ORIGINAL ARTICLE

Loss of Mismatched HLA in Leukemia after Stem-Cell Transplantation

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ABSTRACT

BACKGROUND

Transplantation of hematopoietic stem cells from partially matched family donors is a promising therapy for patients who have a hematologic cancer and are at high risk for relapse. The donor T-cell infusions associated with such transplantation can promote post-transplantation immune reconstitution and control residual disease.

METHODS

We identified 43 patients who underwent haploidentical transplantation and infusion of donor T cells for acute myeloid leukemia or myelodysplastic syndrome and conducted post-transplantation studies that included morphologic examination of bone marrow, assessment of hematopoietic chimerism with the use of short-tandem-repeat amplification, and HLA typing. The genomic rearrangements in mutant variants of leukemia were studied with the use of genomic HLA typing, microsatellite mapping, and single-nucleotide–polymorphism arrays. The post-transplantation immune responses against the original cells and the mutated leukemic cells were analyzed with the use of mixed lymphocyte cultures.

RESULTS

In 5 of 17 patients with leukemia relapse after haploidentical transplantation and infusion of donor T cells, we identified mutant variants of the original leukemic cells. In the mutant leukemic cells, the HLA haplotype that differed from the donor's haplotype had been lost because of acquired uniparental disomy of chromosome 6p. T cells from the donor and the patient after transplantation did not recognize the mutant leukemic cells, whereas the original leukemic cells taken at the time of diagnosis were efficiently recognized and killed.

CONCLUSIONS

After transplantation of haploidentical hematopoietic stem cells and infusion of donor T cells, leukemic cells can escape from the donor's antileukemic T cells through the loss of the mismatched HLA haplotype. This event leads to relapse.

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N Engl J Med 2009;361:478-88.

Transplantation of hematopoietic stem cells from a haploidentical family donor who shares only one HLA haplotype with the recipient is a potentially curative option for patients with high-risk hematologic cancers who lack an HLA-matched donor.[1-3] The major limitation of this strategy is the risk of severe graft-versus-host disease (GVHD), which can result from alloreactions mediated by donor T cells against the recipient's unshared HLA haplotype. Since the publication of studies on extensively T-cell–depleted grafts,[1,2] a variety of strategies have been developed to prevent or control GVHD after transfer of haploidentical T cells.[4,5] The feasibility and efficacy of infusions of haploidentical donor T cells have been established,[6-8] and new immunosuppressive drugs have allowed for the transplantation of haploidentical grafts without depleting them of T cells.[9,10]

The infusion of donor T cells promotes rapid reconstitution of the immune system after transplantation.[6,8] In addition, the graft-versus-leukemia effect mediated by such infusions is an effective form of immunotherapy for hematologic cancers.[11] However, relapses still occur, and the mechanisms involved in such relapses remain elusive.

Genomic or phenotypic alterations of HLA and the antigen-presenting machinery are frequently observed in patients with solid tumors.[12,13] Studies in animal models have shown that these phenomena can be the direct consequence of selective pressure mediated by T cells.[14-16] Moreover, loss of HLA class I surface antigens has been described in patients with melanoma after a partial response to cellular immunotherapy.[17,18] Conversely, alterations involving HLA are rare at the time of diagnosis in patients with hematologic cancers.[19]

Here we show that genomic loss of the recipient's mismatched HLA haplotype, which in principle is targeted by donor T cells, can occur in the leukemic cells of patients who have undergone transplantation of haploidentical hematopoietic stem cells. We suggest that this phenomenon is a mechanism of tumor escape from the selective pressure of a patient-specific graft-versus-leukemia reaction.

