

Brief Report

DONOR-DERIVED ACUTE PROMYELOCYTIC LEUKEMIA IN A LIVER-TRANSPLANT RECIPIENT

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THE long-term survival of donor lymphoid cells in recipients of solid-organ transplants or of fetal lymphocytes that cross the placenta and enter the maternal circulation has been established,¹⁻³ but the fate of other hematopoietic progenitors in organ allografts, especially those of the myeloid lineage, is not known. We describe a case in which acute promyelocytic leukemia developed in a recipient of a liver transplant two years after transplantation. The leukemic clone had genetic and phenotypic markers of the donor, a previously healthy 16-year-old boy who died of a head injury. Our findings indicate that leukemic transformation of donor myeloid cells that were resident in the transplanted liver occurred in this patient.

CASE REPORT

A 57-year-old woman presented with fatigue, palpitations, malaise, and diarrhea. She had a history of primary biliary cirrhosis and had received a liver transplant two years earlier from an unrelated 16-year-old boy who suffered a head injury while playing football and subsequently died of a subdural hematoma. The patient had two episodes of acute graft rejection 2.5 and 17 months after transplantation but never had symptoms or signs of graft-versus-host disease. At the time of the most recent presentation, she was taking prednisone (7.5 mg per day) and cyclosporine (100 mg twice daily) for post-transplantation immunosuppression. The patient had no history of exposure to radiation or alkylating agents and no family history of cancer.

The patient appeared pale and ill and had cushingoid features. There were multiple ecchymoses on her arms and legs. There was no mucosal bleeding or gingival hyperplasia. The hemoglobin level was 10.7 g per deciliter, the white-cell count was 5200 per cubic millimeter, and the platelet count was 41,000 per cubic millimeter. A blood smear showed 18 percent leukemic cells (blasts

and dysplastic promyelocytes). The results of coagulation tests were consistent with findings of disseminated intravascular coagulation and were as follows: prothrombin time, 17.2 seconds (normal range, 11.0 to 13.0); partial-thromboplastin time, 29.7 seconds (normal range, 21.1 to 32.1); fibrin- and fibrinogen-degradation products, 128 mg per liter (normal value, <8 mg per liter); and fibrinogen, 35 mg per deciliter (normal range, 150 to 360 mg per deciliter).

Bone marrow aspiration and biopsy confirmed the diagnosis of the microgranular variant of acute promyelocytic leukemia, with 80 percent blast cells. As determined by flow cytometry, the leukemic cells expressed CD33, CD13, and CD34, but not HLA-DR, results that are consistent with the diagnosis of acute promyelocytic leukemia.

Antibiotics, cryoprecipitate, and heparin (600 U per hour) were administered. All-*trans*-retinoic acid (tretinoin), at a dose of 45 mg per square meter of body-surface area per day, was begun the day after diagnosis. Mild respiratory distress developed several hours before the first dose of all-*trans*-retinoic acid. The patient's clinical status deteriorated rapidly, and within 12 hours severe hypotension, respiratory distress, and metabolic acidosis (pH 6.9) developed. The white-cell count increased to 62,000 per cubic millimeter, consisting predominantly of leukemic cells. The clinical picture was consistent with the presence of sepsis, all-*trans*-retinoic acid syndrome (characterized by fever, respiratory distress, and pulmonary infiltrates, which develop in 25 percent of patients with promyelocytic leukemia during treatment with all-*trans*-retinoic acid⁴), or both. Despite the administration of fluid and vasopressors and mechanical ventilation, the patient's clinical status continued to deteriorate and she died within 24 hours after the first dose of all-*trans*-retinoic acid. Permission for autopsy was denied.

METHODS

Tissue Samples

A sample of the patient's cirrhotic liver, taken at the time of the liver transplantation, was frozen within 48 hours. Liver-biopsy specimens obtained at the time of the two episodes of graft rejection were fixed in formalin and embedded in paraffin within 24 hours. Bone marrow cells aspirated at the time the patient presented with leukemia were stored in 70 percent ethanol at 4°C. Tissue from the donor before transplantation was not available.

Cytogenetic Analysis

Bone marrow samples were transported in RPMI 1640 medium with 15 percent fetal-calf serum. On arrival, cells were cultured for 24 hours at 37°C. Cells were exposed to colcemid (0.05 µg per milliliter) for 30 minutes at 37°C and harvested. Routine slide preparation and G banding were performed.

