Analysis of β-Globin Mutations Shows Stable Mixed Chimerism in Patients With Thalassemia After Bone Marrow Transplantation

By J. Kapelushnik, R. Or, D. Filon, A. Nagler, G. Cividalli, M. Aker, E. Naparstek, S. Slavin, and A. Oppenheim

β-thalassemia major (TM) is caused by any of approximately 150 mutations within the β-globin gene. To establish the degree of chimerism after bone marrow transplantation (BMT), we have performed molecular analysis of β-globin mutations in 14 patients with TM over a period of 10 years. All patients underwent T cell-depleted allogeneic BMT from HLA-identical related donors, using either in vitro T-cell depletion with CAMPATH 1M and complement or in vivo depletion using CAMPATH 1G in the bone marrow collection bag. To date, at different time periods after BMT, seven patients have some degree of chimerism; six of these patients, all blood transfusion-independent, have donor cells in the range of 70% to 95%, with stable mixed chimerism (MC).

HIGH-DOSE CHEMOTHERAPY followed by allogeneic bone marrow transplantation (BMT) is the treatment of choice for a wide range of nonmalignant hematologic diseases, including β-thalassemia major (TM). The pretransplant chemotherapy has a dual purpose: to kill hematopoietic host cells so as to gain bone marrow space and, at the same time, to induce adequate immunosuppression to prevent rejection of donor stem cells.

Despite the significant and continuing advances in conditioning regimens, patients may ultimately develop either persistent host lymphohematopoietic cells or relapse with the basic disease. A number of studies have shown that patients in good clinical condition post-BMT and presenting with normal hematologic parameters may still harbor minimal amounts of residual host hematopoietic cells in their bone marrow and peripheral blood, as determined by highly sensitive techniques. The methods used thus far to detect chimerism after BMT for nonmalignant disorders include detection of sex chromosomes in cases of sex disparity between donor and recipient, analysis of hypervariable regions of the human genome by polymerase chain reaction (PCR) amplification, and ABO typing.

The present work focuses on the detection of point mutations in the β-globin gene of patients with TM undergoing BMT. This innovative technique allows us to establish the degree of chimerism by measuring the level of residual host β-globin genes, using the point mutation as a marker, and permits correlation between the degree of chimerism and the clinical outcome after BMT.

MATERIALS AND METHODS

Patients. The study comprises 14 informative patients with TM (9 males and five females; age range, 1 to 7 years; median, 4 years at presentation) treated during the last decade at the Department of Bone Marrow Transplantation of the Hadassah University Hospital, Jerusalem, Israel. All patients received allogeneic BMT from HLA-identical, related donors. Six pairs were sex-mismatched.

Pretransplant conditioning. All patients received oral busulfan (4 mg/kg/d × 4 days) and intravenous (IV) cyclophosphamide (50 mg/kg/d × 4 days). Thiotaepa IV (5 mg/kg × 1 day) was added for four patients. Total lymphoid irradiation (TLI), 1,000 cGy given in five daily fractions, was performed in 10 patients, and four patients received IV rat anti-human CDW52 (IgG 2b) antibody (CAMPATH 1G) (0.2 mg/kg/d × 4 days) for in vivo depletion of host lymphocytes before chemotherapy.

Graft-versus-host disease (GVHD) prophylaxis. All patients received T cell-depleted bone marrow achieved by treatment either in vitro (nine patients) with CAMPATH 1M, with donor serum as source of complement CAMPATH, or in vivo with CAMPATH 1G directly in the bone marrow collection bag (five patients). In the latter case, depletion was most likely achieved by antibody-dependent cell-mediated cytotoxicity. CAMPATH 1M and CAMPATH 1G were provided by Drs G. Hale and H. Waldmann (Department of Pathology, Dunn School of Pathology, Oxford, UK). The use of CAMPATH 1M in vitro and CAMPATH 1G in the marrow collecting bag has been described previously. Rejection prevention. Cyclosporin A (3 mg/kg/d) was administered intravenously to all patients from day -1 until engraftment (polymorphonuclear cells, >750/mm³).

