Persistence of Mixed Chimerism in Patients Transplanted for the Treatment of Thalassemia

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Molecular genetic techniques permit sensitive assessment of host hematopoiesis after marrow transplantation for thalassemia. Information on this persistence and the cell lines in which it occurs may permit therapeutic intervention in patients at high risk for rejection and/or relapse. The objective of this study, therefore, was to determine the evolution and cell line distribution of persistent mixed chimerism detected in 55 patients treated for β thalassemia. Our findings indicated that rejection occurred in 20 patients, the host component disappeared in 20, and mixed chimerism without transfusion need persisted for 1 to 7 years in 15. In three patients with stable mixed chimerism for 4, 5, and 7 years, host hematopoiesis fluctuated between 25% and 75%. Despite this, donor pattern β-globin chain synthesis maintained hemoglobin levels between 10 and 13.5 g/dL without transfusion. In these three patients, the polymerase chain reaction of the VNTR and the fluorescent in situ hybridization analysis revealed the coexistence of donor and host cells in the different peripheral blood cell subpopulations and precursors studied (CD2, CD4, CD8, and CD19+ granulocytes; glycophorin-A+, erythroid burst-forming units, CD33+, granulocyte-macrophage colony-forming units). We found that rejection and disease recurrence occur in approximately one third of patients with early mixed chimerism. High levels of host type hematoopoiesis can be present in patients not requiring transfusion.

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THALASSEMIA MAJOR is a genetic disease characterized by defective synthesis of hemoglobin A. Thalassemia can be cured only by allogeneic marrow transplantation from an HLA-identical relative.1 The most common cause of treatment failure is graft rejection, which usually accompanies by a regrowth of the patient’s defective marrow and a return of thalassemia. Rarely rejection is not followed by a return of host type hematopoiesis and the patient dies with marrow aplasia. However, rejection is very uncommon later than 6 months after transplantation. Attempts at reducing the rejection rate have usually involved more intensive pretransplant conditioning regimens, which have been accompanied by an increase in regimen-related toxicity and mortality.

Persistence of residual host hematopoietic cells has been observed after marrow transplantation and is commonly referred to as mixed chimerism (MC).2 There is evidence that, in some settings, MC in the marrow is associated with an increased risk of subsequent graft failure and/or disease recurrence.3-5 With the development of extremely sensitive techniques for detecting cells on the basis of their genetic origin, interpretation of this association has become more complicated, in part because of difficulties in identifying which cell lines are chimeric.

From December 1981 through September 1994, more than 700 patients with homozygous β-thalassemia received marrow transplants from HLA-identical related donors in Pesaro. We have reported our clinical experience with marrow transplantation for thalassemia and the prolonged persistence of mixed hematopoietic chimerism in some of the patients, together with speculations on the influence of pretransplant conditioning on MC.6

In this report we address the evolutionary shifts in the proportions of host and donor populations in patients with MC after transplantation and the cellular compartments in which they occur. We hope that this will improve our understanding of the mechanisms involved in the development and maintenance of mixed chimerism and facilitate the detection of events that signal an increased probability of rejection and permit posttransplant therapeutic interventions.

MATERIALS AND METHODS

Patients

From February 1990 through March 1995 the cells of 214 patients receiving transplants from HLA-identical related donors were studied to detect the presence of transient or persistent mixed chimerism during the first 2 months after transplant. Until November 1991, all patients who were transplanted after receiving regimens containing less than 200 mg/kg of cyclophosphamide were included in this study. After this date, all patients were included irrespective of regimen. Seventy-two of these patients have been the subject of this report.7 Sixty-seven patients had MC, and 55 of these had a follow-up of more than 1 year and form the subject of this report.

Disease Class

A system has been described for assigning patients undergoing marrow transplantation for thalassemia to prognostically useful categories.8 Risk factors evaluated were hepatomegaly (>2 cm below the intercostal margin), the presence of portal fibrosis in the pretransplant liver biopsy, and the quality of chelation during the years before transplant. The quality of chelation was characterized as regular when deferoxamine therapy was initiated within 18 months of the first transfusion and administered subcutaneously for 8 to 10 hours continuously for at least 5 days each week. The chelation variable was defined as irregular for any deviation from this requirement.

Class 1 patients had no adverse risk factors, class 3 patients had all three, and class 2 patients had one or two adverse risk factors.
Rejection

The return to the pretransplant pattern of $\beta$-globin chain synthesis and transfusion requirement was determined to indicate rejection.

