Polyclonal Hematopoietic Reconstitution in Leukemia Patients at Remission After Suppression of Specific Gene Rearrangements

By Francesco Lo Coco, Pier Giuseppe Pelicci, Francesca D’Adamo, Daniela Diverio, Giuliana Alimena, Enrico Montefusco, William Arcese, Giuseppe Avvisati, Lidia De Felice, Giovanna Meloni, Clara Nervi, Franco Mandelli, and Giuseppe Saglio

Clonality studies of hematopoietic reconstitution after remission were performed in 24 female patients (pts) with leukemias characterized by specific molecular markers. At diagnosis, 13 pts had promyelocytic leukemia (PML) retinoic acid receptor-α (RAR-α)-rearranged acute promyelocytic leukemia (APL), 8 Philadelphia positive (Ph+) breakpoint cluster region (BCR+), chronic myeloid leukemia (CML), and 3 Ph+ (BCR+) acute lymphoblastic leukemia (ALL). All pts were analyzed at presentation and after Southern blot suppression of specific rearrangements after various treatments, including conventional chemotherapy, autologous or allogeneic bone marrow transplant (BMT), all-trans retinoic acid, and α-2b interferon. DNA from BM samples collected at diagnosis and, during remission phases, were subjected to Southern blot analysis with the M27α probe to detect X chromosome methylation differences, and with BCR, in CML and ALL cases, or PML/RARA probes for gene rearrangements, in APL cases. Twenty-one of the 24 pts had polyclonal methylation patterns at the level of stem cell involvement in hematologic disorders like CML and other myeloproliferative syndromes, acute myeloid leukemia (AML), and myelodysplastic syndromes.1,2

Most human tumors are monoclonal proliferations originating from a single transformed cell, and consistent genetic defects with potential transforming capability have been identified in a variety of neoplastic diseases.3-5 Among hematologic malignancies, significant examples are the BCR/ABL molecular rearrangement of Philadelphia positive (Ph+) chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL)6 and the more recently reported fusion of promyelocytic leukemia (PML) and retinoic acid receptor-α (RAR-α) genes in the t(15;17) translocation of acute promyelocytic leukemia (APL).7,8 Besides their pathogenetic relevance, such rearrangements provide ideal clonal markers of leukemic cells to be exploited for both diagnosis and monitoring of these diseases.9-14

Studies on glucose 6-phosphate dehydrogenase (G6PD) isoenzyme expression15 and of methylation differences at X-linked DNA polymorphic loci16 in heterogeneous females also provide methods to assess clonality and to determine the level of stem cell involvement in hematologic disorders like CML and other myeloproliferative syndromes, acute myeloid leukemia (AML), and myelodysplastic syndromes.15-20

Surprisingly, using the latter methods, several groups have reported that clonal hematopoiesis may also occur in AML patients during the remission phase induced by chemotherapy.21-24 The persistence of clonal hematopoiesis during apparent complete remission has been interpreted according to the theory of a multistep pathogenesis process in human leukemia. In this context, a preleukemic clone maintaining a normal differentiation ability could survive treatment and proliferate once overt leukemia is eliminated.21-24 Alternatively, clonal remissions could be the consequence of the regenerative activity of a single normal precursor cell spared by chemotherapy. However, very little is known about the clinical and prognostic significance of clonal remission in leukemia.24

To address these issues, we decided to investigate the nature of remission after treatment in a series of female patients with Ph+ + CML, Ph+ + ALL, and t(15;17) APL, which were found to be heterogeneous at the X-linked DXS255 locus and who achieved suppression at Southern analysis of specific breakpoint cluster region (BCR)/ABL or PML/RAR-α rearrangements.

MATERIALS AND METHODS

Patient selection. Twenty-four patients (pts), including 13 APL, 8 Ph+ + CML, and 3 Ph+ + ALL cases, were studied. Selection was based on the following criteria: (1) female sex; (2) presence of a specific cytogenetic and/or molecular marker at diagnosis in greater than 90% of leukemic cells; (3) response to treatment with cytogenetic and/or molecular remission, ie, complete cytogenetic conversion to normal karyotype and/or disappearance at Southern blot of the specific gene rearrangement detected at diagnosis; and (4) heterozygosity at the DXS255 locus on the X chromosome.
Therapy. Individual treatments for each of the 24 pts are reported in Table 1. Briefly, 8 pts with APL received as induction therapy conventional chemotherapy (CHT) schedules including an anthracycline (daunorubicin or idarubicin) alone or in combination with cytosine arabinoside (7 + 3 regimen). Following achievement of complete remission (CR), 7 pts received CHT for consolidation and maintenance, and 1 pt received high-dose CHT followed by autologous BMT. Five APL pts received all-trans retinoic acid (ATRA) at oral doses of 45 to 50 mg/m² until the achievement of CR. Of this group, 4 pts received as consolidation conventional CHT and 1 underwent high-dose CHT and autologous BMT.

