Detection of Residual Leukemia by the Polymerase Chain Reaction and Sequence-Specific Oligonucleotide Probe Hybridization After Allogeneic Bone Marrow Transplantation for AKR Leukemia: A Murine Model for Minimal Residual Disease

By William R. Drobyski, Lee Ann Baxter-Lowe, and Robert L. Truitt

Disease relapse after allogeneic bone marrow transplantation (BMT) is a major cause of treatment failure and is thought to evolve from clinically occult residual disease in the recipient. However, the demonstration of minimal residual disease (MRD) in individual patients is of uncertain prognostic significance because the detection of residual disease has not consistently correlated with subsequent relapse. Moreover, the optimal therapeutic approach in patients with MRD after allogeneic BMT is unknown. The study of these issues has been hindered by the lack of clinically relevant animal models. In this report, we characterize a novel murine model for the study of MRD after allogeneic BMT. This model was designed to simulate high-risk BMT in humans in which patients receive transplants in relapse and disease recurrence is the major cause of treatment failure. The H-2-compatible, mixed lymphocyte culture nonreactive murine strains, AKR (H-2k) and CBA (H-2d), were chosen to parallel marrow transplants from HLA-matched siblings, which represent the majority of allogeneic bone marrow transplantations in humans. Male AKR leukemia cells were used in female donor/host chimeras permitting the Y chromosome to serve as a leukemia-specific marker for MRD. Detection of residual male leukemia cells in the peripheral blood of the primary host was facilitated by use of the polymerase chain reaction (PCR) and sequence-specific oligonucleotide probe hybridization (SSOPH). Use of PCR/SSOPH was highly predictive of clinical outcome (relapse or cure) in animals receiving transplants (P < .00002) and detected disease recurrence earlier than comparative flow cytometric analysis studies. This murine model will be useful in evaluating the efficacy of therapeutic strategies aimed at reducing disease relapse posttransplant and can be adapted to other transplant murine tumor systems for the study of MRD.

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The clinical significance of MRD after allogeneic BMT in humans, however, has not yet been well defined. While in some patients documentation of residual disease has been predictive of clinical recurrence, in others the detection of clinically occult malignant cells has not correlated with subsequent relapse. This may be due to the fact that these cells are not clonogenic and, hence, incapable of expansion, or that donor-derived immune effector cells play a role in preventing the progression of disease or facilitate the induction of tumor dormancy. Additionally, the lack of consistent correlation between the presence of MRD and eventual disease relapse has hindered the implementation of therapeutic strategies aimed at augmenting the antileukemia effect of the allogeneic graft in an effort to prevent recurrence.

Animal models have been used to study MRD after allogeneic BMT and support the concept that clinical relapse can evolve from residual malignant cells in transplant recipients. However, these models have been limited by the...
fact that demonstration of MRD in individual animals has generally required use of a bioassay system that necessitates killing the primary host. Consequently, the clinical fate of individual animals cannot be determined. The clinical relevance of these models is therefore questionable because individualized therapeutic interventions cannot be made in animals in whom MRD is detected.

In this study, we report the characterization of a murine AKR leukemia model for the study of MRD after allogeneic BMT using a tumor system analogous to T-cell acute lymphoblastic leukemia in humans. Major histocompatibility complex (MHC)-compatible, mixed lymphocyte culture (MLC) nonreactive murine strains were chosen to simulate HLA-matched MLC-nonreactive transplants in humans. Detection of MRD was accomplished with the polymerase chain reaction (PCR) and sequence-specific oligonucleotide probe hybridization (SSOPH) using a Y-chromosome-specific probe to detect residual male leukemia cells in female donor/host chimeras. The efficacy of this strategy in permitting early detection of relapse and reliably predicting for clinical outcome in individual animals posttransplant was assessed.

MATERIALS AND METHODS

Mice. AKR/J (H-2^d, Thy 1.1^+), AKR/Cu (H-2^b, Thy 1.2^+), and CBA/J (H-2^b, Thy 1.2^+) mice (6 to 20 weeks old) were used in these experiments. AKR/J and CBA/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). AKR/Cu mice were bred in our animal facility. All mice were housed in the American Association for Laboratory Animal Care (AALAC)-accredited Animal Resource Center of the Medical College of Wisconsin. Mice received regular mouse chow and acidified tap water ad libitum.