METHODS

PATIENTS AND TRANSPLANTATION PROCEDURE

We retrospectively identified adults with hematologic cancers who had undergone one or more haploidentical hematopoietic stem-cell transplantations at the San Raffaele Hospital in Milan between 2002 and 2007 and in whom immune reconstitution (defined as an absolute CD3+ cell count >100 per cubic millimeter) had been achieved after infusion of donor T cells. All 43 patients who fulfilled these criteria were included in the study. All had high-risk hematologic myeloid cancers (36 with acute myeloid leukemia and 7 with high-risk myelodysplastic syndrome); 26 patients underwent one transplantation, and 17 underwent more than one. Of these 43 patients, 25 had refractory or relapsing disease at the time of transplantation. In all patients, the conditioning regimen for the first haploidentical hematopoietic stem-cell transplantation was myeloablative. Eight patients received melphalan (140 mg per square meter of body-surface area), thiotepa (13 mg per kilogram of body weight), fludarabine (200 mg per square meter), and antithymocyte globulin (ATG) (Fresenius) (25 mg per kilogram). Thirty-five patients received treosulphan (42 g per square meter), fludarabine (150 mg per square meter), ATG (25 mg per kg), rituximab (500 mg), and total-body irradiation (200 cGy). The median dose of CD34+ cells was 10.2×10^6 per kilogram (range, 2.1×10^6 to 15.5×10^6). Twenty-two patients received donor T cells in one or more infusions after transplantation of CD34+ purified hematopoietic stem cells (median total T-cell dose, 10×10^6 per kilogram; range, 0.01×10^6 to 90×10^6; median time of first infusion, 43 days after transplantation; range, 14 to 61). No prophylaxis against GVHD was administered to those patients either after transplantation or after the T-cell infusions. The remaining 21 patients received an infusion of donor T cells with the stem-cell graft (median total T-cell dose, 438×10^6 per kilogram; range, 83×10^6 to 796×10^6). All 21 patients received prophylaxis for GVHD — 12 patients received 15 mg of methotrexate per square meter for 3 days plus 2 mg of intravenous cyclosporine per kilogram per day, and the remaining 9 patients received 15 mg of mycophenolate per kilogram three times a day plus sirolimus (at a starting dose of 4 mg per day, which was adjusted to achieve a target serum concentration of 8 to 15 ng per milliliter). All participants gave written informed consent in accordance with the protocols approved by the local ethics committee.

CHIMERISM ANALYSES

Hematopoietic chimerism was assessed monthly in samples of bone marrow aspirate with the use

of short-tandem-repeat amplification and genomic HLA typing in parallel, as previously reported[20] (also detailed in the Supplementary Appendix, available with the full text of this article at NEJM.org). For Patients 7, 16, and 43, the analyses were also performed on leukemic blasts purified by a fluorescence-activated cell-sorter (FACS). Results were always compared with those obtained from donor and patient cells before transplantation, which were used as reference controls.

LOSS OF HETEROZYGOSITY

We studied loss of heterozygosity and copy-number variations with the use of polymerase-chain-reaction amplification of 12 highly polymorphic short-tandem-repeat markers spanning the entire length of chromosome 6 and the use of the Illumina Human CNV370-Quad BeadArray or the Affymetrix Human SNP Array 6.0 single-nucleotide-polymorphism (SNP) array. (Details of these methods are provided in the Supplementary Appendix.) For Patients 7, 16, and 43, short-tandem-repeat mapping and SNP analysis were performed on FACS purified leukemic blasts, whereas for Patients 13 and 33, only samples of bone marrow aspirate containing leukemic blasts were available.

IN VITRO EVALUATION OF GRAFT-VERSUS-LEUKEMIA EFFECT

With the use of Ficoll–Hypaque centrifugation, we separated peripheral-blood mononuclear cells obtained from the stem-cell donor for Patient 16, from Patient 16, 85 days after the first hematopoietic stem-cell transplantation and 96 days after the second transplantation, and from a healthy HLA-mismatched subject. For the cells obtained under each of these conditions, 5×10^5 cells were used and plated with 5×10^5 irradiated mononuclear cells (radiation dose, 3000 rad) taken from Patient 16 at the time of diagnosis of leukemia (30% blasts) in 1 ml of Iscove's Modified Dulbecco's medium, supplemented with 10% human serum and 300 IU per milliliter of recombinant human interleukin-2. New medium was added to the cultures every 2 to 3 days, and responder cells were rechallenged with the original stimulator cells at a 1:1 ratio every 10 days. The function of responder cells from the mixed lymphocyte culture was tested after each stimulation with the use of ^{51}Cr-release, enzyme-linked immunospot (ELISpot) and [^{3}H]thymidine-incorporation assays (for details, see the Supplementary Appendix); the target cells were leukemic blasts obtained from Patient 16 at the time of diagnosis or when loss of the patient-specific HLA haplotype was documented.

Figure 1 (facing page). Identification of Mutant Variants of Leukemic Cells Not Detected by Chimerism Analysis with HLA Typing.

Bars show the percentage of leukemic blasts in bone marrow samples from each of the five patients in whom mutant variants of leukemia were detected. Asterisks indicate unavailable or nondiagnostic samples. The pie charts above the graphs show the percentage of chimerism in the corresponding bone marrow aspirate samples, assayed by genomic HLA typing (top row) or short-tandem-repeat (STR) amplification (bottom row). The five patients had a remission after undergoing hematopoietic stem-cell transplantation (HSCT) and receiving a high dose of donor T cells (median dose, 246×10^6 CD3+ cells per kilogram of body weight; range, 90×10^6 to 583×10^6). After transplantation, all five patients eventually had a relapse that was not detected with HLA typing (red boxes). For Patients 13, 33, and 43, the mutant variants were documented during the first and only post-transplantation relapse. In Patients 7 and 16, the first relapse, detected with the use of short-tandem-repeat amplification and HLA typing, was converted to a remission after a second haploidentical transplantation from the original donor. A second relapse followed, and it was not detected by HLA typing.

