Recurrence of Acute Lymphoblastic Leukemia in Donor Cells After Allogeneic Marrow Transplantation Associated With a Deletion of the Long Arm of Chromosome 6

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This report concerns a woman who experienced a relapse of acute lymphoblastic leukemia (ALL) associated with an interstitial deletion of the long arm of chromosome 6 in donor cells more than 4 years after allogeneic bone marrow transplantation (BMT). Direct bone marrow preparations revealed the presence of two leukemic clones 46,XY,del(6)(q23q25) and 45,X,-4,del(6)(q23q25), +8, -15, -21, +i(21q), +mar, the former clearly indicating that male donor cells were involved in the malignant process. Relapse as evidenced by these chromosome anomalies was confined to metaphases from directly prepared marrow cells and phytohemagglutinin (PHA)-stimulated peripheral blood cells. Cytogenetic analyses of T-cell cultures (LTC), T-cell colonies, and early erythropoietic colonies gave predominantly normal donor karyotypes (65 of 69 mitoses) along with three host mitoses and a single donor metaphase carrying the 6q- anomaly. The marrow stroma, as represented by first-passage adherent layer cells from long-term marrow cultures, showed 17 of 19 host metaphases. One of two donor cells found within the stromal elements exhibited a 6q- chromosome. In the subsequent remission mitoses derived from myeloid and lymphoid cells were exclusively of donor origin, and chromosomal abnormalities could no longer be detected. Stromal elements remained host-derived (14 of 16 mitoses).

Recurrence of Leukemia in donor cells after allogeneic marrow transplantation (BMT) is rare. With an estimated 10,000 marrow transplants having been performed worldwide by the end of 1984 only seven such cases have been reported. Three of these patients had suffered from acute lymphoblastic leukemia (ALL) before grafting and morphological examination or cytochemistry and immunophenotyping suggested that the original tumor had recurred after BMT. Furthermore, one patient with ALL and three patients with acute myelogenous leukemia have been described who developed a fatal Epstein-Barr virus-related lymphoproliferative disorder involving donor cells shortly after transplant. This entity clearly differs from recurrent leukemia with respect to the morphological appearance, the clinical course, and the immunological phenotype allowing its identification as a polyclonal or monoclonal tumor of B cell origin.

In this article we report the first case of recurrent ALL in donor cells associated with an interstitial deletion of the long arm of chromosome 6, which is held to be one of the specific chromosome abnormalities in ALL. In addition to karyotyping directly prepared bone marrow cells and peripheral blood lymphocytes from the patient after stimulation with phytohemagglutinin (PHA), we also grew long-term marrow cultures (LTC), T-cell colonies, and early erythropoietic (BFU-E) and granulopoietic progenitors (CFU-C) for chromosomal studies. This gave us the opportunity to perform detailed analyses on the patient’s myeloid and lymphoid compartments and marrow stroma cells to determine whether they were of host or donor origin and how they participated in the leukemic process.

Case Report

In April 1979 acute T-lymphoblastic leukemia (T-ALL, FAB L-2 classification) was diagnosed in a 22-year-old white woman. The diagnosis was based on the morphological appearance of blast cells and on the results of cytochemical staining. Seventy percent of blast forms were focally positive for acid phosphatase (Fig 1A) but lacked myeloperoxidase and naphthol-AS-D-chloroacetate esterase activity (Professor K. Lennert, Institute of Pathology, Kiel, Germany).

Treatment was begun with vincristine, daunorubicin, and prednisone for three weeks with no response. The patient was then treated in accordance with the protocol of the German multicenter ALL/AUL study group, with some modifications. She received vincristine, adriamycin, L-asparaginase, and dexamethasone for four weeks, followed by a combination of cytosine arabinoside, 6-mercaptopurine (6-MP), intrathecal methotrexate (MTX), and cranial irradiation (18 Gy). A remission was induced and the patient was maintained on 6-MP and MTX until consolidation therapy was administered in October 1979.