Cell Samples

Peripheral blood and bone marrow samples were collected in EDTA from the patients and donors before transplant and from all patients at least once between day 15 and day 60, and on days 180 and 365 after transplant. Patients who rejected the transplant were not further investigated. Patients who achieved persistent engraftment (either complete or with mixed chimerism) were studied thereafter during their routine annual follow-up examinations.

Cell Separations and Purification

Cell separations were performed with Dyna Beads (Dynal International, Oslo, Norway) either coupled with the specific antibody (CD3 [Dynabeads #11101], CD4 [Dynabeads #11105], CD9 [Dynabeads #11107], CD19 [Dynabeads #11103]) or coupled in a first step reaction with monoclonal antibody (CD33 [Becton Dickinson, Mountain View, CA], Glycophorin A [Techogenetics, Milan, Italy]), and after washing with antihuman IgG (Dynabeads #11103) or coupled in a first step reaction with specific antibody (CD3 [Dynabeads #11101]). Separation procedures were in accordance with the manufacturers' protocols. The cell products were examined by fluorescence activated cell sorting (FACS) analysis and by rosette formation with microscopy and only samples with more than 95% purity were accepted for further study. Buffy-coat cells were layered on a Percoll 400 gradient. Cells obtained from the 65% to 75% interface were composed of more than 99% granulocytes as confirmed by morphologic examination of cytoplasmic preparations.

Colony-Forming Units

Erythroid burst-forming units (BFU-E) and granulocyte-macrophage colony-forming units (CFU-GM) were grown in 0.3% agar in a humidified incubator gassed with 5% carbon dioxide. Individual and pooled BFU-E and CFU-GM were picked from culture dishes after 14 days.

DNA Extraction and Restriction Fragment Length Polymorphism Analysis (RFLP)

High molecular weight DNA was extracted from peripheral blood (PB) or bone marrow (BM) using standard methods or from BFU-E or CFU-GM colonies with analytic grade mixed bed resin (Biorad, Hercules, CA). Southern blot analyses were performed with hypervariable VNTR probes as previously described. Calibration of the proportions of radioactive signals obtained were performed using 10%, 25%, 50%, 75%, and 90% mixtures of two DNA from individuals with informative VNTR patterns.

Polymerase Chain Reaction (PCR)

To assess the chimeric state in cell subpopulations or in poor DNA samples without usable markers, the highly polymorphic VNTR segments were amplified. Genomic regions thus examined were locus D15S80 and locus D17S30. Each amplified sample contained 25 to 250 ng DNA, 10 mM MgCl2, 10 mM Tris/Cl pH 8.3, 50 mM/L KC1, 1.5 mM/L MgCl2, 0.01% gelation, 2.5 U AmpliTag DNA polymerase (Perkin-Elmer Cetus, Branchburg, NJ), 1 $\mu$mol/L of each primer and 200 $\mu$mol/L of each dNTP. The incubation mixture was cycled 30 times as follows in a 9600 Perkin-Elmer thermal cycler: 60 seconds at 94°C, 60 seconds at 64°C, 60 seconds at 72°C, plus a final extension for 20 minutes at 72°C. After amplification, 10% ultra-thin layer polyacrylamide gels were established for the electrophoresis and stained with silver. To evaluate the size-dependent efficacy of PCR amplification, calibration curves of donor/recipient DNA mixtures were included.

FISH Analysis

FISH analysis was performed on PB, BM, cell subpopulations, and BFU-E and CFU-GM colonies when patient and donor were of different gender. A biotinylated pY3.4 probe for part of the Y chromosome was used in accordance with the manufacturer’s instructions (Oncor, Gaithersburg, MD). Positive and negative controls were included in all tests. Cells separated with Dynabeads were analyzed directly on the membrane surface.

HPLC Analysis of $\beta$-Globin Chain Synthesis

Globin-chain synthesis by marrow or peripheral blood reticulocytes was examined by measuring the incorporation of H3-leucine followed by ionic exchange column or HPLC.

RESULTS

Mixed Chimerism

Fifty-five patients with MC after transplantation were studied. Table 1 describes these patients. Rejection occurred subsequent to the detection of MC in 20 patients, with 7 showing a rapid increase in the proportion of host-type hematopoiesis evolving to graft rejection within
3 months of transplant. In 13 patients this process was slower, with a gradual increase in the proportion of host hematopoiesis culminating in complete rejection between 6 and 24 months after transplant.