RESULTS

CLINICAL OBSERVATIONS

Studies of donor–host hematopoietic chimerism were carried out monthly after transplantation in all 43 patients with the use of short-tandem-repeat amplification and HLA typing in order to look for a reappearance of the host hematopoiesis in the bone marrow, which often predicts relapse.[20] Among the 43 patients, 17 patients — 14 of whom received transplants when they had persistent disease — had a leukemia relapse. In all 17 patients, relapse was confirmed to be of host origin on the basis of short-tandem-repeat chimerism. Surprisingly, in five of these patients, genomic HLA typing of bone marrow cells did not detect host-specific HLA alleles (Fig. 1). In all five patients (Table 1), the leukemic cells at the time of relapse had the same immunophenotype and the same cytogenetic features found at diagnosis, and no new cytogenetic abnormalities were observed. Patient 7 and Patient 43 had GVHD at the time of leukemia relapse (consensus grade 2

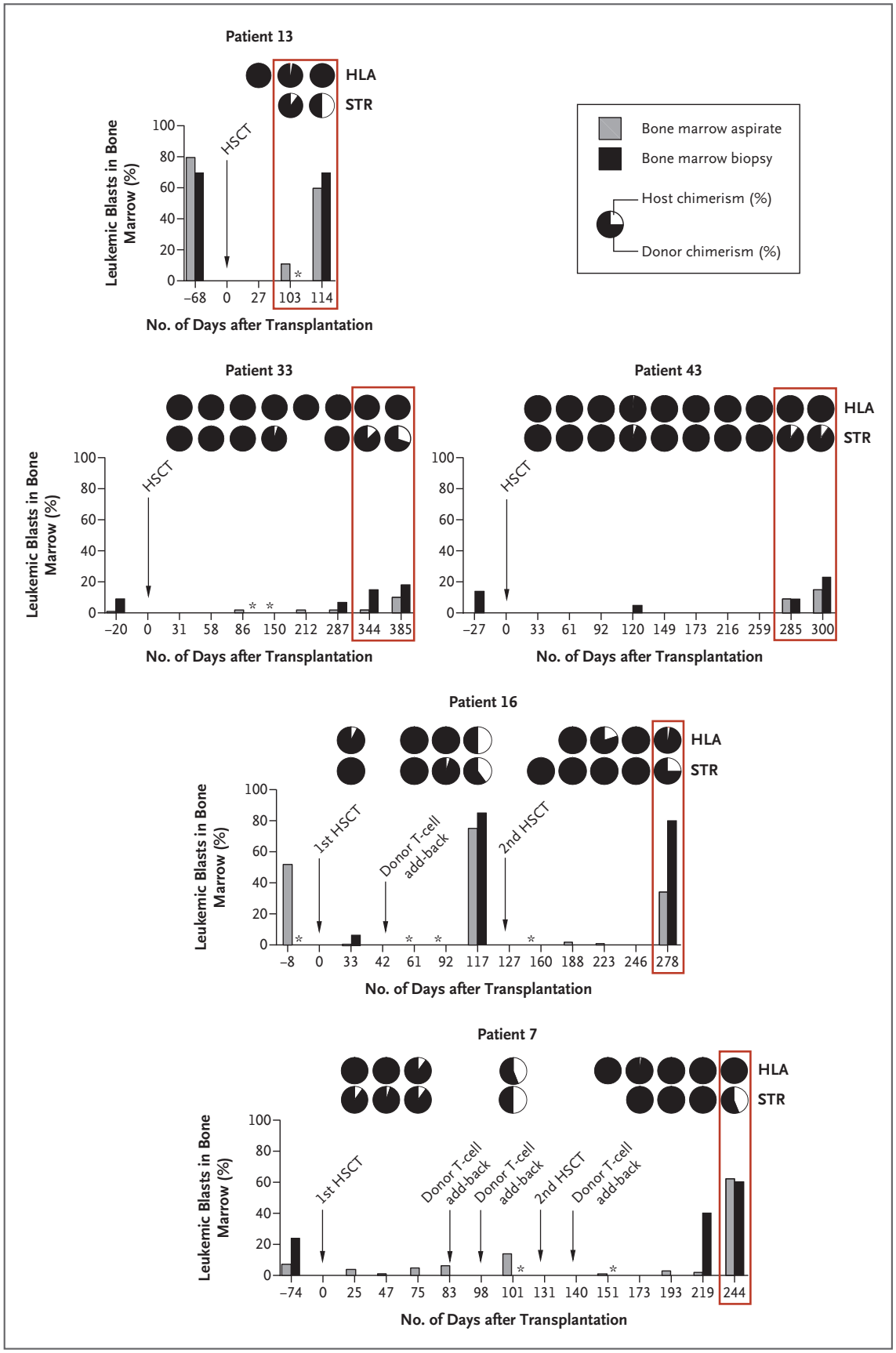


Table 1. Characteristics of the Patients in Whom Loss of the Patient-Specific HLA Haplotype Was Documented.*