Leukemia. T-cell acute lymphoblastic leukemia/lymphoma, which develops spontaneously in AKR/J mice at approximately 8 months of age, was used as a source of tumor. For these experiments, leukemia cells from a retired male AKR/J breeder with advanced spontaneous leukemia/lymphoma were isolated. Peripheral blood from this leukemia-bearing animal was initially injected into female AKR/J mice. A stock of early passage leukemia cells was then prepared by passing peripheral blood from these animals into healthy female AKR/J mice, collecting the spleens during advanced stages, and cryopreserving the pooled spleen cell suspensions. This leukemia designated AKR-M1 was found in preliminary studies to be tumorigenic in both AKR/J (N = 4) and AKR/Cu (N = 3) mice (median survival after injection of 5 x 10^6 cells intravenously [IV] of 40 and 41 days, respectively). The AKR-M1 leukemia cells used in these studies are Thy 1.1^+ and Lyt 2^-; the lethal dose of AKR-M1 leukemia that caused death in 50% of nontransplanted animals (LD_{50}) after IV injection was 10^3 cells (data not shown).

**BMT protocol.** Female AKR/Cu or AKR/J recipient mice received variable doses of AKR-MI leukemia cells by tail vein injection. One week later, host animals received 1.200 cGy total body irradiation (TBI) within 24 hours of BMT as lethal immunosuppression. TBI was administered in a single dose using a Shephard Mark I Cesium irradiator (J.L. Shephard and Associates, San Fernando, CA). The dose rate was 89 cGy/min. All irradiated control mice (N = 12) (without marrow transplantation) died at a median of 12 days after irradiation (range, 7 to 14 days) (data not shown).

BM was flushed from female donor femurs with complete Dulbecco's modified Eagle's medium (CDMEM; GIBCO, Grand Island, NY) plus 5% fetal bovine serum. The marrow plugs were passed through a sterile mesh filter to obtain single suspensions. Marrow cells were then washed, resuspended in fresh medium, and counted. BM cells were always greater than 90% viable by trypan blue dye exclusion. Irradiated recipient mice received a single intravenous injection (0.4 mL) containing 10 x 10^6 female CBA cells. CBA BM usually contained less than 3% mature T cells (CD4^+ and CD8^+ cells) based on flow cytometry studies (data not shown). Thus, murine BM was considered equivalent to human BM that is partially depleted of T cells. This experimental design was used so that all animals would be leukemic at the time of transplant, and therefore would simulate the clinical situation in which allogeneic BMT is performed in patients with relapsed disease.

**Flow cytometric analysis (fluorescence-activated cell sorter [FACS]).** Two-color immunofluorescence was used in some experiments to determine the presence of AKR-M1 leukemia cells in animals receiving transplants. Fluorescein isothiocyanate (FITC)-conjugated anti-Thy 1.2 and phycoerythrin (PE)-conjugated anti-L3T4 (CD4) monoclonal antibodies (MoAbs) were obtained from Becton Dickinson (Mountain View, CA). Donor-derived CD4^+ T cells were defined as L3T4^- Thy 1.2^-, whereas AKR-M1 leukemia cells were defined as L3T4^+ Thy 1.2^+. Blood samples for FACS analysis were obtained from chimeras at selected time points postransplant (vide infra). Erythrocytes were lysed using a warm (37°C) Tris-buffered ammonium chloride solution. Cells were then stained with MoAbs as recommended by the manufacturer for two-color analysis. The cells were analyzed on a FACS Analyzer (Becton Dickinson) equipped with a FACSLite laser and Consort 30 computer support.

**DNA isolation and PCR amplification of murine DNA.** Peripheral blood and spleen cells were obtained from recipient mice at selected time points in the posttransplant period. DNA was isolated using a rapid DNA extraction procedure. Whole blood samples were centrifuged with lysis buffer (0.32 mol/L sucrose, 10 mmol/L Tris, 5 mmol/L MgCl_2, and 1% Triton X-100) to remove red blood cells. After the supernatant was discarded, PCR buffer (50 mmol/L KCl, 10 mmol/L Tris, pH 8.3, 2.5 mmol/L MgCl_2, 0.1 mg/mL gelatin, 0.45% nonide P40, 0.45% Tween 20) and proteinase K (2 mg/mL) were added to the cell suspension. The mixture was incubated at 60°C for 1 hour and then at 95°C for 10 minutes to inactivate the proteinase K. An aliquot of 10 or 20 μL was used for the primer-directed enzymatic amplification of DNA by PCR.