DNA and RNA Extraction

DNA and RNA were extracted from paraffin-embedded tissue specimens according to an adaptation of a previously described method⁵ with the use of the Puregene DNA and Purescript RNA isolation kits (Gentra Systems, Minneapolis). After DNA and RNA extraction, 10 µl of the nucleic acid solution was used for amplification.

Assay for Variable Number of Tandem Repeats and Molecular HLA Typing

A semiquantitative, nonisotopic method involving markers for variable-number tandem repeats (VNTR) and short tandem repeats for monitoring donor-cell engraftment was used as described previously.⁶ With the polymerase chain reaction (PCR), DNA was amplified with primers for DIS80 (chromosome 1), 33.6 (chromosome 1), and SE33 (chromosome 6) and analyzed by electrophoresis on a 6 percent polyacrylamide gel, with visualization by silver staining. We evaluated hematopoietic chimerism semiquantitatively by comparing the intensity of staining of the bands rep-

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representing the alleles we studied. Molecular HLA typing was performed with the reverse-blot sequence-specific oligonucleotide-probe hybridization system developed by Roche Molecular Systems (Pleasanton, Calif.), according to the manufacturer's instructions.⁷⁸

Analysis by Reverse-Transcription PCR

A two-step, nested reverse-transcription PCR (RT-PCR) was used to amplify chimeric *PML-RAR α* messenger RNA (mRNA) and the reciprocal *RAR α -PML* mRNA with published primer sequences.^{9,10} After amplification, the sample was electrophoresed through a 6.5 percent polyacrylamide gel, stained with ethidium bromide, and photographed.

The assay, which detects all three known isoforms of the *PML-RAR α* fusion gene, had a sensitivity of 1 leukemic cell in 1000 normal cells. These isoforms differ only in the *PML* breakpoint. Exon 2 of the *RAR α* gene is joined to exon 6 (long form) or exon 3 (short form) of the *PML* gene.¹⁰ A variable form has also been described.¹⁰ The clinical influence of these isoforms is controversial.¹¹⁻¹⁵ The sensitivity of detection was 0.0001 for the reciprocal *RAR α -PML* fusion gene, which is expressed in approximately 70 percent of patients with acute promyelocytic leukemia.

For all RT-PCR assays, positive control RNA from the NB4 cell line was used. This line exhibits the long form. Negative controls included both water and K562 RNA. A separate amplification of exons 1 and 2 of the *N-ras* gene was used to test RNA integrity.¹⁶ Precautions to eliminate the risk of contamination included the use of separate rooms for procedures performed before amplification, the use of disposable pipette tips that contained filters, and the use of water as negative controls in all amplifications.

Immunohistochemical Analysis

To demonstrate directly that the leukemic cells came from the donor, we developed an immunohistochemical method in which a monoclonal antibody directed against the donor-specific HLA marker B14 was used as a probe for donor cells, and the presence of the t(15;17) translocation was detected by in situ hybridization. The monospecific anti-B14 monoclonal antibody was selected because of the absence of this antigen on recipient cells.

Briefly, air-dried bone marrow smears were fixed in cold acetone for 10 minutes. Endogenous peroxidase activity was blocked with hydrogen peroxide and azide. After a blocking step with 1.5 percent normal goat serum at room temperature, the slides were incubated with a monospecific antihuman HLA-B14 monoclonal antibody (IgM, One Lambda, Canoga Park, Calif.) at 4°C overnight. A biotinylated goat antimouse IgM antibody and avidin-biotin complex labeled with horseradish peroxidase (Vectastatin, Vector Laboratories, Burlingame, Calif.) for 60 minutes at room temperature, followed by metal-enhanced diaminobenzidine (Pierce, Rockford, Ill.) for 60 minutes at room temperature, were used for detection. Counterstaining with hematoxylin was then performed.

Fluorescence in Situ Hybridization

To detect the t(15;17) translocation, we used dual-color fluorescence in situ hybridization with probes (Vysis, Downers Grove, Ill.) for the *PML* and *RAR α* genes on chromosomes 15 and 17, respectively, hybridized to marrow cells in interphase. The melting temperature was 78°C, and the melting time was two minutes. Hybridization was performed overnight at a temperature of 37°C. 4,6-Diamidino-2-phenylindole was used as the counterstain. The Quips mFISH Capture computerized imaging system (Vysis) was used for image analysis.

