Correlation of Occult Clonogenic Leukemia Drug Sensitivity With Relapse After Autologous Bone Marrow Transplantation

By Carole B. Miller, Barbara A. Zehnbauer, Steven Plantadosi, Scott D. Rowley, and Richard J. Jones

Despite initial complete remission rates exceeding 70%, the majority of patients with acute myeloid leukemia (AML) and adults with acute lymphocytic leukemia (ALL) eventually relapse. Improving the therapeutic results in acute leukemia requires detecting, and understanding the biology of, the minimal residual leukemia remaining after therapy and responsible for relapse. To investigate the biologic relevance of an in vitro assay for clonogenic leukemia (leukemia colony-forming units [CFU-L]) as a measure of minimal residual leukemia, we studied 59 consecutive patients with acute leukemia in complete remission undergoing autologous bone marrow transplantation (BMT) with cyclophosphamide-based therapy. CFU-L were cultured from the pretransplant remission marrows in 45 of 58 patients: 35 of 43 patients with AML and 10 of 15 with ALL. Clonal rearrangements, identical to the patients' overt leukemia when available, were detected in the occult CFU-L from four of the eight patients with ALL in whom adequate DNA for analysis could be obtained from the CFU-L. None of the uncultured pretransplant remission marrows from the 15 ALL patients showed clonal gene rearrangements. We also determined the in vitro sensitivity of the occult CFU-L to 4-hydroperoxycyclophosphamide (4HC) and correlated these results with the outcome of the patients. The sensitivity of the occult CFU-L to 4HC was the only factor that predicted relapse following BMT. The actuarial probability of relapse was 18% in the 23 patients whose CFU-L were sensitive to 4HC compared with 77% in the 22 patients whose CFU-L were resistant (P < .001). The only factor that influenced the CFU-L sensitivity to 4HC was the type of leukemia. The CFU-L from the AML patients were more sensitive to 4HC than the CFU-L from the ALL patients (P = .001). Occult CFU-L genetically and functionally represent occult leukemia. Therefore, the CFU-L assay should provide a means for studying the biology of minimal residual leukemia and improving the therapeutic results in patients with acute leukemia.

From The Bone Marrow Transplantation Program, The Johns Hopkins Oncology Center, Baltimore, MD.


Address reprint requests to Richard J. Jones, MD, Room 2-127, The Johns Hopkins Oncology Center, 600 N Wolfe St, Baltimore, MD 21205.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1991 by The American Society of Hematology.

function of occult CFU-L in vitro corresponded to the biologic behavior of minimal residual disease, we evaluated whether the drug sensitivity of the CFU-L correlated with relapse after BMT. We studied the in vitro sensitivity of the occult CFU-L to 4-hydroperoxycyclophosphamide (4HC), a congener of cyclophosphamide that does not require in vivo phosphamide-based therapy.

MATERIALS AND METHODS

Patient selection and treatment. We studied 58 consecutive patients with acute leukemia (43 with AML, 15 with ALL) in CR (less than 5% blasts in the marrow), who underwent autologous BMT between January 1988 and January 1990 (Table 1). All patients gave informed consent for study participation as approved by the Johns Hopkins Joint Committee on Clinical Investigation. Patients with ALL in third remission or AML received busulfan (1 mg/kg every 6 hours orally for 4 days) followed by cyclophosphamide (50 mg/kg/d intravenously for 4 days) and irradiated with lung shielding for the third dose). Before beginning the preparative therapy, BM was harvested, treated ex vivo with 4HC (as a single agent in patients with AML and in combination with vincristine and methotrexate as part of a phase 1 dose-escalation study in ALL patients), and cryopreserved as previously described.11-17

BM cells and 4HC incubations. Samples of the harvested BM were removed before clinical ex vivo treatment (purging). Mononuclear BM cells (density, < 1.078 g/mL) were recovered by Ficoll-Hypaque density centrifugation and T-lymphocyte depleted for the AML and B-lineage ALL assays. For T-lymphocyte depletion, mononuclear BM cells were incubated with sheep erythrocytes at 4°C for 2 hours and then subjected to Ficoll-Hypaque density centrifugation. 4HC (provided by O. Michael Colvin, The Johns Hopkins Oncology Center) was diluted in a medium immediately before each experiment. Mononuclear BM cells were incubated at 2 × 10^7 cells/mL with graded concentrations of 4HC (0 to 60 μg/mL) for 30 minutes at 37°C. The 4HC incubations were arrested by the addition of chilled medium and the cells were washed twice.