The published DNA sequence of the ZFY-2 region of the murine Y chromosome was used to determine primer sequences that would amplify a Y-chromosome-restricted segment. Twenty- and 21-bp oligonucleotides were constructed as primers with the following sequences: 5' TGGAATAGGAGCTGATGCAG 3' (sense strand); 5' GCCTGAACATGTCTTGATGAG 3' (antisense strand). The primers were designed to produce an approximate 350-bp product after PCR amplification.

**Primer-directed enzymatic amplification was performed using PCR. Briefly, 100 μL reaction mixtures containing 10 or 20 μL genomic DNA; 200 μmol/L each deoxynucleotide 5'-triphosphate; 1 μmol/L of each primer; 50 mmol/L Tris-HCl, pH 8.3; 1.5 mmol/L MgCl_2, and 0.01% (wt/vol) gelatin. Samples were boiled for 3 minutes, transferred to a 94°C heat block, and 1 μL of Taq DNA polymerase (Perkin Elmer, Norwalk, CT) was added. Reactions consisted of 35 cycles of denaturation at 98°C, annealing for 30 seconds at 58°C, and polymerization for 20 seconds at 72°C in a programmable heat block (M.J. Research Inc, Watertown, MA). Each sample was characterized by agarose gel electrophoresis and amplified DNA was detected by ethidium bromide staining. Male (AKR-M1) and female (CBA) DNA control samples were included in each PCR experiment.

**Hybridization of PCR-amplified DNA with a sequence-specific oligonucleotide probe.** A 13-base internal oligonucleotide probe derived from the ZFY-2 locus was synthesized with the sequence 5' CTGGTCACTATCC 3'. This probe was labeled by the incorporation
of digoxigenin-deoxyuridine triphosphate (Boehringer Mannheim, Indianapolis, IN) using terminal deoxynucleotidyl transferase. Aliquots of amplified DNA were denatured by incubation in 0.4 mol/L NaOH and 0.6 mol/L NaCl for 5 minutes, neutralized by the addition of 1 mol/L Tris-HCl, and applied to Magnagraph membranes using a dot blot apparatus. Membranes were hybridized in 20% sodium dodecyl sulfate (SDS), 20× SSC, 50× Denhardt’s solution, double-distilled H₂O, herring sperm single-stranded DNA, 1 mol/L NaPO₄, 0.5 mol/L EDTA, pH 8.0, and 1.5 µL of digoxigenin-labeled probe in a hybridization oven at 30°C. Membranes were rinsed in 6× SSC and washed in TMAC solution (3 mol/L tetramethylammonium chloride [TMAC], 50 mmol/L Tris, pH 8.0, 2 mmol/L EDTA, 1% SDS) twice for 20 minutes at 32°C. The membranes were incubated with an antidigoxigenin alkaline phosphatase conjugate and the presence of DNA bound-labeled probe was detected in a colorimetric reaction.

Strategy for the detection of MRD after allogeneic BMT. The strategy to detect MRD posttransplant is dependent on the presence of a marker specific for the leukemia cell population (ie, not found on nonleukemic donor or recipient cells). Due to the absence of a known chromosomal abnormality that might serve as a marker for the AKR leukemia, a model was devised in which the Y chromosome would serve as a “leukemia-specific” marker. Specifically, AKR-M1 leukemia cells derived from a male AKR mouse were used in experiments with female AKR hosts and female CBA BM donors. Detection of male DNA by PCR/SSOPH, therefore, was evidence of residual leukemia in animals receiving transplants. Animals were designated as being free from leukemia when there was no evidence of leukemia by oligonucleotide probe hybridization studies.

In some experiments, flow cytometric analysis (FACS) was also used to detect residual disease. Thy 1.1 markers were used to discriminate leukemic from nonleukemic cell populations; specifically, nonleukemic donor CBA cells were Thy 1.2⁺, whereas AKR-M1 cells were Thy 1.1⁺. Spleens from nontransplanted female AKR/Cu mice were analyzed to determine the “background” percentage of Thy 1.2⁺ L3T4⁺ cells. The average AKR/Cu spleen (n = 4) consisted of 5.4% Thy 1.2⁺ L3T4⁺ cells (95% confidence interval [CI], 2.7 to 8.1). Animals were therefore classified as leukemic by FACS analysis if the percentage of Thy 1.2⁺ L3T4⁺ cells exceeded the upper limit of the 95% CI, ie, greater than 8%.