DNA preparation and PCR. DNA was prepared from peripheral blood according to standard procedures. PCR was performed with Taq polymerase (Appligene, Strasbourg, France) using the conditions recommended by the manufacturer. The following primers, spanning the first and second exons of the β-globin gene from 166 nucleotides (nt) upstream of the cap site to 138 nt in the IVS2 site, were used: P5 (5’ primer), CCAACCTCTAAGCGACGTGCC; and P12 (3’ primer), CTGACTTCCACACTGATGC. The amplification cycle consisted of 1 minute at 92°C, 1.5 minutes at 62°C, and 1.5 minutes at 72°C, giving an amplification product of 799 bp.

Testing for mutant alleles. Before BMT, the β-thalassemia mutation(s) of the patient was identified; in parallel, the β-globin genotype of the donor was determined. Some of the donors were normal, while others were thalassemia carriers. After the transplantation, DNA samples obtained from peripheral blood were tested for chime-

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rism as follows: equal aliquots of the PCR products (5 to 15 μL) were denatured in 0.4 mol/L NaOH/25 mmol/L EDTA for 20 minutes on ice and spotted onto two duplicate nylon membranes together with appropriate controls, namely, donor DNA control and controls for each β-globin mutation (homozygotes, heterozygotes, and normal). Hybridization was performed with excess allele-specific oligonucleotide probes (1 to 2 ng) labeled with [32P] γ-adenosine triphosphate (ATP). For each patient, one membrane was hybridized with the mutant probe and the second, with the respective normal probe. Quantification of the autoradiograms was performed as follows. Until 1993, radioactive counts were determined by cutting the spots from the membrane and measuring in a scintillation counter, since 1993, the radioactivity of the spots is measured directly using a phosphorimage analyzer.

RESULTS

The first objective was to establish the sensitivity and reliability of the method. For dependable quantification of the ratio of normal to mutant alleles, measurements must be performed within the linear range of detection. The PCR is expected to start at an exponential rate and to remain so until one of the reaction components becomes limiting. We, therefore, examined first the progress of the PCR under our conditions. Because the reaction was exponential up to 25 cycles (Fig 1), 23 cycles were used for all subsequent tests.

Linearity of the signal obtained by hybridization depends on the ratio between DNA and probe. The PCR products were spotted onto duplicate membranes at different DNA concentrations; membranes were then hybridized to 1 ng/mL (Fig 2B, closed circles) and 3 ng/mL probes (Fig 2B, open circles). At both probe concentrations, the reactions were linear over a wide range.

Measurement of radioactivity by phosphorimage analysis showed quantification of the results to be highly reproducible. Replicate analyses of a sample, at the same or different DNA concentrations, demonstrated an experimental error of ±1%. Repeated analyses of the same sample at intervals of months were likewise reproducible. We, therefore, consider a 2% hybridization signal as significant. Hence, the method can detect 2% donor (or 2% residual host) cells when both donor and recipient are homozygous, or establish 4% donor cells when the donor is heterozygous (or the recipient is compound heterozygous).

After BMT, peripheral blood samples were analyzed sequentially for detection of the β-globin gene point mutations for all 14 patients. Table 1 lists the clinical parameters of the 14 patients and the outcome of their BMTs. Five patients (43%) were found to have full engraftment (100% donor cells) based on analysis of the β-globin alleles. These five patients are in excellent clinical condition, exhibiting normal growth and development; their hemoglobin levels are comparable with those of the respective donors. In seven patients (50%), mixed chimerism (MC) was detected by the β-globin
gene point mutation method (and not by any of the other engraftment markers); six of these patients have 73% to 96% donor cells with stable MC, are independent of blood transfusions, and are in excellent clinical condition. The seventh patient has 4% to 7% donor cells with only a minimal transfusion requirement; ie, she presents clinically with thalassemia intermedia. The remaining 2 patients (7%) have no detectable donor genes (ie, 100% host cells) and suffer from TM. None of the 14 patients showed signs of acute or chronic GVHD. To date, the period of follow up of the entire group of patients ranges between 6 months and 11 years (median, 4 years).