RESULTS

Cytogenetic Analysis

A male karyotype was found in 22 cells in metaphase (Fig. 1A), all of which contained the t(15;17) translocation characteristic of acute promyelocytic

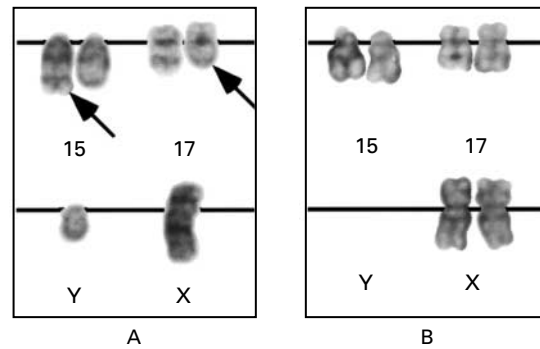


Figure 1. Cytogenetic Analysis.

Panel A shows marrow cells in metaphase with a male karyotype containing the t(15;17) translocation at q22;q12 (arrows), and Panel B shows residual female cells without clonal changes. A translocation to chromosome 5, probably from chromosome 9 (not shown), was also found in all male cells.

leukemia:46,XY,der(5)t(5;9)(p15;q12), t(15;17)(q22; q12). Three residual female cells without clonal changes were also seen (46,XX) (Fig. 1B).

VNTR Assay

Of the three loci examined, *DIS80* had a unique recipient allele and an allele shared with the donor, *33.6* had a unique donor allele and an allele shared with the recipient, and *SE33* had a unique donor allele, a unique recipient allele, and a shared allele. Although no DNA from the donor before the transplantation was available, we demonstrated chimerism by comparing the DNA obtained at the time of the two liver biopsies (after transplantation) with the patient's pretransplantation DNA (Fig. 2 and Table 1). At each locus, the same mixed chimeric pattern was seen in the leukemic marrow, ruling out the possibility that the malignant clone originated from a male blood donor. The *SE33* locus was chosen for semiquantitative analysis of the donor:recipient ratio (Table 1). Two and a half months after the liver transplantation, 65 to 75 percent of the DNA obtained from the liver was of donor origin. Seventeen months after transplantation, this proportion was 45 to 55 percent. This small shift probably represents repopulation of the nonhepatocytic tissue in the donor liver by cells from the recipient. The leukemic marrow had 55 to 65 percent donor DNA.

RT-PCR Analysis

We investigated whether leukemic cells could be detected, before clinical presentation, in the liver-biopsy specimens obtained at the time of graft rejection. As expected, the *PML-RAR α* gene product was detected in cells from the marrow aspirate. The patient expressed the short isoform of *PML-RAR α* (Fig. 3). The expected junction between exon 3 of the *PML* gene and exon 2 of the *RAR α* gene was con-

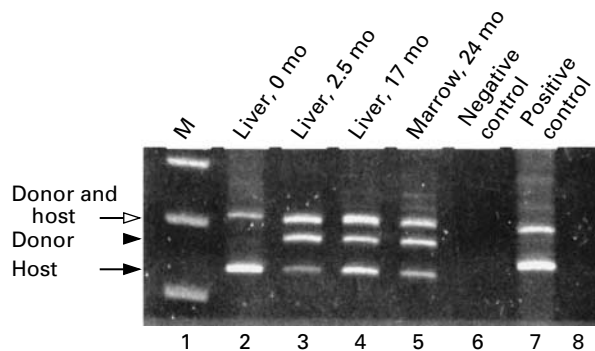


Figure 2. Variable-Number Tandem-Repeat Analysis of Samples Obtained before and after Transplantation.

The results of ethidium bromide–stained polyacrylamide-gel electrophoresis of the PCR product obtained with primers for the SE33 locus are shown. The patient's pretransplantation DNA obtained from a liver-biopsy specimen (lane 2) had two alleles. In contrast, DNA obtained at 2.5 months (lane 3) and 17 months (lane 4) shows a mixture of donor and host cells. The donor shared one allele with the recipient (open arrow) and had a donor-specific allele (arrowhead), whereas the lower band represents a host-specific allele (solid arrow). The same mixed pattern is visible in the bone marrow cells obtained at the time of the diagnosis of acute promyelocytic leukemia (lane 5). M denotes molecular-weight marker (lane 1). The negative control was water (lane 6), and the positive control DNA from an unrelated person (lane 7). Lane 8 is blank.