Statistics. Survival results were analyzed using Kaplan-Meier product limit life table statistics and compared using the log rank test. The concordance of leukemia detection in the peripheral blood versus the spleen by PCR/SSOPH was analyzed with the Kappa Statistic.

RESULTS

Sensitivity and specificity of PCR/SSOPH in the detection of male AKR/J leukemia. A portion of the mouse Y chromosome is composed of two loci that have been termed ZFY-1 and ZFY-2 and that are homologues of the human ZFY gene. The ZFY-2 locus has been shown to be absent from female mice in Southern blot hybridization studies. In initial experiments, PCR studies were performed to confirm that the selected ZFY-2 primer sequences would amplify only male DNA. DNA was obtained from the spleens of male AKR and female CBA mice. PCR amplification of male and female DNA was followed by SSOPH with an internal DNA probe. The results (Fig 1) demonstrate that the predicted 350-bp sequence was found only in PCR-amplified male DNA. Hybridization studies showed that labeled probe bound only to male AKR DNA, confirming the male specificity of the ZFY-2 oligonucleotide probe.

The sensitivity of PCR/SSOPH for detecting minor populations of male DNA was determined by mixing experiments using normal female CBA and male AKR spleen cells. Samples of 10 × 10⁶ cells containing serial 10-fold dilutions of male AKR spleen cells were used for these experiments. DNA was extracted from the mixtures of male AKR and female CBA cells, PCR-amplified, and hybridized as described in Materials and Methods. A negative control contained DNA extracted from female CBA spleen cells only. The results of these studies (Fig 2) indicate that male AKR DNA was detectable at a dilution of 1 in 10⁴. When these experiments were repeated using a total of 1 × 10⁵ cells, the sensitivity of the assay decreased to 1 in 10³ (data not shown).

Exclusion of disease by PCR/SSOPH requires that technical failure of an individual PCR reaction not be interpreted as the absence of leukemia. To obviate this potential problem, we noted that, at the annealing temperature used, which was close to the melting point of primers, PCR amplification of female DNA only resulted in the appearance of irrelevant bands. However, amplified DNA from these reactions did not hybridize to the internal male-specific probe. The presence
of irrelevant bands was also seen in all other reactions (including the mixing experiments), except when there was an extremely strong male PCR signal. In contrast, control samples (no DNA lane 1, Fig 1) or failed PCR reactions (data not shown) yielded no visible bands. Thus, the presence of irrelevant bands confirmed that amplification had occurred and that the extracted DNA did not contain PCR inhibitors. This served as a control for these studies so that an absent hybridization signal could reliably be interpreted as the absence of detectable disease.

Early evidence of disease relapse can be detected in the peripheral blood. The use of sensitive techniques to detect clinically occult residual disease after BMT is dependent on the ability to sample tissues that provide a representative profile of disease status in the recipient. Specifically, if peripheral blood samples are tested for evidence of disease relapse, then it is critical that peripheral blood involvement by leukemia is an early and not a late manifestation of recurrent disease. We therefore investigated whether detection of leukemia in peripheral blood after transplant correlated with early relapse. Prior studies in AKR/J leukemia-bearing mice treated with chemotherapy have shown that the spleen is a major site of residual leukemia in animals that relapse. Based on these data, we reasoned that the presence of leukemia in the spleen would constitute early evidence of disease relapse in recipient mice.

A preliminary study was performed in a nontransplant murine model to determine that leukemia could be detected in animals by PCR/SSOPH. Unirradiated female AKR mice were injected IV with $5 \times 10^4$ AKR-M1 leukemia cells and groups of mice were then killed at weekly intervals. Peripheral blood and spleen samples were obtained and comparatively analyzed for the presence or absence of leukemia by PCR/SSOPH. The data presented in Fig 3A show that AKR-M1 cells were detectable in female CBA mice and that leukemia relapse was evident in both spleen and peripheral blood. Peripheral blood involvement by leukemia was shown to be an early manifestation of relapse as evidenced by concordance between blood and spleen samples in all but one case (15 of 16 samples).

This study was repeated using the transplant model defined in Materials and Methods to determine whether irradiation and BMT would affect the temporal appearance of leukemia relapse in the peripheral blood. Female AKR mice were injected IV with $1 \times 10^5$ male AKR-M1 leukemia cells and one week later were irradiated and received a transplant of female CBA BM. In both experiments, four to eight mice were killed weekly and tested for the presence of leukemia. As shown in Fig 3B, the results were concordant in 14 paired samples from spleen and blood, whereas in two instances leukemia was detected only in the peripheral blood.