In the seven patients with MC, only one was sex-mismatched: a male was transplanted from a female donor (UPN 338, Table 1). For this recipient, PCR for the detection of Y chromosome was positive. The other five sex-mismatched recipients did not have MC.

In the seven patients with MC, the degree of chimerism, as expressed by the host-to-donor DNA ratio, remains relatively stable post-BMT (follow up of 3 to 68 months; median, 18 months). Despite the presence of MC, the six patients with greater than 70% donor cells have red blood cell counts similar to those of their donors; the stable MC in this group of patients, therefore, indicates sustained engraftment and certainly not recurrence of the basic disease.

Thus far, no correlation was found to exist between the pretransplant conditioning regimen or mode of T-cell depletion (TCD) and the incidence of chimerism.

**DISCUSSION**

In this study, we present the detection of the β-globin gene point mutation as an innovative method to confirm engraftment, detect minimal residual disease, and establish degree of chimerism in patients with thalassemia after BMT. Whereas other methods depend on neutral, nonrelated markers for the evaluation of chimerism, the strategy described here is based on the direct measurement of the disease-causing gene, albeit in irrelevant cell lineages.

BMT for thalassemia is usually performed with stem cells from closely related, HLA-matched donors, prohibiting the use of HLA as a marker. The ABO antigens as engraftment markers are of value only in cases of ABO mismatch and, furthermore, have no significance with respect to minimal residual disease. DNA-based gender determination is limited to situations of donor-host sex disparity. The specificity of the β-globin gene point mutation test, on the other hand, makes it applicable for every patient with TM receiving BMT.

The method, based on allele-specific oligonucleotide hybridization, is highly sensitive and capable of detecting an allele even at a level as low as 1%. Thus, in the case of a normal donor, even 1% to 2% residual host cells are detected.
If the donor is a carrier, the detection of residual host cells is less sensitive, because the donor DNA also carries the mutant allele. In such situations, the level of the mutant allele in the absence of residual host cells is already 50%; therefore, the level of detection begins only at 53% to 55% mutant alleles, corresponding to 5% to 10% host cells. Where other engraftment marker techniques have failed, the new method enabled us to demonstrate an incidence of 50% (7 of 14 patients) MC in the recipients of T cell-depleted allografts. Furthermore, the technique demonstrated that MC in the seven patients was stable.

In malignant hematologic diseases using a variety of engraftment marker methods, an MC incidence of 10% to 20% has been reported after unmodified BMT.\(^1\)\(^2\) It should be emphasized that malignant cells may persist among the donor cells in a tumor dormancy-like state. This may be misleading when interpreting data, because MC in these situations is extremely unstable.

In the nonmalignant disorder TM, MC has been evaluated recently in 72 patients by Manna et al\(^3\) using the analysis of the variable number of tandem repeats (VNTR). In that study, the incidence of MC decreased with time (ie, unstable chimerism), reaching 8% at 14 months post-BMT. However, as also noted by these investigators, the VNTR markers do not provide quantitative information on residual host cells. Some investigators have reported that either high-dose irradiation or additional chemotherapy may reduce the incidence of MC\(^4\) in a variety of diseases, whereas others found the development of MC to be independent of the conditioning regimen used.\(^5\)\(^6\) In the above-cited study by Manna et al,\(^3\) the conditioning regimen was shown to influence the frequency of MC post-BMT.