Variable	Patient 7	Patient 13	Patient 16	Patient 33	Patient 43
Sex	Female	Female	Female	Female	Male
Age (yr)	41	33	65	54	53
Donor characteristics					
Sex	Female	Female	Male	Male	Male
Age (yr)	54	55	42	45	25
Relationship	Cousin	Mother	Son	Brother	Son
Diagnosis	Acute myeloid leukemia	Acute myeloid leukemia	Acute myeloid leukemia	Myelodysplastic syndrome	Acute myeloid leukemia
Status at first HSCT	Relapse	Relapse	Relapse	Relapse	Relapse
No. of haploidentical HSCTs from the same donor	2	1	2	1	1
T-cell–replete graft	No (both HSCTs)	Yes	No (first HSCT), yes (second HSCT)	Yes	Yes
Donor–T-cell add-backs	Yes (both HSCTs)	No	Yes (first HSCT), no (second HSCT)	No	No
Total T-cell dose received ($\times 10^{-6}$ CD3+ cells/kg body weight)	113	583	90	289	246
Time from first HSCT to HLA haplotype loss (days)	244	103	278	344	285
CD3+ cell count at time of HLA haplotype loss (per mm^3)†	1860	107	407	3559	2676
Final clinical outcome	Death	Death	Death	Survival — complete response‡	Death
Cause of death	Relapse§	Relapse	Relapse	—	Multiorgan failure¶

* HSCT denotes hematopoietic stem-cell transplantation.
† CD3+ cell counts were determined within 30 days of documentation of HLA haplotype loss, except in the case of Patient 13, in whom it was determined 75 days before documentation of HLA haplotype loss (28 days after HSCT).
‡ Leukemia remission was achieved after a subsequent haploidentical HSCT from a new donor, who was mismatched for the HLA haplotype retained by the mutant leukemia.
§ Patient 7 received a subsequent haploidentical HSCT from a new donor (matched for the HLA haplotype retained by the mutant leukemia), did not have a response to the treatment, and died in relapse.
¶ Multiorgan failure occurred in this patient during preparation for receipt of a haploidentical HSCT from a new donor, who was mismatched for the HLA haplotype retained by the mutant leukemia.