Maintenance therapy was continued until October 1981, when a deteriorating platelet count heralded relapse. The diagnosis of T-ALL was confirmed. In addition to the typical staining pattern for acid phosphatase, the blast cells were also positive for dipeptidylamino-peptidase IV, which is a highly specific marker for T cells at an early stage of differentiation. A second remission was achieved by reinstituting the German ALL/AUL study protocol.

After a conditioning regimen including 12 mg MTX on days 9 and -3, 60 mg/kg cyclophosphamide on days -5 and -4, and 10 Gy total body irradiation on day -1, the patient received an allogeneic BMT from her HLA-identical brother in May 1982. In order to prevent graft-versus-host disease (GVHD), T lymphocytes were removed from the donor marrow using a polyclonal antihuman T-cell globulin, and MTX was administered until day +102. Engraftment was prompt and the further posttransplant course was uneventful except for a mild skin rash not clearly attributable to GVHD, which spontaneously resolved.

On day +878 after BMT a second relapse (first relapse after BMT) occurred. The morphology of the leukemic cells was unchanged when compared with slides from the time of the initial diagnosis or the time of relapse before BMT. Terminal deoxynucleo-

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tidy transferase was positive. Application of the initial treatment regimen was again successful, and a complete remission was verified by bone marrow aspiration.

The patient continued in remission until day +1464, when granulocytopenia and thrombocytopenia developed. The marrow examination on day +1485 revealed 87% blast cells. Morphologically, the blast forms were of lymphoid appearance. They stained positive for PAS (84%), but this time were negative for acid phosphatase (Fig 1B) as well as for myeloperoxidase and nonspecific esterase. The patient was treated with cytosine arabinoside (1.0 g/m² every 12 hours for six consecutive days) and mitoxantrone (10 mg/m² daily for three days). This therapy was tolerated well, and a fourth complete remission was attained. At the time of this writing the patient has stayed in unmaintained remission for six months and is currently leading a normal life.

The patient was advised of the BMT and chemotherapy procedures and the attendant risks in accordance with institutional guidelines, and gave informed consent.

MATERIALS AND METHODS

Blood and bone marrow specimens. Peripheral blood and bone marrow cells obtained after informed consent were collected in 1 mL of alpha medium containing 800 U of preservative-free heparin. In addition to BFU-E and CFU-C, which were grown only from the remission marrow, stromal elements as represented by passaged adherent cells of marrow LTC, T-lymphocyte colonies, and direct preparations of bone marrow metaphases and of unseparated peripheral blood cells stimulated with PHA were all karyotyped at the time of second relapse after BMT (day +1485) and six weeks after the ensuing complete remission had been achieved (day +1557).

Long-term cultures. Initiation and maintenance of long-term marrow cultures and the procedure for detachment of the adherent cell layer followed a protocol described by Coulombel et al. When the adherent layer had grown to confluence (29 days after initiation of cultures from the patient's marrow at relapse and after 27 days of culture of the remission marrow) the nonadherent cells and all of the growth medium were removed from the culture dishes. After trypsinization of the adherent cell layer, the cells were washed twice, suspended in fresh long-term medium, and transferred to new culture dishes. After two additional days of culture most cells had attached to the bottom of the dish, assumed an angulated to spindle-shaped appearance, and were rapidly dividing, as judged by a steady increase in the number of cells per microscopic field. At that stage, cultures were terminated and processed for cytogenetic analysis.

Hematopoietic colony assays. BFU-E and CFU-C were assayed as described with minor modifications. BFU-E were plucked for cytogenetic analysis as early as day 11 and CFU-C on day 7, in order to make sure that a significant number of proliferating cells was present.

T-lymphocyte colony assay. A modification of an assay developed by Aye and Dunne was used. Ficoll-separated peripheral blood cells (final concentration 5 × 10⁹ cells/mL) were plated in 35-mm Petri dishes containing 15% autologous plasma, 1% bovine serum albumin, 0.1% PHA (Wellcome, Dartford, England) 5% PHA-LCM, 10 µg/mL 12-0-tetradecanoylphorbol-13-acetate (TPA) (Sigma, St Louis), and 0.3% agar in Iscove’s modified Dulbecco medium (IMDM). In some experiments, 10 U of recombinant interleukin 2 (Biogen, Geneva) were added. After seven days of culture at 37°C in a humidified atmosphere of 5% CO₂ in air, aggregates containing more than 40 cells were counted as colonies.