In 35 patients with MC, stable functioning engraftment persisted throughout the period of observation. In 20 of these patients, hematopoiesis of host origin disappeared, either within 3 months (6 patients) or over a period of 2 years (14 patients). Persistent host-type hematopoiesis was demonstrated in 15 patients with a minimum follow-up of 1 year and a maximum of 7 years. These patients had persistence of donor type β-globin chain synthesis and maintained hemoglobin levels between 10 g/dL and 13.5 g/dL without red cell transfusions. The pattern of evolution of mixed chimerism and of functional engraftment is illustrated in Fig 1.

**Detailed Examination of Three Long-Term Chimeras**

Of the 15 patients with persisting MC, the three with the most prolonged follow-up have been the subject of more detailed study.

**UPN 572.** A 2-year-old boy was transplanted in 1989 with a donation from his 7-year-old brother who was heterozygous for β-thalassemia. Figure 2 demonstrates the levels of adult β-globin chain synthesis as determined by HPLC, and evolution of MC in the PB and BM over the ensuing 5 years as evaluated by VNTR-RFLP. The proportion of hematopoiesis of donor origin in the PB and BM decreased to less than 20% during the first 2 years after transplant and thereafter increased to a stable level of 50% from the third through the fifth year. During this entire period, the Hb level ranged between 10 and 10.2 g/dL and the level of donor β-globin chain synthesis remained higher than 65%. At 4 years after transplantation, FACS analysis of PB lymphocyte subset distribution showed normal values of CD2+ and high levels of CD57+ cells with a persisting inversion of the CD4+/CD8+ ratio (Table 2). Simultaneous determination of donor/recipient origin of different hematologic lineages was performed by PCR-VNTR on the DNA of PB granulocytes, CD2+ and CD19+ cells, and of BM CD33+ and Glycophorin-A+ cells and BFU-E and CFU-GM colonies (Fig 3). These results all show an approximately 50% proportion of host type cells in the PB cell and BM granulocyte/myeloid precursor analyses and slightly larger proportions of host type cells in the erythroid precursors (pool of 50 BFU-E and Glycophorin-A cells, 75% host type). These findings were confirmed by VNTR-PCR analysis performed on 20 BFU-E colonies singularly picked.

**UPN 322.** This 2-year-old thalassemic girl was transplanted in 1986 with a donation from her HLA-identical 16-year-old sibling who was heterozygous for the thalassemia gene. Mixed chimerism with approximately 50% of DNA of recipient origin was observed at 6 and 7 years after transplant both in PB and in the BM cells (Fig 4). Retrospective RFLP-VNTR analysis of DNA obtained from PB cells cryopreserved at 1 year after transplant showed the same equal
Fig 4. Long-term stable mixed chimerism evaluated by VNTR-RFLP and FISH analysis (—) in the PB and in the BM of case UPN 322. High levels of adult β-globin chain synthesis as determined by HPLC (----). Physiologic levels of peripheral Hb were observed for the whole posttransplant period studied.

Fig 6. Long-term stable mixed chimerism evaluated by VNTR-RFLP and FISH analysis (—) in the PB and in the BM of case UPN 688. High levels of adult β-globin chain synthesis as determined by HPLC (----). Physiologic levels of peripheral Hb were observed for the whole posttransplant period studied.

donor/recipient origin distribution. No marrow samples from that time were available for examination. Seven years after transplant, the proportion of donor precursor erythroid cells determined by FISH analysis for the Y chromosome was 35% in Glycophorin-A+ cells and 30% in a pool of 57 BFU-E agar colonies (Fig 5). This patient showed levels of hemoglobin ranging between 12.5 and 13.5 g/dL from the time of the transplant with production of high levels of donor type β-globin chain synthesis both in the marrow and the peripheral blood. Similar donor/recipient cell distribution was observed in the myeloid compartment. Normal peripheral blood lymphoid cell subset distributions were observed as described in Table 2.

UPN 688. A 19-year-old man with thalassemia was transplanted in 1990 with a donation from his HLA-identical sister heterozygous for β-thalassemia. Six months after transplant FISH analysis demonstrated 75% recipient origin in PB and marrow cells. From this time on, the proportion of host type hematopoiesis decreased and subsequently persisted at a stable level of approximately 50% until 3 years posttransplant (Fig 6). Observations at 3 and 4 years after the transplant showed a different proportion of host hematopoiesis in the BM (75%) from that in the PB (25%). During the entire posttransplant period, the patient had a pattern of β-globin chain synthesis similar to that of the donor with Hb levels of 10 to 11 g/dL. Normal lymphoid subset distribution was observed 4 years after transplant (Table 2) when donor origin hematopoiesis was 84% in CD2+, 83% in CD4+, 78% in CD8+, 86% in CD19+, and 40% in granulocytes (Fig 7). However, in the bone marrow, hematologic lineages showed a poor proportion of donor type of less than 35% both in the erythroid and in the myeloid compartments (Fig 7). Evaluation of donor/recipient origin at 4 years posttransplant of 20 single BFU-E colonies showed 3 colonies of donor origin and 17 of recipient origin.