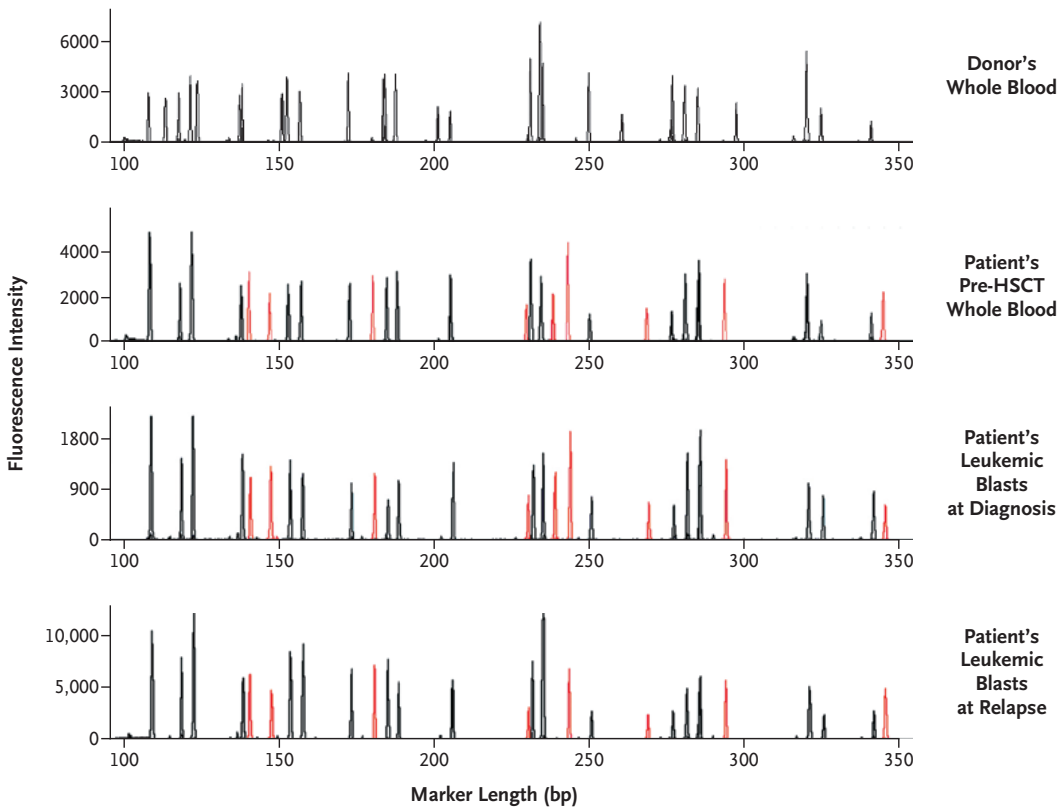


Figure 2. Genomic Loss of the Patient-Specific HLA Haplotype by Leukemic Cells after Transplantation in Patient 16.

Panel A shows electropherograms of the polymerase-chain-reaction products (amplified with the AmpFISTR Profiler Plus kit) used to interrogate nine highly polymorphic short-tandem-repeat loci on different chromosomes. Patient-specific markers, identified on the basis of comparison with samples from the stem-cell donor, are shown in red. At the time of both diagnosis and relapse, the short-tandem-repeat profile of leukemic blasts was patient-specific. Panel B shows the results of strip-based genomic HLA-DRB probing for Patient 16 (Dynal RELI SSO kit). Note the absence of signals for the patient-specific HLA-DRB alleles (corresponding to the red vertical lines) in the leukemic cells obtained at relapse. Band 39 of the typing strip (indicated by the asterisk) can be accounted for by both DRB3*0101 (donor-specific) and DRB1*0701 (patient-specific) and was thus negative only in the mutant leukemic cells at relapse, in accordance with loss of DRB1*0701. Similar results were obtained for the genomic typing of HLA-A, HLA-B, HLA-C, and HLA-DQB1 in Patient 16, as well as for all five HLA loci in the other four patients with leukemic relapse that was not detected by HLA typing. HSCT denotes hematopoietic stem-cell transplantation.

and grade 1, respectively). None of the other three patients had GVHD after transplantation.

GENOMIC CHARACTERIZATION OF THE MUTANT LEUKEMIC-CELL VARIANTS

To determine why recipient HLA alleles were not detected at the time of relapse, leukemic blasts from the five patients, obtained at the time of diagnosis and at the time of relapse, were purified with the use of FACS and subjected to genomic HLA typing and short-tandem-repeat analysis. Although the blasts obtained at the time of relapse were of patient origin (Fig. 2A), they did not harbor any of the patient-specific HLA alleles for the five loci tested (Fig. 2B). Instead, they carried only the HLA haplotype shared by the donor and the recipient. In contrast, blasts obtained at the time of diagnosis were heterozygous for the same loci. These findings show that genomic loss of the patient-specific HLA haplotype occurred in vivo after transplantation.