With this assay more than 95% of the colonies develop in close vicinity to the agar surface and can easily be harvested by thorough floating of the agar surface with phosphate-buffered saline (PBS). Ninety-four percent of the cells derived from colonies grown from the patient's blood stained positive with the pan T-cell monoclonal antibody Leu-4 (Becton Dickinson, Mountain View, CA) but lacked surface markers known to be present on myeloid, monocytic, and B cells.

Cytogenetic studies. Direct preparations of bone marrow cells and of unseparated peripheral blood cells stimulated with PHA were stained with Giemsa, and R- and Q-banded as described previously.

For chromosome analyses of the passaged stromal cells grown from LTC one of the following methods was used. If the adherent cell layer was thin with numerous gaps between individual cells, the chromosome preparation was performed in the culture dish by adding Colcemid (Demecolcin; CIBA, Wehr, FRG) (final concentration 0.06 µg/mL) directly to the long-term medium. After one hour, all of the medium was removed and the adherent cells were washed twice with 0.075 mol/L KCl, 4 mL of which were left in the dish for 20 minutes. The hypotonic solution was replaced with fixative (3:1 methanol:acetic acid) and finally removed. After two

Fig 1. Acid phosphatase reaction of the patient’s blast cells at diagnosis (A) and at the time of last relapse (day +1485 post BMT) (B).
washes with fixative the culture dishes, with the passed stromal cells remaining in situ, were gently dried, followed by cytogenetic analysis.

If the cells forming the passed stromal cell layer had grown to confluence before chromosomal analysis could be undertaken, Colcemid was added to the culture dish as described, but the layer was then rinsed with PBS, trypsinized,\(^1\) and handled as described.\(^2\) Suitable metaphases were G- or Q-banded according to published methods.

BFU-E were prepared by adding Colcemid (final concentration 0.045 μg/mL) to the culture dish for one hour at 37°C. Individual BFU-E were plucked, put into tubes containing 0.1 mL 0.075 mol/L KCl for resuspension, transferred onto polylysine-coated microscope slides\(^3\) and kept in hypotonic solution for 35 minutes. Fixative was then dropped onto the area where the colony had been placed and the slide was air-dried and stained as described. Only single colonies in which two or more metaphases could be analyzed were accepted.

CFU-C and T-lymphocyte colonies were treated similarly except for chromosome analysis, which proved necessary to pool colonies, in order to obtain a sufficient number of mitoses.

*HTLV-I antibodies.* Sera were tested for the presence of antibodies against the human T-lymphotropic virus type I (HTLV-I) by enzyme-linked immunosorbent assay (ELISA)\(^4\) and Western blot analysis. For the Western blot, HTLV-I was used as antigen that previously had been concentrated from cultured medium supernatant of MT-2 cells. The virus preparation was separated on SDS-PAGE and blotted to nitrocellulose. The blot was saturated with 1.5% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.05% gelatin (Cohen—buffer) for two hours at room temperature. Diluted sera (1:50 to 1:200 in PBS) were incubated overnight, washed, and then incubated with antihuman IgG (1:1000), conjugated to peroxidase, and reacted with diaminobenzidine.

RESULTS

Chromosome analyses performed on the patient’s bone marrow and the donor’s lymphocytes before BMT had shown normal female and male karyotypes, respectively. Thirteen and 605 days after BMT, cytogenetic studies on the patient’s marrow or peripheral blood cells revealed a male pattern with no abnormalities.

