteers formed diffusely associated blast colonies containing TdT+ CD2+CD3+CD5+CD7+CD25+ mature “activated” T lymphocytes. Since a blast colony was defined as >20 tightly associated TdT+ T-cell precursor cells and no TdT' cells were detected on cytospin preparations of pooled colonies, it was assumed that none of the observed colonies in these cases was composed of TdT' T-lineage ALL blasts.

Controls included BM specimens from two healthy volunteers, one high-risk T-lineage ALL patient in partial relapse, two newly diagnosed T-lineage ALL patients, and one high-risk remission B-lineage ALL patient.

Abbreviation: ND, not determined.

TdT+CD2+CD3(surface)+CD5+CD7+CD25+. Hence, colonies in these seven cases most likely comprised IL-2 responsive normal mature T-cell populations. In the remaining five patients (UPN 643, 654, 665, 882, and 1081), whose fresh leukemic BM blasts at diagnosis expressed the CD3 antigen on their surface, the vast majority of colonies from day 7 cultures of FACS sorted CD3+CD5,7+ lymphoid cells were spherical, and were formed by 20 to 100 tightly associated round cells with a distinct cell border of high refractility. Isolated colony cells displayed blast morphology with a highly irregular nuclear membrane and prominent nucleoli, and, as detailed later in this section, they had the composite immunophenotype TdT+CD2+CD3+CD5+CD7+CD25+, consistent with T-lineage ALL (Fig 2). The number of CD5,7+ T-lineage LPC per 10^8 MNCs in the remission BM, which was calculated from the percentages of CD3-CD5,7+ and CD3+CD5,7+ lymphoid cells and the sum of the number of blast colonies in these fractions, showed a marked interpatient variation, ranging from less than 233 to 214,047 (median, 8,568). These findings indicated that the remission BMs from our high-risk remission T-lineage ALL patients contained less than 0.0002% to 0.2% (median, 0.0086%) residual T-lineage LPC.

In parallel experiments shown in Table 2, we analyzed “negative control BMs” from two healthy volunteer donors and one high risk remission B-lineage ALL patient. The percentages of CD3-CD5,7+ lymphoid cells in these control BMs ranged from 0.5% to 2%. FACS sorted CD3-CD5,7+ lymphoid cells yielded 0 to 26 20- to 30-cell colonies per 10^5 cells cultured, which were composed of small round TdT- lymphoid cells with a regular nuclear membrane. These colony cells were not further characterized because of the limited cell numbers. By comparison, the percentages of CD3+CD5,7+ lymphoid cells ranged from 2.7% to 8.8%. FACS sorted CD3+CD5,7+ lymphoid cells yielded 10 to greater than 100 100- to 200-cell colonies, which were composed of diffusely associated small mature lymphocytes. The composite immunophenotype of isolated colony cells was TdT-CD2+CD3+CD5+CD7+CD25-, consistent with a normal “activated” T-cell marker profile (data not shown). Also
Fig 1. Detection of residual CD3- LPC in remission BM samples of high risk T-lineage ALL patients. (A) Wright-Giemsa stained preparations of FACS sorted CD3- CD5.7+ T-cell precursors from BM samples of four high-risk remission T-lineage ALL patients (UPN 654, 665, 767, 1081). (B) Wright-Giemsa stained preparations of colony blasts from day 7 cultures of FACS sorted T-cell precursors. Note the blast morphology with a basophilic cytoplasm, irregular nuclear membrane, chromatin structure, nucleoli and vacuolation.
Fig 2. Coexistence of CD3- and CD3+ LPCs in remission BM samples of high-risk T-lineage patients. (A) CD3-CD5.7+ LPCs; (B) CD3+CD5.7+ LPCs from UPN 643 (1): day 7 blast colonies in PHA-LCM plus rIL-2 stimulated cultures. (2) Wright-Giemsa stained cells pooled from day 7 blast colonies. Note the immature blast morphology with a basophilic cytoplasm, irregular nuclei, prominent nucleoli and vacuolation. (3-5) The immunophenotypic features of colony blasts: (3) nuclear TdT; (4) surface CD5, and (5) surface CD7.
analyzed were "positive control BMs" from one T-lineage ALL patient in partial relapse containing 12% blasts and two newly diagnosed T-lineage ALL patients containing 96% to 98% blasts. The percentages of CD3+CD5,7+ lymphoid cells in these positive control BMs ranged from 18.6% to 86.2%. FACs sorted CD3+CD5,7+ lymphoid cells yielded 0 to 294 20- to 30-cell colonies per 10^5 cells cultured, which were composed of TdT+CD5+CD7+CD25+ blasts. By comparison, the percentages of CD3+CD5,7+ lymphoid cells ranged from 0.9% to 27.8%. FACs sorted CD3+CD5,7+ lymphoid cells yielded no blast colonies in one of these samples. In the remaining two cases, FACs sorted CD3+CD5,7+ lymphoid cells yielded 175 to 1,360 compact 20- to 50-cell colonies per 10^5 cells cultured, which were composed of TdT+CD5+CD7+CD25+ blasts.