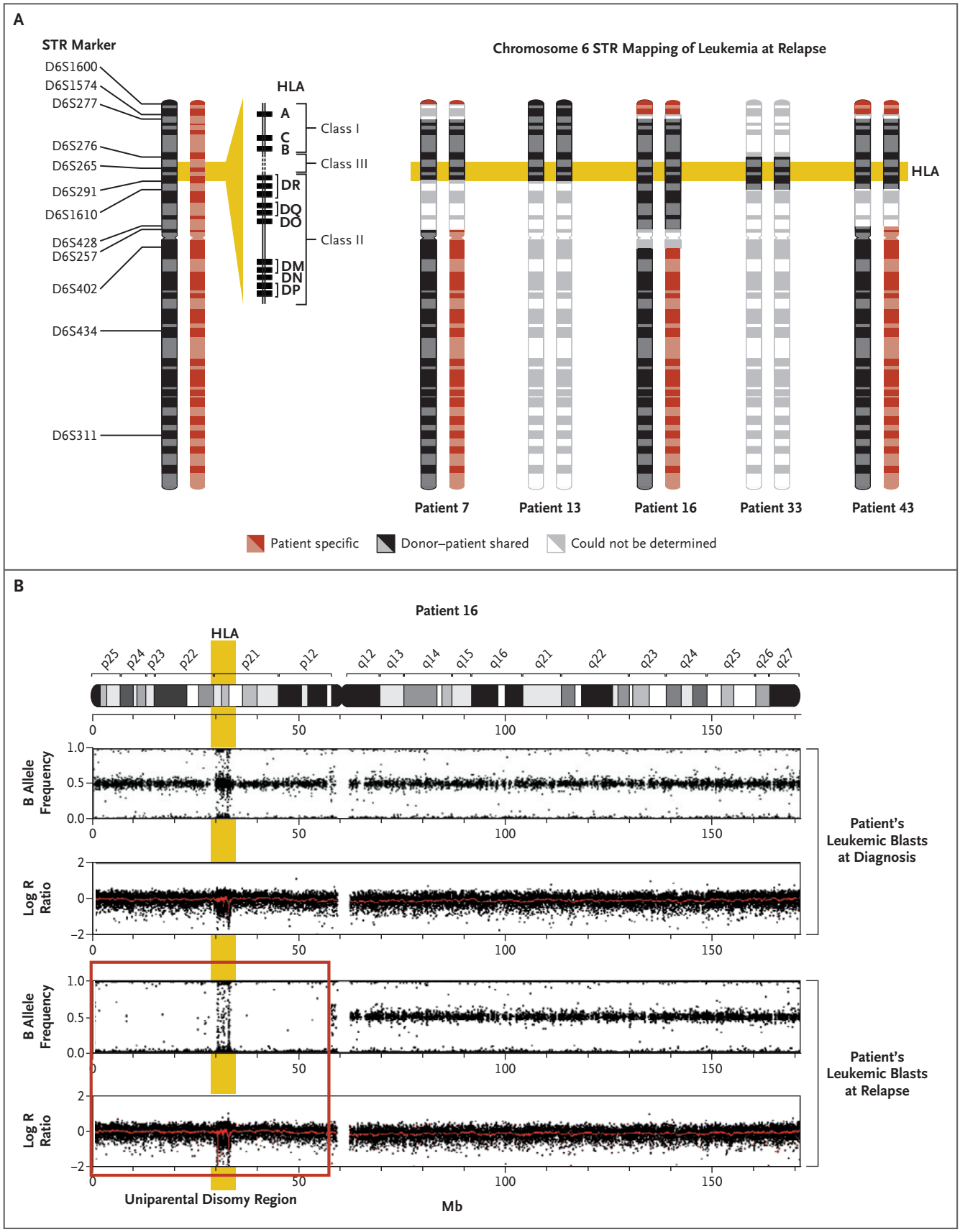
To determine the extent and the mechanism of the loss of heterozygosity in the patients with relapsed leukemia, we performed microsatellite mapping and SNP arrays of chromosome 6, including the HLA region. In all five patients, the analyses showed loss of heterozygosity involving the short arm of chromosome 6, encompassing the HLA region and causing loss of the patient-specific haplotype (Fig. 3, and Fig. 1 of the Supplementary Appendix). Analysis of copy-number variations showed no deletions in chromosome 6p, a finding that is in line with the observed karyotype. On the basis of these results, we concluded that loss of the HLA haplotype was due to acquired partial uniparental disomy of chromosome 6 (i.e., substitution, for the "lost" haplotype, of a corresponding region from the homologous chromosome).

Figure 3 (facing page). Loss of the Patient-Specific HLA Haplotype through Extensive Rearrangements in Chromosome 6, Leading to Acquired Uniparental Disomy.

A schematic reference map (Panel A, left side) shows 12 highly polymorphic short-tandem-repeat (STR) markers spanning chromosome 6 that were used to assign the patient-specific (dark red and light red) and shared (black and dark gray) haplotype of the original leukemia, along with an expanded view of the HLA region (yellow). STR mapping of leukemic cells at relapse is shown in ideogram representations (Panel A, right side) of both chromosomes 6, in the five patients with documented loss of the HLA haplotype. Chromosome regions for which no unequivocal reconstruction was possible are shown in light gray and white. In all five patients, genomic rearrangements encompassing the entire HLA region (yellow) led to homozygosity for the HLA haplotype shared between donor and patient. Results from the Human CNV370-Quad BeadArray (Panel B) show the leukemic blasts obtained at diagnosis and at relapse, with the loss of the patient-specific HLA haplotype, in Patient 16. Two plots are included for each time point. The upper plot shows the frequency of the B allele, which indicates the allelic composition of each single-nucleotide polymorphism (SNP). BB genotypes have a B allele frequency of 1, AB genotypes a frequency of 0.5, and AA genotypes a frequency of 0. The lower plot shows the log R ratio, which is a measure of the copy number for each SNP. The mean of the log R ratio is shown as a red line. The HLA region in chromosome 6p21.3 (yellow) shows a smeared signal pattern because of the high density of highly homologous SNPs, as is usually observed in samples from healthy donors. The lack of heterozygous genotypes across the p arm of chromosome 6 in the absence of an alteration in copy number in the leukemic cells at relapse indicates a large area (>50 Mb) of uniparental disomy (outlined in red).