Attempts to investigate the chromosomes at the time of first relapse after BMT (day +878) were unsuccessful because no mitoses were obtained either with or without the methotrexate synchronization technique. On day +1039, when a remission had been induced but reappearing clusters of immature lymphoid cells within an otherwise normal marrow suggested the resurgence of leukemia, 40 of 41 mitoses were male (46,XY); one metaphase lacked one of the sex chromosomes and one chromosome 7(44,X,—7).

The results of detailed cytogenetic analyses done at the time of third relapse (second relapse after BMT) (day +1485) and the following remission (day +1557) are summarized in Table 1.

At relapse, 17 of 20 marrow cells analyzed after direct preparation were male. In two of these, the long arm of chromosome 6 was partially missing, the karyotype being 46,XY, del(6)(q23q25) (Fig 2). A more detailed view of both chromosomes 6 demonstrating the interstitial deletion of one of the chromosomes is given in Fig 3. The same abnormality was also found in metaphases obtained from PHA-stimulated peripheral blood cells (two mitoses), T-cell colonies, and passaged adherent layer cells (one mitosis each). A second new clone characterized by a loss of one of the sex chromosomes in combination with several other abnormalities including a 6q— chromosome (45,X,—4, del(6)(q23q25), +8, —15, —21, +i(21q), +mar) was found in the remaining three metaphases obtained after direct marrow preparation. Because recipient and donor had no differing fluorescent polymorphisms, we were unable to decide if this clone was host- or donor-derived. The five mitoses found after stimulation of peripheral blood cells with PHA and the vast majority of karyotypes originating from T-cell colonies were male (66 of 69 mitoses), three mitoses were female. On the contrary, 17 of 19 metaphases from the passaged adherent cell layer were of female origin, two mitoses were male, with one of these bearing the 6q— anomaly.

After a remission had been achieved, exclusively normal male karyotypes were found in the myeloid and the lymphoid compartment. The marrow stroma showed fourteen host metaphases; two mitoses were of donor origin.

Both patient and donor lacked antibodies against HTLV-I, as judged by ELISA and Western blot analysis.

DISCUSSION

A deletion of the long arm of chromosome 6 was first described in 1976 as a specific structural anomaly found in the leukemic cells of patients with ALL.\(^2\) This initial report was confirmed by the Third International Workshop on Chromosomes in Leukemia\(^2\) where 6% of 216 cases of ALL carrying any chromosome abnormalities exhibited a 6q—chromosome. At least 25 cases have now been reported,\(^26\)-\(^29\) the majority of which seemed to belong to the non-T cell non-B cell subgroup of ALL with isolated cases being of T cell origin.

Table 1. Results of Chromosome Studies Performed at Second Relapse After BMT (Day +1485) and in Remission (Day +1557), Grouped According to the Origin of Cells Taken for Cytogenetic Analysis

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Direct Bone Marrow</th>
<th>PHA-stimulated Peripheral Blood</th>
<th>T-Cell Colonies</th>
<th>BFU-E</th>
<th>CFU-C</th>
<th>Passaged Adherent Layer From LTC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relapse</td>
<td>Remission</td>
<td>Relapse</td>
<td>Remission</td>
<td>Relapse</td>
<td>Remission</td>
</tr>
<tr>
<td>46,XY</td>
<td>15</td>
<td>33</td>
<td>3</td>
<td>56</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>46,XY,6q—</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45,X,6q—</td>
<td>3</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>46,XX</td>
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<td>3</td>
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*Abbreviation: LTC, long-term cultures.
*Full karyotype: 45,X,—4,del(6)(q23q25), +8, —15, —21, +i(21q), +mar.
†Eleven mitoses of female origin carried nonclonal abnormalities.
In the present case the discovery of an interstitial deletion of the long arm of chromosome 6 in male metaphases adds substantial evidence that the malignant transformation had actually occurred in donor cells. As the mitotic activity of various hematopoietic cell lineages after direct preparation of marrow cells may vary greatly according to the culture conditions in use, one cannot exclude the possibility that the demonstration of a normal karyotype in a leukemic marrow merely indicates that only members of the residual normal hematopoiesis have been analyzed while the leukemic cells went undetected because of their blocked mitotic activity. This could also have happened in cases where normal donor metaphases were found at relapse after BMT, and the donor cells might then have represented the residual normal population originating from the marrow graft, while the leukemic clone—possibly of host origin—was missed. The detection of a disease-specific chromosome abnormality can avoid such ambiguities and gives more direct proof that donor cells were involved in the leukemic process.

