Characterization of Mixed Chimerism in Patients With Chronic Myeloid Leukemia Transplanted With T-Cell–Depleted Bone Marrow: Involvement of Different Hematologic Lineages Before and After Relapse

By Etienne Roux, Kaveh Abdi, Daniel Speiser, Claudine Helg, Bernard Chapuis, Michel Jeannet, and Eddy Roosnek

We have characterized mixed chimerism (MC) in five patients with chronic myeloid leukemia (CML) who received transplants with T-cell–depleted bone marrow (BM) and who relapsed within 4 years after transplantation. To study the possible relation of MC with relapse, we purified different populations of leukocytes and analyzed their donor/receptor origin by a method based on polymerase chain reaction amplification of minisatellite DNA regions. Our results show that before relapse, all hematopoietic recipient cells are T cells, whereas monocytes, B, and natural killer (NK) cells are of donor origin. This observation does not appear to be specific for CML as similar results were found in two control patients with acute myeloid leukemia (AML). At the time of (CML) relapse, recipient granulocytes, monocytes, and erythrocytes appeared and progressively replaced the respective lineages of donor origin. No other lineages seemed to be involved as B cells and NK cells remained of donor origin and no significant changes in the number of recipient T cells were detected. In this respect relapse of CML after BM transplantation (BMT) seems not to be very different from the primary disease in chronic phase before transplantation. Furthermore, we conclude that after BMT, an association between mixed chimerism before relapse and the (CML) relapse does exist because both phenomena are consequences of T-cell depletion of the BM graft. However, this correlation might well be indirect as the MC caused by the recipient T cells appears to be independent of the one caused by the recurrent disease.

We have previously shown that in chronic myeloid leukemia (CML) patients who received a T-cell–depleted BM, before relapse, host hematopoietic cells are detected essentially in the mononuclear cell fraction. However, at the time of hematologic relapse granulocytes change to host type while the mononuclear cells remain of mixed origin. To characterize this phenomenon further, we have determined the evolution of MC in monocytes, T, B, and natural killer (NK) cells in five CML patients who relapsed within 4 years after transplantation.

We show that in CML patients after BMT, MC after relapse is mainly caused by reappearance of malignant host-derived granulocytes, monocytes, and erythrocytes. Furthermore, when patients receive transplants with a T-cell–depleted BM, an MC caused by persisting healthy host T cells can occur before relapse.

MATERIALS AND METHODS

Patients. Seven patients treated for acute myeloid leukemia (AML) or CML were transplanted with a Campath-1Mi5 T-cell-depleted BM from an HLA-identical sibling. Diagnosis and clinical outcome are listed in Table 1.

Preconditioning. Total body irradiation consisted of 2 daily fractions of 200 cGy for 3 days. The lymphoid irradiation (including the spleen) was composed of 2 daily fractions of 150 cGy for 2 days. Chemotherapy and further preconditioning are described in Table 1.

Cell samples. Cytogenetic analysis was performed on BM samples. All other analyses were performed on peripheral blood after ficoll (Pharmacia, Uppsala, Sweden) separation of the mononuclear cells from the granulocytes. Days of the (blood) sampling point BMT were: 31 (BM), 45, 73, 87, 109, 185, 410 (BM), and 432 for unique patient number (UPN) 1; 94, 327, 360 (BM), 747, 900, 1,040, and 1,119 for UPN 10; 52, 271, 733, 910, 1,297 (BM), 1,344, and 1,731 for UPN 21; 53, 185, 361 (BM), 419, 551 (BM), 741, 936, and 1,196 for UPN 22; and 78, 101, 119, 182, 419, and 953 for UPN 23.

Analysis of cells before separation. All mononuclear cells were analyzed for expression of CD4 or CD8 (T cells), CD14 (monocytes), CD16 (NK cells), and CD19 (B cells). To assure that in our BM-transplanted CML patients, as in normal individuals, these cell surface markers could be considered as lineage specific and that no other important cell population was ignored, the following controls were included in our fluorescence-activated cell sorter (FACS) analysis: (1) Of all samples the percentage of cells expressing at least one of these markers was determined by staining with a combination of all antibodies specific for the respective cell surface markers. This always resulted in staining over 90% of the cells. (2) CD4+ and CD8+ cells were analyzed for coexpression of CD3 by double immunofluorescence. Except for a low percentage of CD3+/CD8+ cells, all CD4+ and CD8+ expressed the CD3 molecule. Furthermore, the percentage of CD3+/CD4+ cells always was below five. (3) For at least one sample after relapse, double fluorescence was performed to exclude coexpression of more than one of the lineage-specific markers on leukemic cells. No coexpression of CD3, CD14, CD16, or CD19 was detected.