The present study indicates that the high incidence of MC may be related to TCD before BMT, despite increasing the conditioning regimen by either TLI or monoclonal anti-human lymphocyte antibody.\(^7\)\(^8\)\(^9\)\(^10\) Indeed, TCD has been reported to increase relapse and rejection rates in malignant diseases because of a lack of donor anti-host lymphocyte-mediated reaction.\(^11\)\(^12\) The fact that MC has remained stable to date (longest follow up, 68 months) suggests that MC in patients with thalassemia need not lead to rejection of the graft or recurrence of the disease. It should be emphasized that in our patients with stable MC, hematopoiesis is normal, even in those patients with only 70% donor cells. In this context, it should also be noted that the patient with approximately 5% residual donor cells displays clinical symptoms of thalassemia intermedia at more than 4 years post-BMT. Judged by other engraftment assays, such a patient might be considered to harbor 100% host cells.

A similar observation, ie, that stable MC permits functional donor hematopoiesis, has recently been described in a murine model, in which correction of \(\beta\)-thalassemia was achieved in mice transplanted with normal congenic bone marrow.\(^13\)\(^14\)\(^15\) The mice were conditioned by sublethal total

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### Table 1. Chimerism and Outcome of BMT

<table>
<thead>
<tr>
<th>UPN</th>
<th>Age at BMT (yrs)</th>
<th>Conditioning Regimen/TCD</th>
<th>(\beta)-Globin Genotype</th>
<th>Months Post-BMT</th>
<th>% Donor</th>
<th>Clinical Outcome</th>
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<tbody>
<tr>
<td>50</td>
<td>2</td>
<td>BuCy, TLI/1M</td>
<td>FS44/FS5</td>
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<td>100</td>
<td>Alive and well</td>
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<td>IVS1, 1/IVS1, 1</td>
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<td>90</td>
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</tr>
<tr>
<td>170</td>
<td>2</td>
<td>BuCy, TLI/1M</td>
<td>IVS2, 1/IVS2, 1</td>
<td>20</td>
<td>100</td>
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<tr>
<td>184</td>
<td>1</td>
<td>BuCy, TLI/1M</td>
<td>IVS1, 110/IVS1, 110</td>
<td>24</td>
<td>5</td>
<td>Thalassemia intermedia</td>
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<td>223</td>
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<td>IVS1, 110/IVS1, 110</td>
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<td></td>
<td></td>
<td>3</td>
<td>97</td>
<td>Alive and well</td>
</tr>
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Abbreviations: BuCy, busulfan cyclophosphamide; TLI, total lymphoid irradiation; BuTCy, busulfan thiotepa cyclophosphamide; 1M, CAMPATH 1M; 1G, CAMPATH 1G.
body irradiation, and, notwithstanding the resulting mixed red blood cell chimerism, correction of anemia was recorded. Quantitation of donor-type early hematopoietic progenitor cells (CFU-S) demonstrated that the correction of the anemia arose from a minority of normal immature bone marrow cells. The investigators concluded that successful BMT for \( \beta \)-thalassemia does not necessarily require total ablation of endogenous host cells. Because the \( \beta \)-globin gene point mutation marker is completely independent of HLA, sex, blood type, and VNTR, it constitutes a sensitive and accurate tool for determination of chimerism in patients with TM after BMT, as evidenced by the high proportion of patients with MC detected in the present study.

In conclusion, our study suggests that the \( \beta \)-globin gene point mutation assay is ideal for early detection of engraftment, rejection, or recurrence of TM after BMT. The incidence of MC in this report was higher than might have been expected from the studies published to date, which leads us to surmise that posttransplant MC with respect to nonmalignant diseases is underestimated. This may be attributed to the fact that the methods currently in use to detect residual host cells and MC are of limited sensitivity. One should also consider that TCD may play a critical role in the occurrence of MC. In light of this possibility, the mode of GVHD prophylaxis as practiced today should be reconsidered. Future studies will be required to address the issue of cell-mediated immunotherapy to eliminate residual host cells in patients with TM after BMT. Finally, a crucial point of this report concerns the stable MC exhibited by our patients, as detected by the \( \beta \)-globin mutation assay. These findings imply that total eradication of the host hematopoietic system to ensure sustained engraftment in patients with TM is not an absolute necessity.

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