FUNCTIONAL STUDY OF THE GRAFT-VERSUS-LEUKEMIA RESPONSE

Since HLA mismatches can elicit robust T-cell responses,[21,22] we investigated whether loss of the HLA haplotype allowed the mutant leukemic cells to escape immunosurveillance by the donor's T cells. We stimulated mononuclear cells obtained at different times after hematopoietic stem-cell transplantation from a representative patient (Patient 16) with cells obtained from the patient at the time of diagnosis. Mononuclear cells from the stem-cell donor and from a healthy HLA-mismatched subject served as controls. After three rounds of stimulation with leukemic cells, T cells accounted for more than 85% of the cultures (data not shown). These T cells consistently produced a robust response to the original leukemic cells, as determined by tests for cytotoxicity, interferon-γ release, and proliferation. Leukemia-reactive T cells from the stem-cell donor and the patient after transplantation specifically targeted the patient-specific HLA molecules, as could be seen when we tested them against a panel of HLA-typed target-cell lines (data not shown). The same T cells did not respond to leukemic blasts harvested at relapse, whereas T cells from the healthy HLA-mismatched subject did respond to the blasts harvested at both time points (Fig. 4).



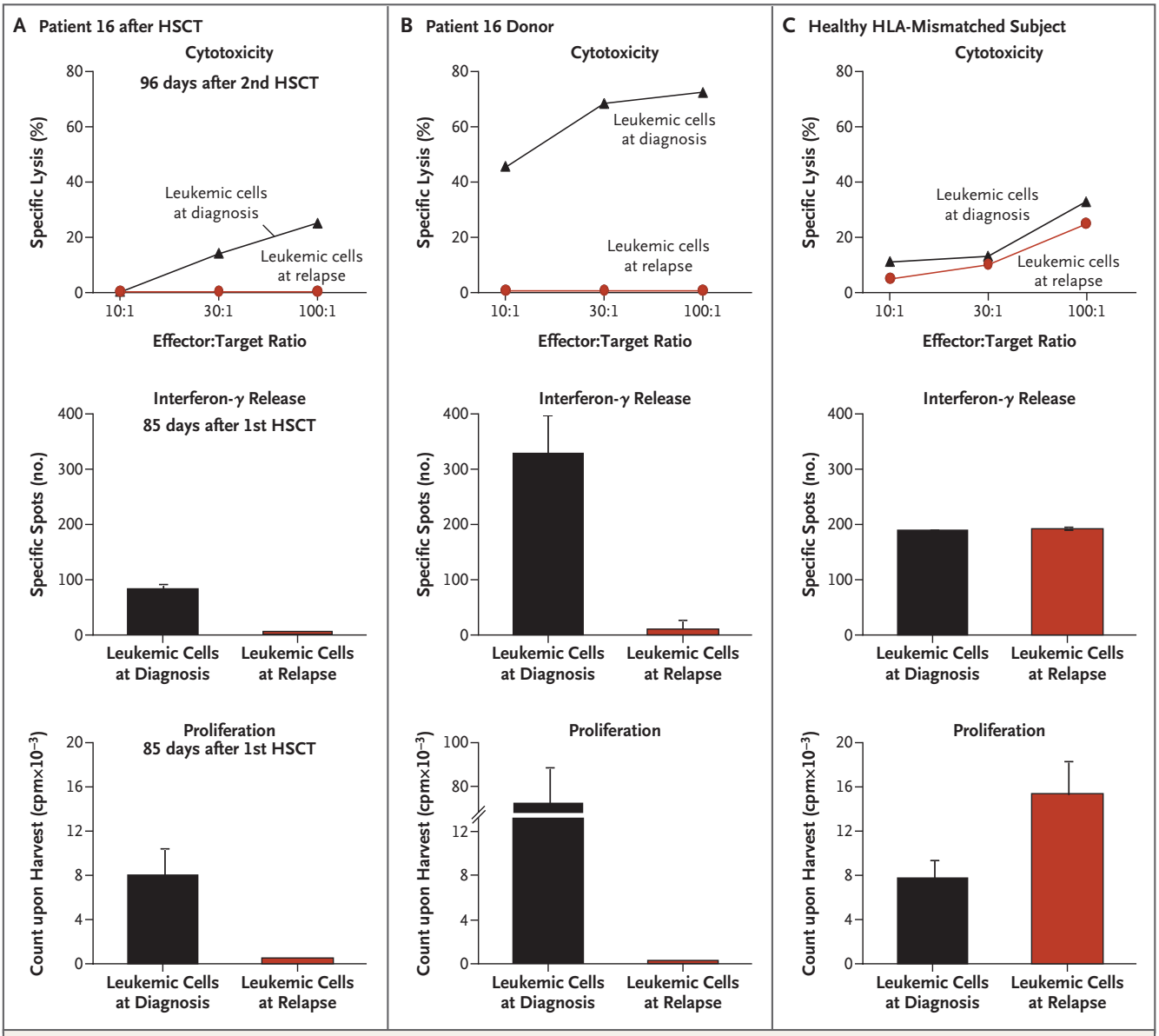


Figure 4. Immune Escape of Mutant Leukemic Cells in Patient 16.