Effects of ex vivo marrow purging on residual leukemic blasts in autografts. As detailed in the Materials and Methods, autografts were purged ex vivo with a combination of T101/CD5-Ricin and G3.7/CD7-Ricin immunotoxins plus 4-HC. The rationale of combining immunotoxins with 4-HC has been published in previous reports.13,14

To accurately determine the destruction of residual T-lineage LPC in the autografts, we used multiparameter flow cytometry, cell sorting, and LPC assays, as described in the Materials and Methods. When CD3+CD5,7+ and CD3+CD5,7+ T-lineage lymphoid cells from postpurge autograft samples were isolated by sterile multiparameter cell sorting and assayed for T-lineage ALL blast colony formation in the LPC assay system, no blast colonies were observed in the postpurge samples from 11 of 13 patients. In the remaining 2 patients (UPN 665 and 701), there were 7 (98% reduction in clonogenicity as compared with prepurge samples) and 124 (no reduction in clonogenicity as compared with prepurge samples) blast colonies per 10^5 cells plated (Table 3). The estimated numbers of T-lineage LPC in the postpurge autografts ranged from less than 14 LPC/10^8 MNC (ie, 0.000014% of MNC) in UPN 675 to 1,488 LPC/10^8 MNC (ie, 0.00148% of MNC) (Table 3). Hence, 0.8 logs to greater than 3.4 logs (82.6% to >99.96%) of residual LPC were eliminated from autologous remission autografts by the applied combined immunochemopurging protocol. Since all analyzed cases were in complete remission, a more accurate measurement of the log kill efficacy of the purging procedure was hampered by the limit of sensitivity, which is determined by the numbers of residual LPC in prepurge autograft samples.

BMT, engraftment, and posttransplant complications. As detailed in the Materials and Methods section, the first nine patients were prepared for BMT with single dose TBI plus high dose Ara-C and the subsequent five patients were prepared with hyperfractionated TBI plus high dose cyclophosphamide. The posttransplant course of the patients is detailed in Table 4. The median BM cell dose of the autografts was 0.54 × 10^8 MNC/kg (range, 0.23 to 0.78 × 10^8 MNC/kg). By multiplying the number of LPC per 10^8 MNC and the cell dose (MNC per kilogram) of the reinforced autografts, we estimated the number of reinforced T-lineage LPC per kilogram. These values ranged from less than 8 LPC/kg in UPN 675, 735, and 836 to 655 LPC/kg in UPN 701 (Table 4). Thirteen of 14 patients engrafted, as defined by a WBC greater than 1,000/µL for 3 consecutive days, at a median of 23 days (range, 18 to 62 days) post-BMT. The median time to reach an absolute neutrophil count (ANC) greater than 500/µL was 27 days (range, 16 to 63 days) (Table 4). One patient (UPN 735) had an ANC >500/µL at day 28 but died at 38 days in respiratory failure secondary to aspergillus pneumonitis. Two patients (UPN 692 with initial engraftment at 62 days and UPN 701 with initial engraftment at 30 days) had poor hematologic recovery with persistent leukopenia, anemia, and thrombocytopenia. UPN 692 received an untreated "back-up" autograft containing 1.0 × 10^8 nucleated cells/kg on day 98 post-BMT and UPN 701 received an untreated back-up autograft containing 0.2 × 10^8 nucleated cells/kg on day 105 post-BMT. Despite the reinfusion of untreated back-up BM, these