On the other hand the demonstration of the 6q- anomaly supports the diagnosis of ALL at the time of last relapse. The absence of the typical acid phosphatase reaction at that time, however, when compared with the unequivocal staining pattern of blast cells at diagnosis casts some doubt as to whether it really was the original leukemia that recurred after BMT. Indeed, for the only additional case of late donor cell leukemia reported so far, Witherspoon et al presented evidence that the leukemia occurring six years postgrafting arose from a cell that was different from that of the original leukemia. The long latent period between transplantation and expression of the leukemic transformation after BMT would also support their view that other than the previously discussed mechanisms might be responsible for the development of leukemia in donor cells late after allogeneic BMT. For example, it has been proposed that more than one oncogene may be required for the ultimate transformation.

Regarding the mechanisms of relapse in donor cells after BMT, two aspects of our case deserve further comment. First, the oncogene c-myb, probably coding for a protein that is important in the early differentiation steps of hematopoietic cells, has been localized to the long arm of chromosome 6 within bands q22-24, which is in the region of the breakpoints found in most cases of ALL associated with a 6q- chromosome. What particular implications, if any, this observation might have in our case is not clear. Second, adult T-cell leukemia, which is endemic in southwestern Japan but sporadically found in other parts of the world, seems correlated to abnormalities of chromosome 6. More than half of 30 Japanese patients carrying high titers of HTLV-I antibodies exhibited a 6q- chromosome. As both leukemic
clones found in our patient showed the 6q− anomaly and blast cells were T cell derived, at least before BMT, it seemed reasonable to test for antibodies against HTLV-I. Both recipient and donor were negative for HTLV-I antibodies, however, and thus we cannot explain the relapse in donor cells as a result of transmission of virus particles from recipient to donor tissue.

Little is known about the kinetics of relapse after BMT and in particular how different lymphohematopoietic lineages and the marrow stroma are involved. Using chromosomal studies of direct marrow preparations and mitogen-stimulated blood cultures, Lawler et al defined various chimeric states related to leukemic relapse after grafting. The pattern they reported in patients with relapsed ALL was the coexistence of recipient and donor metaphases in the marrow while lymphoid cells from the peripheral blood remained of donor type. Our case fits this description in its lymphoid parts, while the fact that all dividing cells found after direct marrow preparation were of donor origin would appear a rare exception to the rule. Incomplete chimerism in cells from T-cell colonies obviously is not infrequent, as we observed this phenomenon in three of 12 patients early after BMT (manuscript in preparation).

The origin of the marrow stroma after BMT is still controversial. While several investigators have demonstrated that marrow fibroblasts remain host-derived, Keating et al reported that cells from the adherent layer of long-term cultures became progressively donor in origin with increasing time after grafting. This is in contrast to the findings in our patient (Table 1). The only explanation we have for this discrepancy is that Keating et al analyzed cells from the original adherent layer, while we used passaged stromal elements for cytogenetic studies. It is well known that the adherent layer of human LTC contains a substantial number of hematopoietic progenitors. In line with these findings we were able to grow CFU-C (45 colonies per 10^6 mononuclear cells) from the patient's original adherent layer cells after culture of her remission marrow. Although colony-forming cells were no longer obtained from the adherent layer cells after a single passage, we believe that the few donor mitoses found after cytogenetic analysis of the first-passage stromal elements reflect the persistence of hematopoietic cells of the donor within a host-type marrow stroma rather than the existence of an incomplete chimerism within this compartment.

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REFERENCES

Recurrence of acute lymphoblastic leukemia in donor cells after allogeneic marrow transplantation associated with a deletion of the long arm of chromosome 6

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