Mononuclear cells harvested from Patient 16 at two time points after hematopoietic stem-cell transplantation (HSCT) (Panel A), from the haploidentical stem-cell donor (Panel B), and from a healthy HLA-mismatched subject (Panel C) were tested against leukemic blasts taken from the patient at diagnosis (black) and at relapse (red) after three rounds of specific stimulation. T-cell–specific lysis of leukemic blasts (top row) was determined with the use of a ^{51}Cr-release assay at different effector:target ratios. T lymphocytes releasing interferon-γ in response to leukemia were detected as specific spots in an enzyme-linked immunospot (ELISpot) assay (middle row). Cell proliferation in response to the relevant stimulator cells (bottom row) was determined after a 72-hour coculture by means of 16 hours of pulsing with [^{3}H]thymidine. Counts per minute at the time of harvest are shown on the y axis. In all three assays, T cells from the patient after transplantation and from the stem-cell donor reacted to the leukemia at diagnosis but not to the leukemia at relapse, which had lost the patient-specific HLA haplotype. Conversely, T cells from the healthy HLA-mismatched subject responded equally to leukemia cells harvested at both time points. T bars denote the standard deviation among experimental replicates.

DISCUSSION

Genomic instability is a hallmark of myeloid cancers, and it has been shown to be associated with loss of heterozygosity without loss of genetic material (uniparental disomy), even in leukemic cells with a normal karyotype.[23-25] Our data indicate that this loss of heterozygosity can

confer a selective advantage on leukemic cells, which become able to escape immunologic pressure from alloreactive donor T cells.

Genomic loss of the patient-specific HLA haplotype occurred in 5 of 17 patients (29%) whose disease relapsed. The frequency of this event suggests the value of assessing the HLA genotype of the leukemic cells in cases of relapse after transplantation to identify alternative donors whose T cells could eliminate escape mutants. The last two patients in whom we documented loss of the patient-specific HLA haplotype were candidates for a subsequent transplantation of haploidentical hematopoietic stem cells from a different donor, who was mismatched for the HLA haplotype retained in leukemic cells. Remarkably, one of the two patients is alive and in complete remission more than 16 months after the second transplantation.

Previous reports have proposed that natural killer cells are the main determinant of the graft-versus-leukemia effect after haploidentical hematopoietic stem-cell transplantation with T-cell depletion.[26,27] The escape mechanism we describe, which relies on uniparental disomy, did not affect the overall expression of cell-surface class I HLAs (see Fig. 2 of the Supplementary Appendix), nor did it consistently evoke reactivity by the total population of natural killer cells (see Fig. 3 of the Supplementary Appendix). In losing specific HLA alleles, leukemic blasts may have gained susceptibility to alloreactive natural killer cells that carry as their sole inhibitory receptors immunoglobulin-like receptors that are specific for the lost haplotype.[28] The reportedly low frequency of this subpopulation of natural killer cells in adults, particularly during the initial months after haploidentical hematopoietic stem-cell transplantation with infusion of donor T cells,[29] may explain why natural killer–cell alloreactivity failed to prevent a disease relapse.

In the five patients we describe, other mechanisms of T-cell–mediated alloreactivity, such as reactions against minor histocompatibility antigens[30] or immunization against inherited paternal antigens,[31] apparently did not provide protection against the mutated variants of leukemic cells, suggesting that in these patients, major HLA mismatches were the pivotal targets of the antileukemic response, and their loss was sufficient to allow relapse.

Taken together, our data indicate that immune escape by leukemic cells from a graft-versus-leukemia effect after haploidentical hematopoietic stem-cell transplantation can lead to relapse. The phenomenon we observed is likely to be the consequence of selective pressure mediated by alloreactive donor T cells, further strengthening the biologic rationale for the use of T-cell adoptive immunotherapy. Loss of the patient-specific HLA haplotype is easy to diagnose and has important implications for selecting a treatment that is suitable for relapse after transplantation.

Supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC 44/2007), the Cariplo Foundation (2007.5486), the Telethon Foundation (GGP08201), the European Commission (FP7-ICT-2007-216088), the Italian Ministry of Health, and the Italian Ministry of Research and University.

Dr. Bonini reports receiving consulting fees from MolMed. No other potential conflict of interest relevant to this article was reported.

We thank Drs. Giovanni Tonon, Filippo Martinelli Boneschi, and Alessandro Bulfone for their valuable suggestions on the genomic characterization of the mutated leukemia; Dr. Alessio Palini for supervision of cytometric analyses and cell sorting; and Drs. Lisbeth Guethlein and Kevin Goudy for their review of earlier versions of this article.

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