Red blood cell (RBC) phenotyping was performed according to standard techniques used for the determination of blood groups.

From the Division of Immunology and the Division of Onco-Hematology, Hôpital Cantonal Universitaire, Geneva, Switzerland. Submitted June 8, 1992; accepted August 27, 1992.

Address reprint requests to Eddy Roosnek, PhD, Unité d’Immunologie de Transplantation, Hôpital Cantonal Universitaire, 24, rue Michel-du-Crest CH-1211 Genève 4, Switzerland.

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Purification of blood cell subpopulation. At least $8 \times 10^6$ cryopreserved cells were used to purify the different subpopulations. Whenever after relapse CD14$^+$ cells were very abundant (>70%), these cells were depleted by antibody and complement treatment before separation of the other lineages. For the purification of CD14$^+$ these cells were depleted by antibody and complement treatment (CD16$, \text{UPN } 21 \text{ time point } 4 \text{ and } \text{CD16}^+/\text{CD19}^+$ cells UPN 23) either coupled with the specific antibody (CD4, Dynal No: 111.07; CD19, Dynal No: 11006). Separation procedures were performed according to the manufacturer’s protocol. Two samples (CD16$, \text{UPN } 21 \text{ time point } 4 \text{ and } \text{CD16}^+/\text{CD19}^+$ cells UPN 23) were purified by FACS (FACSTAR PLUS; Becton Dickinson, Mountain View, CA) after being stained with anti-CD16 (mouse-IgG) and/or anti-CD19 (mouse-IgG$_2a$) developed with subclass specific antisera (Southern Biotechnologies, Birmingham, AL).

Exclusion of samples from analysis. Results were excluded from analysis (see Figs 2 and 3) for one of the following reasons: (1) a subpopulation represented less than 2% of the mononuclear cells (UPN 10, CD16: samples 4, 5; UPN 22, CD19: sample 1); (2) the purified sample contained more than 5% of free cells and/or a significant part of cells was attached to one magnetic bead only (UPN 1, CD19: sample 5; UPN 10, CD16: sample 2, 3, CD19: sample 5; UPN 21, CD16: sample 1, CD19: sample 1); (3) none or very little DNA was obtained after polymerase chain reaction (PCR) amplification (UPN 1, CD8: sample 1, CD16: sample 1, CD19: sample 1; UPN 8, CD16: sample 1, CD19: sample 1; UPN 9, CD19: sample 1; UPN 10, CD19: sample 1; UPN 23, CD4: sample 1).

Preparation of DNA and PCR analysis. To remove DNA from cells other than those attached to the beads, samples were DNAase I treated (Boehringer, Mannheim, Germany; 10 minutes room temperature, 500 U/mL) and washed three times before DNA extraction. High molecular weight DNA was extracted directly from cells attached to the magnetic beads and analyzed by PCR as described previously.14 In short, DNA was isolated from nuclei following Triton X-100 (Fluka, Buchs, Switzerland) solubilization. Amplifiers for 33.1, YNZ22, and ApoB VNTR have been described.7-19 Incubation mixtures were cycled 28 times as follows for ApoB amplifiers: 50 seconds 95°C, 50 seconds 60°C, 60 seconds 72°C. Amplification products were digested with S1 nuclease19 and separated on 1% agarose gels. Southern blotting was performed on nylon membranes (ApoB and YNZ22) or dried gels (33.1). Precautions taken to avoid contaminants were as follows: amplification was always performed with at least two R/D pairs presenting distinguishable patterns as well as with pretransplant DNA.

MC was evaluated by gel scanning of the autoradiograms. A calibration curve of mixtures of pretransplant DNAs was included in the quantification protocol to take into account the size-dependent efficacy of PCR amplification.