A composite analysis of both studies showed that the presence or absence of leukemia in peripheral blood and spleen was concordant in 29 of 32 samples ($k = 0.81$). In only one instance was leukemia present in the spleen without being detected in the blood. Results were consistent at both early and late time points after transplant. These data demonstrate that serial sampling of peripheral blood in this tumor system can be used to detect early evidence of recurrent leukemia.

Effect of pretransplant leukemia cell inoculum on disease relapse. In the previous study, the majority of mice undergoing BMT 1 week after the injection of $1 \times 10^6$ male AKR/J leukemia cells did not develop detectable leukemia by 3 to 4 weeks posttransplant (5 of 8 mice PCR negative, Fig 3B). Because these animals were periodically killed for concurrent analysis of blood and spleen cells, they could not be observed for clinical outcome. However, these data suggested that the majority of mice would not have relapsed after allogeneic BMT at this leukemia cell dose. To substantiate this PCR data, a leukemia bioassay was performed. Spleens from all week 4 animals (3 of 4 PCR negative) were processed into single cell suspensions and 20 to $40 \times 10^6$ cells from each animal were injected into individual young AKR hosts. These
A MURINE MODEL FOR MRD AFTER ALLOGENEIC BMT

Table 1. Proportion of Mice Positive for Leukemia by PCR/SSOPH After Receiving Graded Pretransplant Leukemia Cell Inoculums

<table>
<thead>
<tr>
<th>Leukemia Cell Dose</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Blood</td>
<td>Spleen</td>
<td>Blood</td>
</tr>
<tr>
<td>3 x 10^6</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>1/3</td>
</tr>
<tr>
<td>6 x 10^6</td>
<td>0/3</td>
<td>1/3</td>
<td>1/3</td>
<td>3/3</td>
</tr>
<tr>
<td>9 x 10^6</td>
<td>0/3</td>
<td>3/3</td>
<td>2/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

Six female AKR mice in each group received graded doses of male AKR leukemia cells 1 week before transplant. Female AKR hosts were then conditioned with 1,200 cGy TBI and received 10 x 10^6 MHC-compatible female CBA BM cells. Three animals each were then tested for leukemia by PCR/SSOPH at weeks 1, 2, and 4 posttransplant. The three mice in each group tested at week 1 were killed at week 2 for concurrent analysis of both spleen and blood samples. The remaining three mice in each group were tested at week 4 for the development of leukemia.

“Secondary” animals were followed for survival. Three recipients whose spleen donors had been PCR negative survived greater than 120 days; the fourth animal, whose spleen donor was PCR positive, died of leukemia (data not shown). Based on the bioassay and PCR data, we reasoned that this leukemia cell inoculum was insufficient to cause relapse in the majority of animals and could be eradicated by the BMT conditioning regimen alone. It is important to bear in mind that the aim of the study was to develop a model to simulate high-risk BMT in which relapse is the major cause of treatment failure and evolves from MRD in the recipient posttransplant. This necessitated that most animals develop recurrent leukemia posttransplant; although, to assess the predictive value of PCR/SSOPH for clinical outcome, a minority of animals had to remain free of leukemia to determine whether PCR negativity predicted for cure.

A study was therefore performed to determine the optimal pretransplant leukemia cell inoculum. Cohorts of six female AKR animals were each injected with either 3 x 10^6, 6 x 10^6, or 9 x 10^6 AKR-M1 leukemia cells and received transplants as previously described with female CBA BM only. Three animals each were then monitored weekly for the presence of leukemia as determined by PCR/SSOPH. The data in Table 1 shows that at 1 week posttransplant, none of the recipients in any of the dose groups had detectable leukemia. However, between 2 and 4 weeks posttransplant, all animals receiving 6 to 9 x 10^6 tumor cells developed leukemia as detected by PCR/SSOPH, whereas only a minority of animals receiving 3 x 10^6 cells were positive after 4 weeks. These data indicate that, by administering a higher pretransplant tumor burden, the rate of relapse could be increased and that the optimal pretransplant leukemia cell inoculum that would lead to relapse in the majority, but not all, animals was between 3 x 10^6 and 6 x 10^6 cells. A dose of 4 x 10^6 cells, therefore, was chosen for subsequent experiments.