DISCUSSION

At present we cannot distinguish clearly between immunologic rejection, thalassemic relapse, and a combination of both. We very rarely see patients with marrow aplasia following loss of donor engraftment; when this occurs we presume the rejection is solely immunologic in origin. However, the most common graft failure is accompanied by a return of thalassemic hematopoiesis, and this may be due to either immune rejection or "relapse" or a combination of both. Concurrent sequential marker studies of lymphoid and erythroid cell lineages might be useful in examining these possi-
bilities, but we do not have enough of such data at present. Moreover, the concept that graft failure is the outcome of either immune or “relapse” phenomena may be flawed, or immune-mediated rejection may itself be a quantitative phenomenon. We believe our current understanding of this situation is inadequate for intelligent speculation and we have defined “rejection” with this in mind.

The presence of hematopoietic cells of donor as well of recipient origin has often been observed after transplant, especially in patients receiving T-cell-depleted marrow as treatment for hematologic diseases. However, the influence of mixed chimerism on graft rejection is still a matter of debate. The development of techniques capable of providing some measure of quantitation has permitted a more detailed examination of this problem. We have shown that graft rejection probabilities were higher in thalassemic patients with mixed chimerism and proportional to the amount of donor type hematopoiesis present in the recipient.

In the present study, we have observed the evolution of mixed chimerism in a group of 55 thalassemic transplanted patients with MC. Twenty patients evolved to full donor engraftment, 15 remained persistent mixed chimeras, and 20 rejected the transplant. In patients who rejected the transplant, it has been possible to observe two different patterns of MC evolution and reappearance of thalassemia. Seven patients showed early rejection, while in 13 patients, graft failure developed over a period of 24 months. It is likely that immunologic mechanisms regulate the donor/recipient cellular interaction and these may influence the time when rejection is recognized.

It was interesting to observe that some patients with late graft failure no longer had functional grafts (with absent or very low β-globin chain production), although donor cell hematopoiesis still existed. On the other hand, some patients had persistent host type hematopoiesis (sometimes at levels as high as 80%) but remained transfusion independent, with normal β-globin chain synthesis and the normal functioning graft. Roux et al. observed that for patients who remained leukemia free after transplant, mixed chimerism was restricted to the T cells, while at the time of relapse it was possible to identify mixed chimerism in different cell sub-populations. We could find no difference in the donor/recipient origin of cell subsets within the PB in the three long-term mixed chimeras at 4, 4, and 7 years after transplant, except in patient UPN 688 who had different proportions of host type cells in lymphoid cells and granulocytes. It is unclear why this patient showed a higher proportion of host type hematopoiesis in the BM than in the PB both at 3 and 4 years after transplant.

A possible explanation for the high β-globin chain production in these three long-term mixed chimeras is that it could be due to different donor/recipient distribution between the erythroid and myeloid precursors. However, analysis of mixed chimerism in single or pooled BFU-E and CFU-GM colonies or in Glycophorin-A and CD33+ cells did not support this hypothesis. In fact, patients UPN 322 and UPN 688 showed no difference in the proportions of host and donor cells between myeloid and erythroid compartments, while patient UPN 572 had a slightly higher proportion of donor precursor cells in the myeloid compartment than in the erythroid compartment. Another possible explanation could be found in enhanced apoptosis of recipient thalassemic erythroblasts compared with donor erythroblasts. van den Bos et al. demonstrated that murine transplantation for β-thalassemia with normal congenic BM cells after sublethal total body irradiation resulted in partial RBC chimerism and correction of anemia, concluding that successful transplantation in thalassemia does not require ablation of endogenous BM.

Our results are in agreement with these findings, showing that, despite the presence of large amounts of residual host hematopoiesis widely distributed in the different hematologic lineages and including the erythroid precursors, patients with persistent MC produced sufficient donor β-globin chain synthesis and Hb. These findings may have implications for the development of gene therapy for thalassemia suggesting that small quantities of transformed cells may be sufficient to correct the genetic error.

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MIXED CHIMERISM IN THALASSEMIA AFTER BMT

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