Colony assays. After incubations with 4HC, the mononuclear BM cells were cultured for CFU-L and granulocyte-macrophage colony-forming units (CFU-GM) using conditions we have previously described.18 Briefly, washed marrow cells were cultured at 2 × 10^5 cells/mL in 1.32% methylcellulose containing 30% fetal bovine serum, 1% bovine serum albumin, 10% phytohemagglutinin-stimulated lymphocyte-conditioned media, 10^-7 mol/L 2-mercaptoethanol, and a medium. Additionally, 10^5 feeder cells/mL were added to the ALL cultures. Normal donor peripheral blood mononuclear cells (density, < 1.078 g/mL), obtained by Ficoll-Hypaque density centrifugation, depleted of adherent mononuclear cells by incubation in petri dishes at a concentration of 10^6 cells/mL for 2 hours at 37°C, and irradiated to 3,000 cGy with a dual 60Co source, served as feeder cells for the ALL cultures. The cell culture suspensions (1 mL) were plated into 35-mm petri dishes and incubated in a humidified atmosphere containing 7.5% CO2 at 37°C. Quadruplicate cultures were scored with an inverted microscope. CFU-L were scored on days 3 through 6 and CFU-GM on days 13 or 14. A colony was defined as an aggregate of 40 or more cells. CFU-L colonies can be distinguished from normal progenitor colonies in that the CFU-L appear early in culture (day 2 to 4) and reach maximal size by day 3 to 6.11 In addition, colony morphology allows differentiation from normal CFU-GM and T-cell colonies in that CFU-L are compact, homogeneous colonies, while normal colonies are sparse by day 6 and contain cells very heterogeneous in size and shape.11

Individual colonies were plucked and cytospins were prepared. Cytospins were stained with a modified Wright-Giemsa stain for morphologic examination. Immunoperoxidase staining of colonies was performed with monoclonal antibody (MoAb) MT2, which marks most mature T and B cells but few if any ALL or AML cells.18

Analysis of clonal gene rearrangements. To confirm that CFU-L arise from the patient's leukemic clone, individual ALL colonies (about 500 to 5,000 per patient) were plucked and pooled to provide enough cells (2 × 10^4 to 1 × 10^5) for Southern blot analysis. The uncultured mononuclear marrow cells (density, < 1.078 g/mL) from these patients were similarly analyzed. High molecular weight DNA was prepared and digested with restriction endonucleases EcoRI or BamHI with buffer conditions as recommended by the manufacturer (Bethesda Research Laboratories, Gaithersburg, MD). We used methods for agarose gel electrophoresis, alkaline transfer to nylon membranes,21 DNA hybridization,22 and random primer radiolabeling23 previously described. Molecular probes consisted of the constant region of the T-cell receptor β chain (TCRβ)24 or the J-region of the Ig heavy chain (IgH) gene.25
Yeast transfer RNA (BRL, Gaithersburg, MD) was used as a carrier to recover DNA from small numbers of cells.

Statistical evaluations. The dose-response of CFU-L and CFU-GM to 4HC was determined by plotting the logarithm of the surviving fraction of CFU-L or CFU-GM against 4HC concentration (Fig 1). Each data point represented the mean of the four plates. The sensitivity of CFU-L and CFU-GM to 4HC was defined as the estimated slope of the resulting linear regression line.

The major statistical endpoints of this study were event-free survival and time to relapse following BMT. Event-free survival was defined as the time from the day of BMT until relapse or death from any cause. Time to relapse was defined as the time from the day of BMT until relapse with deaths being censored. Event-time distributions were estimated using the method of Kaplan and Meier. For event-time distributions, point estimates are reported ±95% confidence intervals. The following prognostic factors were analyzed for their effects on relapse and death: age (as a continuous variable), sex, type of leukemia, preparative regimen, remission number (first remission versus second and third), duration of first remission for patients in second and third remissions (as a continuous variable), time from achieving remission until day of transplant (as a continuous variable), and sensitivity of CFU-L to 4HC (as a continuous and dichotomous variable). For categorical factors, the differences between event-time distributions were tested using the log-rank statistic. The prognostic significance of continuously distributed variables on the event-time distributions was assessed using the proportional hazards model with hazards ratios expressed per unit change. To adjust for the effect of several prognostic factors simultaneously, the multivariate proportional hazards model was used. Hazard ratios exceeding 1.0 indicate an increased risk of death or relapse in the presence of the prognostic factor. Similarly, hazard ratios less than 1.0 indicate a lower risk of death or relapse with the prognostic factor. To determine the influence of prognostic factors on dichotomous outcomes, univariate and multivariate logistic regression models were used to estimate the odds ratio. Differences in the sensitivity of CFU-L or CFU-GM to 4HC between categories (ie, AML v ALL) were tested by the Wilcoxon rank-sum test. All P values reported are two-sided. Analyses were performed as of August 1, 1990.