PCR/SSOPH is superior to FACS for detection of MRD and predicts for clinical outcome. Mixing experiments (see above) demonstrated that PCR/SSOPH was able to detect AKR-M1 leukemia cells at a sensitivity of 1 in 10^3 to 10^4 cells. These data suggested that PCR/SSOPH would be more effective than conventional FACS analysis at detecting leukemia in vivo. To determine if PCR/SSOPH allowed for earlier diagnosis of relapse than FACS analysis, the two techniques were compared in mice receiving allogeneic BMT. Female AKR/J mice were injected with 4 x 10^6 AKR-M1 leukemia cells. One week later, host animals were conditioned with 1,200 cGy TBI and received a transplant of 10 x 10^6 BM cells from female CBA mice. Cohorts of nine mice each were screened for the presence of leukemia in the peripheral blood on alternating weeks beginning 2 weeks after BMT. Equal aliquots of blood, approximately 100 µL, were used for detection of leukemia by FACS and PCR/SSOPH. During weeks 2 through 5 posttransplant, this volume of blood typically contained between 10^6 to 10^7 nucleated cells (data not shown), which is within the cell range that was tested in mixing experiments (see above). Animals testing positive by either FACS or PCR were not tested further because, in preliminary studies, repeated retroorbital venipuncture was associated with high mortality. Mice initially testing negative were screened a second time 2 weeks later. The results shown in Table 2 demonstrate that male leukemia could not be detected in any animal at 2 weeks after BMT. However, by week 3, five of nine animals were positive by PCR/SSOPH, whereas none of these mice had evidence of leukemia by FACS. Leukemia was not detectable in the peripheral blood by FACS until 4 weeks after transplant. By week 5, the results of both PCR and FACS analysis were concordant in the four animals tested. These data showed that PCR/SSOPH was able to detect leukemia at least 1 week earlier than flow cytometric studies. Additionally, at week 4, when leukemia was detectable by both PCR and FACS, PCR was still the more sensitive.

Table 2. Comparative Analysis of the Sensitivity of FACS Versus PCR/SSOPH for the Detection of MRD After Allogeneic BMT

<table>
<thead>
<tr>
<th>Mice</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Leukemia, d 38</td>
</tr>
<tr>
<td>I-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Survived &gt;60 d</td>
</tr>
<tr>
<td>I-3</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Leukemia, d 27</td>
</tr>
<tr>
<td>I-4</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Leukemia, d 50</td>
</tr>
<tr>
<td>I-5</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Leukemia, d 47</td>
</tr>
<tr>
<td>I-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Leukemia, d 46</td>
</tr>
<tr>
<td>II-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Leukemia, d 45</td>
</tr>
<tr>
<td>II-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Leukemia, d 36</td>
</tr>
<tr>
<td>II-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Leukemia, d 34</td>
</tr>
<tr>
<td>III-1</td>
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<td>-</td>
<td>+</td>
<td>Leukemia, d 34</td>
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<tr>
<td>III-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Survived &gt;60 d</td>
</tr>
<tr>
<td>III-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Leukemia, d 34</td>
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<td>+</td>
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<td>III-5</td>
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<td>+</td>
<td>Leukemia, d 40</td>
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<tr>
<td>IV-2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Leukemia, d 34</td>
</tr>
</tbody>
</table>
| IV-3 | -      | -      | -      | -      | Died, d 53 (PCR-)
| IV-4 | -      | -      | -      | +      | Leukemia, d 36 |

Female AKR mice were injected with 4 x 10^6 male AKR-M1 leukemia cells and 1 week later were irradiated and received a transplant of female CBA BM. Cohorts of mice were then screened on alternate weeks, beginning with week 2, for the presence of leukemia by either FACS or PCR/SSOPH. All animals were observed for clinical outcome. (+) and (−) denote animals testing positively or negatively for leukemia, respectively.
testing positive (m, n = 31) or negative (j, n = 4) for the presence of leukemia in the peripheral blood by PCR/SSOPH in the posttransplant period. Data include mice presented in Table 2 plus those from an additional replicate experiment (Total n = 35).

The median time from PCR positivity to leukemic death in 28 evaluable animals that relapsed was 15 days (range, 3 to 23 days). Three animals that expired during retroorbital venipuncture were not evaluable. This corresponded approximately to a 2-week window between the detection of leukemia and death. In each experiment, untreated female AKR hosts received 4 × 10^6 AKR-M1 leukemia cells to confirm tumorigenicity (N = 7). All developed leukemia and died at a median of 30 days after injection (range, 28 to 44 days).