TABLE 1. RESULTS OF DNA AND RNA ANALYSIS.

SAMPLE	PERCENTAGE OF DONOR DNA*	<i>PML-RARα</i> FUSION GENET
Recipient's liver, pretransplantation	0	NA
Donor liver, 2.5 mo after transplantation	65–75	Negative
Donor liver, 17 mo after transplantation	45–55	Negative
Recipient's bone marrow, 24 mo after transplantation	55–65	Positive

*Results were obtained by variable-number tandem-repeat analysis.

†Results were obtained by the RT-PCR. NA denotes no amplifiable RNA.

firmed by direct sequencing of the PCR product (data not shown). In contrast with the marrow aspirate, the two liver-biopsy specimens obtained 21.5 and 7 months before the diagnosis of acute promyelocytic leukemia did not have a detectable *PML-RAR α* fusion-gene product, in spite of the intact RNA integrity as judged by the presence of amplifiable *N-ras* (Fig. 3 and Table 1). Therefore, leukemic infiltration of the liver at the time of the two liver biopsies was ruled out within the limits of sensitivity of this assay (1 leukemic cell in 1000 cells).

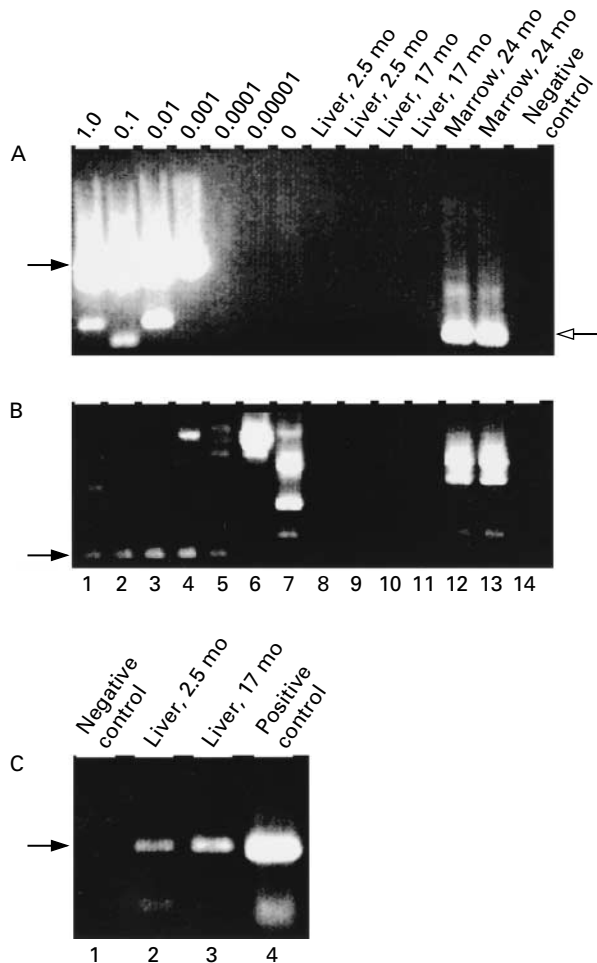


Figure 3. RT-PCR for the *PML-RAR α* Fusion-Gene Product (Panel A) and the Reciprocal *RAR α -PML* Fusion-Gene Product (Panel B).

To determine sensitivity, RNA from an acute promyelocytic leukemia cell line (NB4) was mixed with normal marrow RNA in decreasing proportions: 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, and 0 in lanes 1 through 7, respectively, in Panels A and B. The sensitivity of the PCR for the *PML-RAR α* fusion gene was 0.001, whereas that of the PCR for the *RAR α -PML* fusion gene was 0.0001. The PCR products from the liver-biopsy specimens obtained 2.5 months (lanes 8 and 9) and 17 months (lanes 10 and 11) after transplantation and from the bone marrow cells obtained at 2 years (lanes 12 and 13) — the time of the diagnosis of acute promyelocytic leukemia — were run in duplicate. In addition, to demonstrate RNA integrity, the *N-ras* gene product was amplified in two liver-biopsy specimens that were negative for both the *PML-RAR α* and *RAR α -PML* fusion-gene product (lanes 2 and 3 in Panel C). K562 cells were used as positive controls (lane 4). The solid arrows indicate the expected sizes of the long version of the *PML-RAR α* (500 bp) fusion-gene product, as well as the products of the reciprocal *RAR α -PML* (77 bp), and the *N-ras* (250 bp) reactions. The NB4 cell line used as a positive control contains the long version of the *PML-RAR α* fusion gene. The open arrow indicates the short isoform of the *PML-RAR α* fusion gene (150 bp) detected in our patient.