RESULTS

CFU-L were cultured from 45 of 58 patients: 35 of 43 patients with AML and 10 of 15 patients with ALL. The median number of CFU-L cultured per 2 × 10⁹ cells plated was 97 (range, 12 to 952) in the patients with ALL and 280 (range, 28 to 962) in the patients with AML. Plucked CFU-L colonies primarily contained immature cells with similar morphology to the patients’ original leukemia. The cells from the plucked CFU-L colonies were negative for MT2 antibody staining.

To confirm that occult CFU-L originate from the patient’s occult leukemia, CFU-L from patients with ALL were analyzed for the presence of clonal gene rearrangements. None of the uncultured pretransplant remission marrows from the 15 ALL patients showed clonal gene rearrangements. The plucked and pooled occult CFU-L colonies cultured from the remission marrows provided DNA sufficient for Southern blot hybridization (0.5 to 1.0 µg) in eight of the 10 patients with ALL from whom CFU-L were grown. In four of these patients, clonal gene rearrangements were observed in the cultured CFU-L. Identical rearrangements were found in the occult CFU-L and overt leukemia from the two patients in whom overt leukemia was available for comparison (Figs 2 and 3). No clonal gene rearrangement was detected in the CFU-L from four patients. In three of these four patients, diagnostic marrow specimens were not available for demonstration of a gene rearrangement. The TCRβ and IgH probes were both tested on the pooled, plucked CFU-L from these four patients; however, only BamHI was used for digestion.

In the 45 patients from whom occult CFU-L were grown, CFU-L sensitivity to 4HC was prospectively correlated with outcome following autologous BMT. The other prognostic factors analyzed for their effect on outcome in these patients are shown in Table 2. Using the sensitivity of CFU-L to 4HC as a continuous variable, it was the only predictor of time to relapse in univariate and multivariate analyses (hazard ratio < 10⁻⁶, P = .01, multivariate proportional hazards model). To categorize patients for survival analyses, a CFU-L sensitivity to 4HC of 0.1 was found to maximally separate relapers and nonrelapers. CFU-L from 23 patients (22 with AML and one with ALL) had a sensitivity to 4HC of ≥ 0.1 and were classified as sensitive (Fig 1A); CFU-L from 22 patients (13 with AML and nine with ALL) had a sensitivity to 4HC less than 0.1 and were classified as resistant (Fig 1B). Importantly, although the median CFU-L sensitivity to 4HC was 0.12 (range, 0.1 to
The actuarial probability of relapse and event-free survival at 28 months (median follow-up 16 months, minimum follow-up 6 months) in all 58 patients was 51% (95% confidence interval, 37% to 70%) and 38% (24% to 52%), respectively (Fig 4). The actuarial probability of relapse (Fig 5B) was 18% (4% to 58%) in the 23 patients whose occult CFU-L were sensitive to 4HC compared with 77% (56% to 93%) in the 22 patients whose occult CFU-L were resistant (hazard ratio = 12.7, \( P < .001 \)), multivariate proportional hazards. Patients whose CFU-L were sensitive to 4HC also had a significantly better probability of event-free survival than patients whose CFU-L were resistant (Table 2, Fig 5A). In addition to CFU-L sensitivity to 4HC, age was also found to influence the event-free survival (Table 2). Multivariate analysis showed that the CFU-L sensitivity to 4HC (hazard ratio = 5.0, \( P = .002 \)) and age (hazard ratio = 1.025, \( P = .046 \)) were independent prognostic factors for event-free survival. Furthermore, the CFU-L sensitivity to 4HC remained the only factor that predicted relapse and event-free survival after autologous BMT in the AML and ALL patients when analyzed separately. The actuarial probability of relapse was 18% (4% to 58%) in the 22 patients with AML whose CFU-L were sensitive to 4HC compared with 86% (57% to 99%) in the 13 patients with AML whose CFU-L were resistant to 4HC (hazard ratio = 15.8, \( P < .001 \)), multivariate proportional hazards. Likewise, the actuarial probability of event-free survival was 66% (38% to 84%) compared with 11% (1% to 35%) for the AML patients whose CFU-L were either sensitive or resistant to 4HC, respectively (hazard ratio = 4.6, \( P = .006 \), multivariate proportional hazards). Although the numbers of patients with ALL are relatively small, the only ALL patient whose CFU-L were sensitive to 4HC survives event-free at 15 months after BMT, while the actuarial
CLONOGENIC ASSAY AND OCCULT LEUKEMIA