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Table 3. Detection of Residual LPC in High-Risk Remission T-Lineage ALL Autografts After Ex Vivo Purging

<table>
<thead>
<tr>
<th>UPN</th>
<th>Lymphoid Cells (% of MNC)</th>
<th>CD3+CD5,7+ Fraction</th>
<th>CD3+CD5,7+ Fraction</th>
<th>Mean No. TdT+CD5,7+ Blast Colonies/10^5 FACs Sorted Cells</th>
<th>CD5,7+ LPC/10^8 MNC</th>
<th>Log Kill</th>
</tr>
</thead>
<tbody>
<tr>
<td>643</td>
<td>46.2</td>
<td>8.6</td>
<td>0.2</td>
<td>0</td>
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<td>&lt;88</td>
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<tr>
<td>654</td>
<td>48.5</td>
<td>3.0</td>
<td>1.9</td>
<td>0</td>
<td>0</td>
<td>&lt;313</td>
</tr>
<tr>
<td>665</td>
<td>28.4</td>
<td>2.8</td>
<td>2.8</td>
<td>7</td>
<td>0</td>
<td>196</td>
</tr>
<tr>
<td>675</td>
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<td>0.8</td>
<td>0.6</td>
<td>0</td>
<td>0</td>
<td>&lt;14</td>
</tr>
<tr>
<td>692</td>
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<td>1.9</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td>&lt;19</td>
</tr>
<tr>
<td>701</td>
<td>28.6</td>
<td>1.2</td>
<td>0.7</td>
<td>258</td>
<td>0</td>
<td>1,488</td>
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<tr>
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<td>1.8</td>
<td>0</td>
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<tr>
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<tr>
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<tr>
<td>1051</td>
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<td>0.4</td>
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<td>0.0</td>
<td>0</td>
<td>0</td>
<td>&lt;259</td>
</tr>
</tbody>
</table>

Samples from purged autografts were examined for the presence of residual T-lineage LPC as described in the Materials and Methods. The number of CD5,7+ LPCs were determined as described in the legend of Table 2. Log kill values were obtained by comparing on a logarithmic scale the number of CD5,7+ LPC per 10^8 MNC in prepurge samples shown in Table 2 with the number of CD5,7+ LPC per 10^8 MNC in postpurge samples shown in this table. 
patients continued to have poor BM function and died of hemorrhage at 139 days and 131 days post-BMT, respectively. Nine patients relapsed at 37 to 510 days (1.2 to 17 months, median, 65 days/2.2 months) post-BMT and 6 have subsequently died of leukemia (Table 4). Two patients (UPN 817 and UPN 836) are alive and disease free at 28 and 26 months post-BMT.

Sequential analysis of the composite immunophenotypes of leukemic blasts obtained from T-lineage ALL patients at diagnosis, relapse, or during remission before BMT, and at relapse after BMT. As illustrated in Table 5, the composite immunophenotypes of TdT+ T-lineage ALL colony blasts in cultures of either CD3-CD5,7+ or CD3+CD5,7+ FACS sorted remission BM lymphoid cells showed striking similarities to those of fresh BM blasts at the time of diagnosis, relapse before BMT, and/or relapse after BMT. For example, in UPN 643, the composite immunophenotype of colony blasts in cultures of FACS sorted CD3+CD5,7+ lymphoid cells was CD1+CD2+CD3+CD5+CD7+CD19+CD25+, which is similar to that of fresh BM blasts obtained at relapse before or after BMT. In view of the pronounced immunophenotypic heterogeneity of T-lineage ALLs, the observed similarities between the composite immunophenotypes of colony blasts in LPC assays and the composite immunophenotypes of freshly obtained primary BM provided suggestive evidence that the T-lineage LPC assays may detect those LPC clones that are biologically relevant in vivo. These intriguing findings prompted us to investigate the correlation between the numbers of LPC in remission BMs and the probability of remaining in remission post-BMT.