RESULTS

MC of different lineages. We have analyzed MC in the different lineages of the mononuclear cell fractions of seven patients who were transplanted for leukemia (for details see Table 1). Cells were separated on basis of expression of CD4 or CD8 (T cells), CD14 (monocytes), CD16 (NK cells), and CD19 (B cells). These markers are lineage specific and are expressed by more than 90% of the mononuclear cells before as well as after relapse (for detailed controls see Materials and Methods). After the cell separation procedure, the donor/recipient origin of the cells was determined by PCR amplification of minisatellite DNA regions, a method that enables one to discriminate between almost every donor/recipient pair, even when only a very small number of cells are available.14,17,20,21

Figure 1 shows the three distinct patterns of chimeraism that can be observed in different cell lineages of one patient. First, (T) cells can be of donor as well as of recipient type, resulting in a stable MC that can persist for more than 3 years after transplantation (Fig 1B). Secondly, cells can be of donor origin only and remain so even after cytogenetic relapse (Fig 1C). Finally, cells can be of donor origin before relapse, of mixed origin early during relapse, and of recipient origin after relapse (Fig 1D).

MC before relapse. Figure 2 shows the analysis of MC in the different mononuclear cell lineages of all five CML patients studied. Before cytogenetic relapse (indicated by the arrows) all recipient cells are confined to the T-cell lineage. Both CD4$^+$ as well as CD8$^+$ cells are of mixed origin and, although the number of recipient T cells may differ significantly between patients (compare UPN 21, UPN 23, and UPN 1 with UPN 10 and UPN 22), the number of host CD4$^+$ and CD8$^+$ cells in one individual remains more or less constant. In contrast, CD16$^+$, CD19$^+$, and CD14$^+$ cells are of donor origin only. Similar results were obtained in two AML patients (Fig 3). Again, host cells were restricted to the CD4 and CD8 subtypes suggesting that our findings are not unique to CML.

MC during the relapse. Except for the interval between transplantation and relapse which in our study varies between 3 months and 4 years, all five patients show a very similar
pattern of relapse. At the time of cytogenetic relapse the first recipient monocytes are detected in the blood. Then, while the total number of monocytes remains more or less constant, recipient monocytes begin to replace the donor cells of the same lineage. After a period of 6 months to 2 years, the monocytes are 90% to 100% of recipient type (Fig 1D; Fig 2). At that time, with the exception of UPN 21, the total number of monocytes has increased 2- to 10-fold and form
the major mononuclear cell lineage. Remarkably, no important changes in the origin of the other mononuclear cell lineages are observed. Up to more than 1 year after the cytogenetic relapse, T cells remain of mixed origin, while B cells and NK cells are of donor type (Fig 2).

**Analysis of granulocytes, erythrocytes, and BM Philadelphia-positive metaphases.** Table 2 compares the donor/recipient origin of granulocytes and erythrocytes with the origin of monocytes and the number of Ph+ metaphases in the BM. Although the data are not complete enough to justify a final conclusion as granulocyte data before relapse are lacking, they strongly suggest that both the granulocyte and the erythrocyte lineage are implicated in CML relapse in the same manner as monocytes are. At the time when in the BM the Ph+ metaphases are predominant, all three lineages have become of recipient origin. When some Ph+ metaphases are still present, some donor monocytes (UPN 21, UPN 22) and donor granulocytes (UPN 21) can be detected.

**DISCUSSION**

After BMT, when the patient’s hematopoietic system has been reconstituted by the donor marrow, recipient hematopoietic cells sometimes persist. Especially when the donor marrow has been depleted of mature T cells,\(^6,14,22-24\) this can be a common finding. This presence of hematopoietic cells of donor as well as of recipient origin, defined as MC has been described under many different circumstances.\(^1,14,23,25,26\) The parameters that cause this state of MC and its possible clinical consequences, however, have remained a matter of debate. One reason for this might be that the definition of MC has always been a very broad one and very few studies present enough similarly defined cases of MC to allow a good comparison. For instance, patients have been reported to be “mixed chimeras” at the moment of appearance of the first donor marrow derived erythrocytes some weeks after transplantation.\(^11\) Other investigators\(^17\) use the term “mixed chimeras” for patients who relapse after transplantation and whose recipient cells probably reflect the recurring leukemia. Furthermore, many different techniques (with different sensitivities) have been used analyzing different cell types in patients who were treated with different preconditioning regimens.\(^1,13,23,25-28\)

We have previously described the occurrence of MC in a group of 20 patients who had undergone BMT as a treatment for leukemia. All patients had received an antileukemia chemotherapy followed by a transplantation preconditioning that included total body irradiation. In addition, the Campath-1M antibody had been used as the T-cell–depletion method, which consistently leads to an effective depletion \((2.1 \pm 0.8 \text{ log for the seven patients described in this report})\) and con-