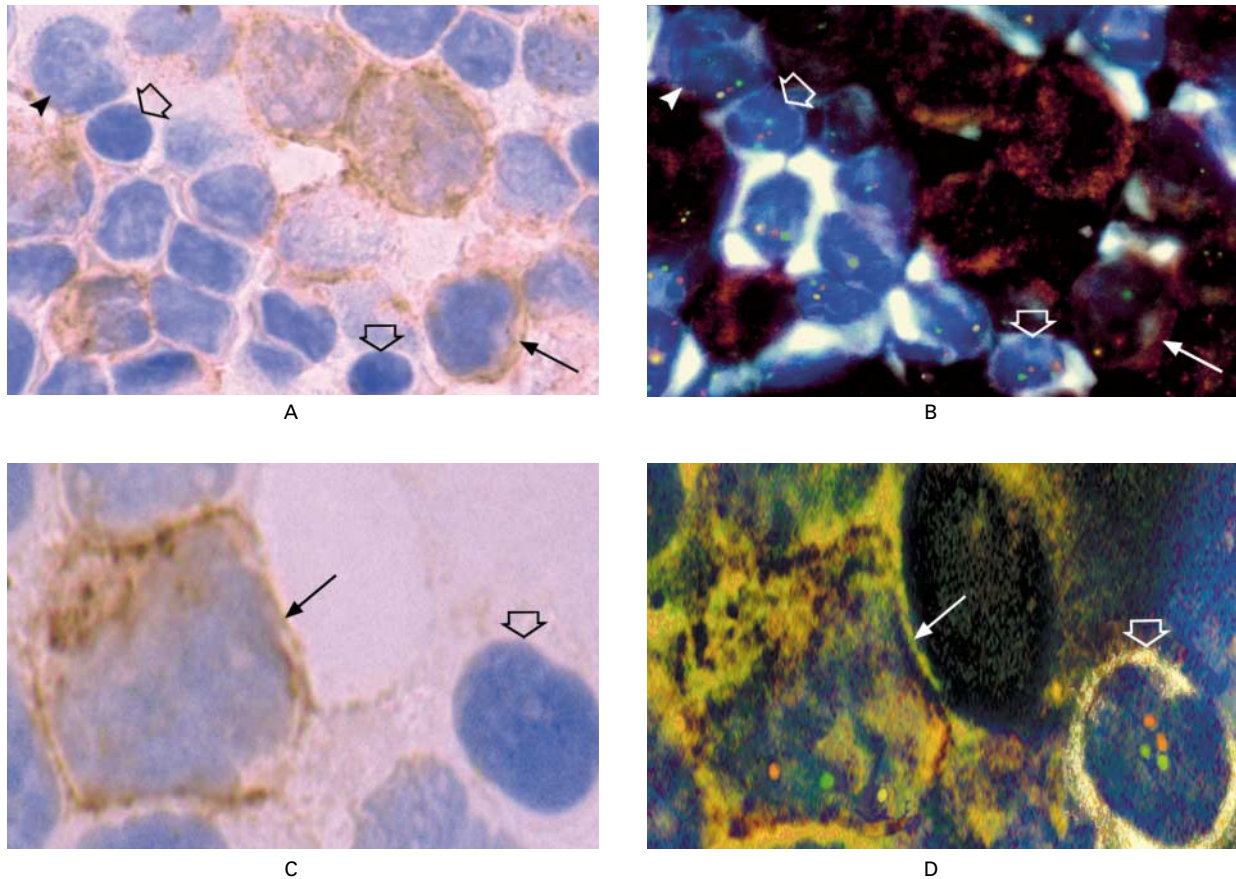


Figure 4. Analysis of the Leukemic Bone Marrow by Immunohistochemistry and Fluorescence in Situ Hybridization.