Fig 4. Actuarial probability of event-free survival (A) and relapse (B) after autologous BMT in the entire 58 patients with acute leukemia. The proportion event-free at 28 months is 38% (24% to 62%) and the probability of relapse is 51% (37% to 70%).

The relapse rate was 67% (38% to 92%) and event-free survival was 17% (1% to 43%) in the patients with ALL whose CFU-L were resistant to 4HC. The actuarial probabilities of relapse (61.1% v 49.3%, P = .48) and event-free survival (35.9% v 40.3%, P = .78) were not different between the 13 patients from whom CFU-L were not cultured and the 45 patients from whom CFU-L were grown, respectively.

The only factor that influenced the CFU-L sensitivity to 4HC was the type of leukemia (Table 3). The occult CFU-L from the AML patients were more sensitive to 4HC than the occult CFU-L from the ALL patients (median sensitivity of 0.1 v 0.05, P = .001, rank-sum test), while the median CFU-GM sensitivity was similar in the patients with AML and ALL (0.038 v 0.033). The CFU-L sensitivity to 4HC was independent of the remission number and previous exposure to cyclophosphamide. The type of leukemia remained a significant prognostic factor for the CFU-L sensitivity to 4HC in multivariate analysis (odds ratio = 13, P = .02).

DISCUSSION

As recently described by others,10,11 we found that CFU-L colonies cultured from remission marrows demonstrated clonal gene rearrangements identical to the patients' overt leukemia at diagnosis. In addition, the CFU-L assay is a

Table 3. Influence of Patient Characteristics on CFU-L Sensitivity to 4HC

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of Patients</th>
<th>Odds Ratio</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>—</td>
<td>0.99</td>
<td>.77</td>
</tr>
<tr>
<td>Duration of first remission</td>
<td>—</td>
<td>1.01</td>
<td>.48</td>
</tr>
<tr>
<td>Time from remission to transplant</td>
<td>—</td>
<td>0.98</td>
<td>.94</td>
</tr>
<tr>
<td>Previous cyclophosphamide exposure</td>
<td></td>
<td>0.90</td>
<td>.87</td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
<td>13.5</td>
<td>.02</td>
</tr>
<tr>
<td>No</td>
<td>36</td>
<td>0.57</td>
<td>.30</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>25</td>
<td>1.08</td>
<td>.88</td>
</tr>
<tr>
<td>ALL</td>
<td>10</td>
<td>1.08</td>
<td>.88</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>1.08</td>
<td>.88</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>1.08</td>
<td>.88</td>
</tr>
</tbody>
</table>

Odds ratios (from univariate logistic regression) show the relative risk for having CFU-L resistant to 4HC (slope <0.1). For categorical variables, the odds ratio is for the second variable listed relative to the first.
more sensitive method for detecting minimal residual disease than standard techniques for detecting gene rearrangements, because the clonal gene rearrangements could not be detected in the uncultured BM from which the CFU-L were grown. However, improving the therapeutic results in acute leukemia requires not only improving the detection of minimal residual leukemia but also understanding its biology. The drug sensitivity of overt CFU-L cultured from patients with acute leukemia at initial diagnosis has been shown to predict remission induction, but generally has not correlated with remission duration. This finding is not surprising because the cells ultimately responsible for relapse probably represent only a very small fraction of, and their biology may be quite distinct from, the overt leukemia at diagnosis. We found that the sensitivity of the occult CFU-L to 4HC in vitro did predict relapse following autologous BMT with cyclophosphamide-based therapy. Hence, occult CFU-L not only arise from, but also functionally represent, the minimal residual leukemia responsible for relapse. The ability to culture CFU-L from remission marrows should provide enriched populations of cells for studying the biology (drug sensitivity, mechanisms of drug resistance, growth characteristics and requirements) of the minimal residual leukemia that was "resistant" to primary therapy.