DISCUSSION

The aim of this study was to develop a clinically relevant animal model for the evaluation of MRD after allogeneic BMT. The model was designed to simulate high-risk BMT in humans in which patients receive transplants in relapse and disease recurrence is the major cause of treatment failure. The H-2-compatible MLC nonreactive murine strains AKR (H-2^k) and CBA (H-2^q) were chosen to simulate HLA-matched sibling transplants, which represent the majority of allotransplants in humans. AKR recipients received CBA BM only, which does not contain enough T cells to promote graft-versus-host disease (GVHD) and is analogous to the situation in humans in which recipients receive BM grafts depleted of mature T cells. Consequently, there is no GVH-associated antileukemia effect in this BMT model and animals that were made leukemic before conditioning had a high rate of relapse (31 of 35) in the posttransplant period. Because the AKR leukemia possesses no marker specific for the malignant cell population to distinguish it from normal cells, a male AKR leukemia cell line was used. This permitted the Y chromosome to serve as a leukemia-specific marker in female donor/host transplants. Residual leukemia was detected in recipient animals by PCR/SSOPH, which was both sensitive and specific for the detection of male AKR-M1 leukemia cells. The superiority of PCR/SSOPH versus FACS analysis was demonstrated by the earlier detection of MRD in the primary host after marrow transplantation. PCR/SSOPH was also highly predictive of clinical outcome in all animals (P < .00002) and detected relapse approximately 2 weeks before death.

Disease relapse in humans is generally diagnosed when there is clinical evidence of the reemergent malignant cell population. However, the detection of MRD before clinical relapse is possible using sensitive molecular biologic techniques, such as the PCR, that are able to exploit specific chromosomal abnormalities or gene rearrangements in hematologic neoplasms as markers of disease recurrence. PCR has been used for the detection of MRD in a variety of hematologic malignancies, including chronic myelogenous leukemia (CML),^13,14^ Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL),^25^ B- and T-cell ALL^8,26^ and follicular lymphomas bearing the (14, 18) translocation.^27^ The most extensively studied of these diseases has been CML, in which the chimeric BCR-ABL RNA transcript has been used as a marker for disease recurrence. In several studies, patients in clinical remission after allogeneic BMT for CML have been followed-up posttransplant using PCR as a prognostic tool to predict relapse.^13,14,28^ The results of these studies
have been conflicting and, to date, use of PCR has not been able to identify accurately patients destined to relapse. Similarly, in the other disease states, detection of residual malignant cells by PCR has not been able to predict for clinical recurrence in all patients.

Determining which patients with MRD will ultimately relapse is also important from a therapeutic perspective. Studies in humans have begun to explore the use of immunotherapeutic approaches to augment the GVL effect posttransplant to eradicate clinically occult disease in BMT patients. Hauch et al. have demonstrated that in patients who have undergone allogeneic BMT for CML, the ability of their peripheral blood mononuclear cells after activation by interleukin-2 (IL-2) to lyse host-derived CML targets directly correlated with subsequent clinical relapse. These findings suggest that donor-derived immune effector cells are responsible in part, for mediating an antileukemia effect and this effect can be enhanced by the administration of cytokines such as IL-2 or α-interferon posttransplant in an effort to increase GVL reactivity. However, clinical concerns exist that these strategies may cause significant side-effects, including the inhibition of hematopoiesis or the exacerbation of GVHD, especially in recipients of T-cell-replete marrow grafts. The use of IL-2 in allogeneic BMT, for example, has been thus far limited to patients receiving T-cell-depleted BM grafts. The present inability to predict relapse on an individual basis has also meant that some patients who are destined to be cured may unnecessarily receive treatment. The optimal therapeutic approach in these patients remains undefined.