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**Table 2. Origin of Monocytes, Granulocytes, Erythrocytes, and Percent of Ph+ Metaphases After BMT**

<table>
<thead>
<tr>
<th>UPN</th>
<th>Days pBMT</th>
<th>Monocytes</th>
<th>Granulocytes</th>
<th>Erythrocytes</th>
<th>Ph+ Metaphases</th>
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<tbody>
<tr>
<td>21</td>
<td>52</td>
<td>0</td>
<td>D</td>
<td>0</td>
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<td>0</td>
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<tr>
<td></td>
<td>733</td>
<td>0</td>
<td>D (910)</td>
<td>0 (910)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1,344(^*)</td>
<td>90</td>
<td>80(^t) (1,297)(^t)</td>
<td>R (1,731)</td>
<td>70 (1,317)</td>
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<tr>
<td>22</td>
<td>53</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
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<td>185</td>
<td>0</td>
<td>0 (361)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>419</td>
<td>0</td>
<td>ND</td>
<td>Ni</td>
<td>12 (551)</td>
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<td></td>
<td>741</td>
<td>40</td>
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<td></td>
<td>936</td>
<td>70</td>
<td>90</td>
<td>66</td>
<td></td>
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<td>100</td>
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<td>87</td>
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<td>109</td>
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<td>188</td>
<td>40</td>
<td>60</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>432</td>
<td>100</td>
<td>R</td>
<td>100 (410)</td>
<td></td>
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</table>

Abbreviations: D, donor; R, recipient; Ni, not informative; ND, not done.
\(^*\) Blood samples drawn after relapse are underlined.
\(^t\) Expressed as percentage of host cells.
\(^t\) Whenever for this particular analysis a blood sample was used drawn at a day that differed more than 1 week from the day mentioned in the column “Days pBMT,” the day of blood sampling is mentioned in parentheses.
We do not favor such a hypothesis for two different reasons: that because of their abundance before chemotherapy, lymphoid cells remain normal unless a lymphoid blast crisis occurs. Therefore, the capacity to partially repopulate the peripheral circuit arose. Whenever a conditioning regimen is used that ablates all recipient BM activity, the leukocytes of recipient’s origin can only be T cells as repopulation by other lineages is dependent on recruitment from the BM.

At the time of relapse, the percentage of MC will increase as in addition to the recipient T cells present, leukemic cells reappear. Which type of cell lineages will be involved is dependent on the type of leukemia. We think that for CML, the evolution of relapse after BMT is not so much different from the development of the disease before transplantation. Like in the chronic phase of the primary disease, where at the beginning only the myeloid and erythroid lineages are leukemic,13-15 at the time of relapse after BMT only these lineages will be implicated and hence change to cells of patient origin. In contrast, except for the recipient T cells that were already present, lymphocytes will remain of donor origin also after relapse. This reflects the situation during a chronic phase, where lymphoid cells remain normal unless a lymphoid blast crisis occurs.

An alternative explanation for the fact that relapse occurs preferentially in myeloid and erythroid lineages would be that because of their abundance before chemotherapy, leukemic cells of these lineages have a higher chance to survive. We do not favor such a hypothesis for two different reasons: Firstly, all five patients show exactly the same pattern of relapse despite considerable differences in the time between BMT and relapse. Secondly, one patient (UPN 10), who received a transplant in first chronic phase and therefore had no lymphoid leukemic cells before transplantation, suffered from a lymphoid blast crisis 3½ years after BMT (4 months after the last sample analyzed in Fig 2). This shows that at least in one case the relapse must have been caused by a Ph+ pluripotent stem cell that had survived treatment.

We do not know whether our findings are representative for all CML relapses in patients transplanted in chronic phase. However, if relapses after BMT would always affect monocytes, granulocytes, and erythrocytes rather than one lineage only, it would indicate that Ph+ stem cells are much less therapy sensitive than more mature, already lineage committed leukemic precursors.

This could explain why, after BMT, CML patients relapse more frequently and why a graft-versus-leukemia effect (GVL) of the BM graft is essential for leukemia-free survival.26 One could argue that the persistence of recipient T cells after transplantation reflects full tolerance of the graft toward the host and therefore indicates the absence of such a GVL. Therefore, although in itself independent, MC before relapse and the relapse itself will often be associated and its evolution in the first months post-BMT might be a way to evaluate the risk of a subsequent relapse.

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