The detection of clonal gene rearrangements in the occult CFU-L colonies was limited by the small numbers of cells for DNA analysis, obtained by pooling plucked colonies. Sufficient DNA was extracted for analysis in only eight of 15 patients with ALL, and the yield of DNA allowed digestion with more than one restriction enzyme in only one patient. Digestion with multiple enzymes is preferred because a clonal rearrangement may not always be identified with only one enzyme due to comigration of genomic and rearranged alleles. In addition, the detection of gene rearrangements in the ALL colonies was hindered because most patients referred to our institution for BMT are in CR and overt leukemia samples, necessary for identifying the clonal gene rearrangement, are usually not available. Identification of clonal gene rearrangements, as evidence of minimal residual ALL, is optimized by prior elucidation of the rearrangement that is characteristic of the disease at diagnosis. We have no genetic proof that the CFU-L from AML patients represent leukemia, because defined gene rearrangements are rare in AML and we have been unable to reproducibly perform cytogenetics on CFU-L. However, we believe that the CFU-L colony morphology and growth characteristics, combined with the differing normal CFU-GM and occult CFU-L drug sensitivity and the correlation of occult CFU-L drug sensitivity with relapse, are convincing evidence that we are indeed cloning occult AML.

We were unable to culture occult CFU-L from 13 of 58 patients in CR before BMT. The clinical outcome in these patients was similar to those patients from whom occult CFU-L were cultured. Therefore, it is unlikely that the inability to culture occult CFU-L from patients was the result of a smaller leukemia burden, below the assay's limit of detection. More likely, the inability to culture occult CFU-L from some patients represented leukemias that will not clone in vitro, because the fraction of patients without CFU-L growth in our study is similar to the fraction of occult leukemias (about 20%) that will not grow in CFU-L assays. Our results also suggest that CFU-L growth in vitro is not the biologically poor prognostic factor that others have reported.

There may be trends for some factors (ie, remission number or type of leukemia) to predict relapse in univariate analyses (Table 2) that may not reach statistical significance because of the relatively small sample sizes. No factor that we analyzed, other than the occult CFU-L sensitivity to 4HC, was an independent prognostic factor for relapse after autologous BMT in multivariate analysis. It may not be surprising that remission number and type of leukemia are not independent risk factors for relapse, because these factors are probably strongly correlated with the drug sensitivity of the occult CFU-L. In fact, the type of leukemia influenced the sensitivity of CFU-L to 4HC. Occult CFU-L from patients with ALL were significantly more resistant to 4HC than occult CFU-L from patients with AML, with the CFU-L from all but one of the ALL patients being classified as resistant. This relative resistance of occult ALL in vitro is consistent with the relative clinical resistance of ALL to BMT as indicated by the substantially higher relapse rates that our center and others have seen after BMT for ALL when compared with AML. The reasons for the relative resistance to 4HC of the occult CFU-L from patients with ALL are unclear. It does not appear to be the result of greater prior cyclophosphamide exposure than the AML patients, and the normal progenitors from the ALL patients are not more resistant to 4HC. In addition, the ALL patients do not appear to be more heavily pretreated than the AML patients, as measured by their number of remissions before BMT.

No firm conclusions regarding the necessity for ex vivo purging in the treatment of acute leukemia with autologous BMT can be drawn from our results. Although occult CFU-L can be cultured from the majority of marrow grafts before clinical purging, it is possible that cryopreservation of the grafts or immune mechanisms can eradicate the residual leukemia present in the harvested marrow. Furthermore, because cyclophosphamide-based therapy is used for both the systemic treatment and the purging in our BMT studies, the correlation between occult CFU-L sensitivity to 4HC and relapse may be a reflection of either the in vivo or the ex vivo treatment of the leukemia, or both.

REFERENCES


15. Rowley SD, Davis JM, Plantadosi S, Jones RJ, Yeager AM, Santos GW: Density-gradient separation of autologous bone marrow grafts after ex vivo purging with 4-hydroperoxycyclophosphamide. Bone Marrow Transplant 6:321, 1990


Correlation of occult clonogenic leukemia drug sensitivity with relapse after autologous bone marrow transplantation

CB Miller, BA Zehnbauer, S Piantadosi, SD Rowley and RJ Jones