A representative field in Panel A shows that most cells were primitive myeloid forms (solid arrow and solid arrowhead) — that is, large cells with moderate amounts of cytoplasm, some with bilobate nuclei. Residual normal cells in the field (probably lymphocytes) had dense chromatin (open arrows). (A Wright's-stained aspirate revealed that 80 percent of the nonerythroid cells were blasts or dysplastic promyelocytes.) Arrows point to typical examples of staining (Panels A and C) or patterns revealed by fluorescence in situ hybridization (Panels B and D). Some leukemic cells showed intense brown staining for the HLA-B14 antigen (Panels A and C, solid arrows), others showed moderate or minimal staining, and some showed no staining (solid arrowhead, Panel A). Morphologically normal cells (open arrows, Panels A and C) were negative for the HLA-B14 antigen. In Panels C and D, a higher magnification of a different field than shown in Panels A and B shows an intensely staining leukemic cell (solid arrow) and a non-staining normal erythroid cell (open arrow). The fields shown in Panels B and D correspond to those in Panels A and C after hybridization with probes for the *PML* and *RAR α* genes on chromosomes 15 (green) and 17 (red), respectively. Morphologically normal cells have two of each chromosome (Panels B and D, open arrows), whereas leukemic cells have a fusion signal (Panels B and D, solid arrow and solid arrowhead) and are therefore missing one of each chromosome signal. Some cells have incomplete hybridization (Panels A and B, $\times 5000$; Panels C and D, $\times 10,000$).

Molecular HLA typing

The results of serologic HLA typing of the donor were as follows: HLA-A1,26; HLA-B14,57; and HLA-DR6,7. (The former DR6 type has since been split into DR13 and DR14 by higher-resolution typing.) Molecular DRB1 typing of DNA derived from the recipient's cirrhotic liver before transplantation showed homozygous DRB1*08. Typing of DNA from the two liver-biopsy specimens obtained after transplantation and the leukemic marrow showed the presence of DRB1*08 from the recipient and DRB1*13,07

from the donor. The limited amount of tissue available made further molecular typing of the recipient impossible. However, HLA-B typing of the leukemic marrow showed the presence of HLA-B*14,57 (the known type of the donor) and HLA-B*07,49 or 50 (for technical reasons, it was not possible to differentiate between HLA-B*49 and HLA-B*50 in this chimeric specimen). Since a person can have only two alleles at any locus, the recipient had to have HLA-B*07,49 or 50. These results again confirm the presence in the leukemic marrow of cells with genetic

markers of the donor and the recipient. Furthermore, the donor and the recipient were incompatible at both tested HLA loci.

Immunohistochemical Analysis and Fluorescence in Situ Hybridization

Leukemic cells stained for HLA-B14 (from the donor) at the surface; however, the intensity of staining varied from very dense to undetectable (Fig. 4). Analysis of 100 cells showed that 30 cells were negative for HLA-B14, whereas 70 cells were positive. Of the 30 HLA-B14-negative cells, 7 had evidence of a t(15;17) translocation and 23 did not (Table 2 and Fig. 4). The 7 cells with a hybridization signal indicating a *PML-RAR α* fusion gene were leukemic cells morphologically, whereas the other 23 cells were not. Of the 70 HLA-B14-positive cells, 45 clearly had a fusion signal indicative of a t(15;17) translocation (Fig. 4). In the other 25 HLA-B14-positive cells, the hybridization signal was obscured by the HLA-B14 staining on the surface (Table 2); however, all 70 HLA-B14-positive cells were leukemia cells morphologically. Review of the entire smear revealed that all cells with normal morphologic features, including erythroid precursors, lymphocytes, eosinophils, and megakaryocytes, were negative for HLA-B14, and all cells exhibiting a normal hybridization pattern were negative for HLA-B14.

DISCUSSION

This report describes the development of donor-derived acute promyelocytic leukemia in a female recipient of a liver transplant from a male donor. The presence of unique sex-chromosome and HLA markers, which enabled identification of donor and recipient cells, and a leukemia-specific marker — the t(15;17) translocation — provided an opportunity to pinpoint the origin of the leukemic cells in donor myeloid cells that had been transferred to the recipient in the graft two years earlier.