Correlation between probability of remaining in remission post-BMT and patients' pre-BMT characteristics. We examined the potential influence of the residual leukemia burden at the time of BMT, as measured by our new MRD detection assay system and expressed as the number of LPC/10⁸ MNC in the remission BM samples before BMT, on the probability of remaining free of relapse and DFS, after BMT. To this end, we divided the patients into two groups composed of those with LPC numbers less than (group 1) versus those with LPC numbers equal or greater than (group 2) the calculated median of 8,568 LPC/10⁸ MNC. Figure 3 depicts the product-limit estimates for the probability of remaining in remission, and DFS, of these two groups. The Kaplan-Meier estimates and standard errors of the probability of remaining in remission after BMT were 14 ± 13% for group 2 patients and 53 ± 25% for group 1 patients, P = .006. An advantage in DFS was observed for group 1 (DFS: 0 ± 0% for group 2 vs 33 ± 19% for group 1, P = .008). These correlations suggest a predictive value of the new MRD detection assay for the outcome of high risk remission T-lineage ALL patients undergoing autologous BMT. However, they are preliminary and have to be interpreted with caution because of the limited sample size. In contrast to the numbers of LPC, the percentage of TdT+ cells, or percentage of lymphoblasts in remission BM immediately before BMT did not correlate with the probability of relapse after BMT.

Notably, eight of nine patients who relapsed post-BMT had no remaining LPC detected in their autografts after ex vivo purging (Table 3). In the remaining patient (UPN 665), 2.7 logs of contaminating LPC in the autograft were killed by the purging protocol and there were only 196 residual LPC/10⁸ MNC (0.0002%) in the purged autograft. Hence, no apparent association existed between the outcome and the log kill efficacy of purging, the numbers of LPC/10⁸ MNC in purged autografts, or the estimated numbers of LPC/kg infused to the patients.

**DISCUSSION**

The present report details the results obtained in 14 patients with high T-lineage ALL who underwent autologous BMT in complete remission for treatment of their residual disease. In agreement with previous reports, recurrence of leukemia was the leading cause of treatment failure and was experienced by 9 of 14 patients, indicating that the
residual leukemia in the majority of high-risk remission T-lineage ALL patients is resistant to radiochemotherapy.

Until more effective preparative regimens become available, laboratory methods that more accurately assess and monitor the quality of remission would allow patients to be transplanted at a time when their residual disease burden is minimal, and the probability of a given preparative regimen to eradicate residual disease is optimal. Therefore, considerable effort has been invested in developing highly sensitive MRD detection methods capable of detecting very small numbers of contaminating leukemic blasts in remission BM samples, which are not discernible by conventional morphologic examinations or immunophenotyping.23–30

In this study, we used a novel quantitative MRD detection assay system that combines fluorescence activated multiparameter flow cytometry and cell sorting with LPC assays to analyze remission BM samples from T-lineage ALL patients for the presence of residual clonogenic leukemic blasts. Notably, high numbers of residual LPCs detected in remission BM before BMT appeared to indicate a poor prognosis, providing evidence for the biologic significance and clinical value of in vitro T-lineage ALL LPC assays and suggesting that the in vitro clonogenic blasts detected by LPC assays likely represent the in vivo counterparts of the in vivo clonogenic T-lineage ALL blasts. Two possible explanations for the observed association between LPC numbers in remission marrow and outcome after autologous BMT are (1) LPC numbers may accurately reflect the total leukemia burden or its clonogenic pool, which determines outcome, or (2) the assay system favors the outgrowth of drug/radiation resistant leukemic clones, and the number of such clones will likely affect outcome. Whether the MRD data generated in this new assay system will add useful information and predictive strength to established prognostic parameters, such as WBC at diagnosis and remission number, will be the subject of a separate long-term study on a larger patient population.