Animal models have been used in an attempt to simulate MRD after allogeneic BMT in humans and to explore potential therapeutic approaches whereby antileukemia reactivity might be enhanced to eradicate residual malignant cells. Slavin et al. using the BCL1 leukemia model, demonstrated that mice, conditioned with total lymphoid irradiation and cyclophosphamide and then receiving a transplant of histoincompatible BM, could be "cured" of disease. However, in approximately half of these "cured" mice that did not have evidence of clinical disease, leukemia could be detected after transfer of spleen cells to secondary hosts. These studies showed that residual disease in the primary host could persist in the posttransplant period in a dormant state and appeared to be regulated by an ongoing donor-derived immune effector cells. This response could be further enhanced by the administration of cytokines such as IL-2 in the posttransplant period. Truitt and Atasoylu have demonstrated in an AKR leukemia model that the kinetics of elimination of MRD differ depending on the intensity of the pretransplant conditioning regimen. AKR recipients receiving equivalent doses of B10.BR BM and spleen cells eliminated leukemia earlier posttransplant (18 days vs 30 days) when conditioned with 1,200 cGy as opposed to 900 cGy of TBI. In the majority of those animals destined to relapse, residual leukemia could not be detected within the first 3 weeks by flow cytometric studies and required the use of a bioassay. Collectively, these studies demonstrated that, as in humans, clinical relapse can evolve from an MRD state and immunotherapeutic approaches may be effective in eradicating residual disease.

While animal models such as these have provided information pertaining to antileukemia reactivity after allogeneic BMT, they have certain limitations. First of all, these models use bioassays to document disease status posttransplant. This requires that the primary host be killed and, thus, it cannot be observed for clinical outcome. Also, the cells that are adoptively transferred into secondary hosts to confirm the presence or absence of disease may not be representative of the disease status of the individual animal. In some instances, this is due to the pooling of spleen cells from multiple primary hosts before injection into secondary indicator animals so that determining the status of the original animals is problematic. Additionally, bioassays typically sample only one organ in the primary host, which may not correlate with leukemia burden in the entire animal. The growth of residual disease in the primary host may also be affected by donor-derived immune effector cells that regulate leukemic cell growth and may be responsible for preventing clinical relapse. Weiss et al. demonstrated that the adoptive transfer of spleen cells from individual animals into multiple secondary hosts resulted in leukemia in only a fraction of adoptive recipients. This was attributed to the concurrent transfer of donor immunocompetent cells, which prevented leukemic growth in some secondary recipients. However, the fact that not all secondary recipients developed leukemia made it difficult to determine the true status of the primary host or the potential contribution of donor immune cells.

A second limitation is that leukemia relapse is typically simulated by the administration of leukemia cells at variable time points in the posttransplant period. This is done to determine the animals' ability to resist a transferred leukemia cell challenge that is assumed to be indicative of GVL reactivity. This approach is distinguishable from the clinical situation in humans in which disease relapse evolves from residual malignant cells that survive the conditioning regimen and are never completely eradicated. In these patients, a GVL effect is due to the dynamic interplay between residual tumor cells and donor-derived immune effector cells with antitumor reactivity that are present immediately after BMT. The antileukemia effect that conveys resistance to a transferred leukemia cell challenge post-BMT, therefore, may not be synonymous with a GVL effect that evolves in the setting of MRD.

In the present study, these limitations were obviated by the development of a model in which MRD was detected posttransplant in the peripheral blood of animals that were leukemic before allogeneic BMT. The use of PCR/SSOP allowed each recipient animal to be observed for clinical outcome without the need for bioassays. Sampling of the blood in the primary host for evidence of recurrent disease was based on the premise that peripheral blood involvement was an early manifestation of relapse. Prior studies by Valeriote et al. using an AKR leukemia model demonstrated that the spleen and liver are the major sites of residual disease in chemotherapy-treated mice who subsequently relapse. We therefore considered the presence of leukemia in the spleen to represent early relapse after BMT. A comparative analysis
showed that leukemia was detected in the peripheral blood either before or contemporaneous with its appearance in the spleen in 16 of 17 cases, supporting the use of peripheral blood sampling as a screening approach for the detection of early relapse.

The AKR leukemia is a tumor system that has been previously shown to be useful in the study of GVL/GVH reactivity and MRD after allogeneic BMT.15,24 The observations that GVL reactivity is not exclusively dependent on the clinical expression of GVHD15 and that donor-derived effector cell populations with relative leukemia, as opposed to host specificity, exist and play a role in mediating antileukemia reactivity16,17 were both made using this system. These findings have since been confirmed in humans18,25 and indicate that animal models can provide a conceptual framework with which to address these clinically relevant questions. This AKR murine leukemia model may therefore be useful in the investigation of MRD and of new therapeutic strategies aimed at augmenting the GVL effect after allogeneic BMT and provide a preclinical foundation for the application of these approaches in humans. The strategy used here to detect residual leukemia cells is also one which can be adapted to other transplant and nontransplant murine tumor systems for the study of MRD and tumor dormancy as well as to determine the efficacy of specific treatment modalities in eradicating malignant cells.

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