Several lines of evidence support the notion that the leukemic cells in the recipient came from the donor of the liver. First, cytogenetic analysis demonstrated that all leukemic cells with the t(15;17) translocation, which is pathognomonic of acute promyelocytic leukemia, had a male karyotype, whereas all cells without this translocation had a female karyotype. Second, molecular HLA typing of the leukemic marrow at two loci (HLA-B and HLA-DRB1) confirmed that both donor and recipient cells were present and showed the presence of all expected donor-specific alleles. Third, in the bone marrow, blasts and dysplastic promyelocytes expressed the donor-specific HLA marker B14, whereas residual normal hematopoietic cells did not. Fourth, leukemic cells that expressed the donor-specific HLA-B14 antigen also harbored the t(15;17) translocation, as detected by fluorescence in situ hybridization, whereas morpho-

TABLE 2. RESULTS OF IMMUNOHISTOCHEMICAL STAINING AND FLUORESCENCE IN SITU HYBRIDIZATION.*

RESULT	HLA-B14-POSITIVE CELLS (N=70)		HLA-B14-NEGATIVE CELLS (N=30)	
	MORPHOLOGICALLY NORMAL	MORPHOLOGICALLY LEUKEMIC	MORPHOLOGICALLY NORMAL	MORPHOLOGICALLY LEUKEMIC
	no. of cells			
Signal for fusion gene present	0	45	0	7
Normal signal present	0	0	23	0
Signal masked	0	25	NA	NA

*NA denotes not applicable.

logically normal cells did not. Finally, quantitative VNTR analysis detected the presence of 55 to 65 percent donor DNA in the recipient's marrow, corresponding approximately to the extent of leukemic involvement estimated by morphologic examination.

Leukemia originating in donor cells has been described after bone marrow transplantation in 23 cases.¹⁷⁻³⁶ Since all these cases entailed donor transplants from relatives of the recipients, mechanisms for the development of donor-cell leukemia have been thought to involve inherited genetic factors,^{35,37} transmissible viral agents,^{17,18,20,21,24,25,29,33} or the persistence of an undefined environmental factor in the marrow affecting leukemic cells.^{17,18,20,26,28,33} Since our patient had a liver graft from an unrelated donor and no preexisting malignant condition, these conjectures are not relevant.

Hematopoietic stem cells are present in the liver, a site of hematopoiesis in fetuses and, under certain pathologic conditions, in adults.³⁸ Although donor-derived lymphoid cells are able to survive over the long term in the recipient of a solid-organ transplant,¹ the fate of other hematopoietic lineages is unknown. Multilineage engraftment is the best indication of the persistence of multipotent stem cells, but it is very rare after solid-organ transplantation. The only well-described case³⁸ of multilineage engraftment was a patient who underwent liver transplantation, had severe graft-versus-host disease 32 days after transplantation, and died of graft-versus-host disease on day 135. Microchimerism is another recently described phenomenon, in which foreign cells remain detectable in the host for years.^{2,3,39} Normal hematopoietic cells of donor origin were not detectable at the time of diagnosis of acute promyelocytic leukemia in our patient, because normal hematopoietic elements expressing the donor-specific HLA-B14 antigen were not found (Table 2 and Fig. 4) and cytogenetic analysis showed no male cells with a normal karyotype (Fig. 1). The presence of donor-derived leukemia two

years after liver transplantation suggests the prior existence of normal donor-type myeloid precursors that underwent leukemic transformation in the recipient.

To test the validity of this conclusion, we tried to ascertain the timing of the postulated leukemic transformation. Although donor tissue was not available for testing, a complete blood count and automated differential cell count obtained before liver donation did not suggest the presence of any hematologic disorder. RT-PCR, which can detect 1 leukemic cell in a population of 1000 normal cells, failed to detect the *PML-RAR α* fusion gene product in two liver-biopsy specimens obtained 2.5 and 17 months after transplantation. This result rules out gross leukemic infiltration of the liver at the time of the biopsies, but does not rule out the possibility of undetectable involvement in the liver or the presence of leukemic cells in another organ, such as the bone marrow.

The donor and recipient were unrelated and were incompatible for both tested HLA loci. The ability of an HLA-incompatible leukemic clone to proliferate in this patient is remarkable. Factors that may have contributed to the survival of donor leukemic cells include the immunocompromised state of the patient and the disruption of the *PML* gene in the donor cells. In mice, the *PML* gene controls the expression of major-histocompatibility-complex (MHC) class I genes.⁴⁰ Our finding of low-to-variable expression of the donor-specific HLA-B14 antigen on leukemic cells is compatible with interference with expression of MHC class I genes, possibly from disruption of the *PML* locus. Reduced expression of HLA class I antigens on acute promyelocytic leukemia cells may have contributed to the lack of a response by the recipient's immune system.

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