Unlike cytogenetic or molecular biologic analyses of the remission BM samples for the presence of clonal populations of leukemic blasts,21–30 our assay system for minimal residual leukemia does not depend on the presence of clonal chromosomal abnormalities or the availability of clonospecific probes. The opportunity is thus provided to routinely use this more generally applicable assay system to predict the outcome of high risk remission T-lineage ALL patients. In addition, a quantitative analysis of remission BMs for the presence of clonogenic ALL blasts using multiparameter flow cytometry and LPC assays will probably yield biologically more relevant information about the quality of remission than a qualitative analysis for the presence of ALL blasts, using methods that do not discriminate between leukemic cells of different proliferative capacities, such as the amplification of DNA sequences flanking the crossover sites of certain clonotypic chromosomal translocations, or amplification of rearranged T-cell receptor gamma or delta chain sequences using the PCR technique.28–30

A potential limitation of autologous BMT is the likely presence of residual leukemia blasts in the remission autografts, which could contribute to leukemic relapse. Therefore, most centers have used autografts that were harvested...
in complete remission when the residual leukemia burden in the BM is believed to be minimal, and which were purged ex vivo using immunologic or pharmacologic methods in an attempt to eradicate occult leukemic cells. In the present study, autografts were purged ex vivo using the [T101/anti-CD5-Ricin + G3.7/anti-CD7-Ricin + 4-HC] combined immunochemotherapy protocol. This purging protocol was previously found to be highly effective against clonogenic blasts of several in vitro established T-lineage ALL cell lines, as well as against fresh LPCs from T-lineage ALL patients.\(^\text{13,14}\) Furthermore, the anti leukemia efficacy of this combined protocol was found to be superior to the anti leukemia efficacy of alternative purging protocols which used immunotoxins alone or 4-HC alone.\(^\text{13,14}\) Thirteen of 14 patients engrafted at a median of 23 days verifying the repopulation capacity of autografts treated by this new purging protocol. Consistent with our previous reports,\(^\text{13}\) the [T101-Ricin + G3.7-Ricin + 4-HC] combined immunochemotherapy protocol was very effective against residual T-lineage LPC in the remission autografts and killed 98% to 100% (maximum kill, >3.4 logs, >99.96%) of contaminating LPC in 10 of 11 evaluable patients. However, despite effective ex vivo purging of autografts, nine patients relapsed at a median of 2.2 months post-BMT. There was no apparent association between the outcome and the log kill efficacy of purging, the numbers of residual LPC in purged autografts, or the estimated numbers of LPC reinfused to the patients. These findings suggest that in our series of high-risk remission T-lineage ALL patients, the primary reason for the recurrence of leukemia after autologous BMT was not the reinfusion of leukemic blasts in autografts due to inefficient ex vivo purging, but rather the inability of the preparative regimens to eradicate the therapy refractory residual in vivo leukemia burden of the patients.

The predictive value of the described MRD assay system for the outcome of high-risk remission T-lineage ALL patients undergoing autologous BMT supports our hypothesis that the in vitro clonogenic T-lineage ALL blasts represent the in vitro counterparts of the in vivo clonogenic T-lineage ALL LPCs. Until more effective preparative regimens are developed to successfully eliminate the residual leukemia burden in high-risk remission ALL patients, recurrent disease will most likely continue to be the leading cause of failure and death after BMT. An accurate assessment of the quality of BM remission before BMT using this novel MRD detection assay might allow us to determine the optimal timing for BMT and the need for additional therapy to achieve a more substantial remission with less residual leukemia burden.

ACKNOWLEDGMENT

We thank Kevin Waddick, Virginia Kuebelbeck, and Mridula Chandan-Langlie for technical assistance, and JoAnn Mattson for preparation of the manuscript.

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Autologous bone marrow transplantation in high-risk remission T-lineage acute lymphoblastic leukemia using immunotoxins plus 4-hydroperoxycyclophosphamide for marrow purging

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