All pts with CML were initially treated with α-2b interferon (IFN) (Hoffman-La Roche, Nutley, NJ) according to reported protocols for at least 12 months. After hematologic response and partial karyotypic conversion (15% to 75% Ph+ BM metaphases) after 1 year, 3 pts underwent BM harvesting and autologous BMT, whereas the 5 remaining pts achieved a complete Ph+ suppression and were maintained on IFN therapy. Finally, the 3 pts with Ph+ ALL were treated with CHT according to the GIMEMA 0288 protocol and, after CR, received allogeneic BMT from HLA-identical siblings.

DNA samples. DNA was obtained from BM aspirates at diag-

Table 1. DXS255 Methylation Patterns at Diagnosis and at Remission Following Suppression of Specific Rearrangements

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Disease</th>
<th>Methyl Allele at Diagnosis</th>
<th>Therapy</th>
<th>Time of Sampling (from CR*)</th>
<th>Source of DNA</th>
<th>Methylated Allele(s)</th>
<th>Follow-Up</th>
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<tr>
<td>1</td>
<td>13</td>
<td>APL</td>
<td>A</td>
<td>CHT</td>
<td>5 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 15+ m</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>APL</td>
<td>B</td>
<td>ATRA/CHT</td>
<td>7 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 10+ m</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>APL</td>
<td>A</td>
<td>ATRA/CHT</td>
<td>1 mo</td>
<td>BM</td>
<td>A, B</td>
<td>Rel. (5 m) dead</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>APL</td>
<td>B</td>
<td>CHT</td>
<td>1 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 17+ m</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>APL</td>
<td>A</td>
<td>CHT</td>
<td>5 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 22+ m</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>APL</td>
<td>B</td>
<td>ATRA/CHT</td>
<td>4 mo</td>
<td>BM</td>
<td>A, B</td>
<td>Rel. (6 m) dead</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>APL</td>
<td>A</td>
<td>CHT</td>
<td>5 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 20+ m</td>
</tr>
<tr>
<td>8</td>
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<td>A</td>
<td>CHT</td>
<td>4 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 6+ m</td>
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<td>9</td>
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<td>B</td>
<td>CHT</td>
<td>8 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 30+ m</td>
</tr>
<tr>
<td>10</td>
<td>29</td>
<td>APL</td>
<td>A</td>
<td>Auto BMT</td>
<td>44 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 63+ m</td>
</tr>
<tr>
<td>11</td>
<td>22</td>
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<td>A</td>
<td>CHT</td>
<td>11 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 29+ m</td>
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<tr>
<td>12</td>
<td>51</td>
<td>APL</td>
<td>B</td>
<td>ATRA/Auto BMT</td>
<td>2 mo</td>
<td>BM</td>
<td>A, B</td>
<td>4 mo</td>
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<tr>
<td>13</td>
<td>63</td>
<td>APL</td>
<td>A</td>
<td>ATRA/CHT</td>
<td>15 mo</td>
<td>BM</td>
<td>A</td>
<td>15 mo, T cells A</td>
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<tr>
<td>14</td>
<td>69</td>
<td>CML</td>
<td>A</td>
<td>IFN</td>
<td>14 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 20+ m</td>
</tr>
<tr>
<td>15</td>
<td>67</td>
<td>CML</td>
<td>A</td>
<td>IFN</td>
<td>53 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 60+ m</td>
</tr>
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<td>16</td>
<td>42</td>
<td>CML</td>
<td>A</td>
<td>IFN</td>
<td>48 mo</td>
<td>PB</td>
<td>A, B</td>
<td>CCR 67+ m</td>
</tr>
<tr>
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<td>CML</td>
<td>B</td>
<td>IFN</td>
<td>39 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 59+ m</td>
</tr>
<tr>
<td>18</td>
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<td>CML</td>
<td>B</td>
<td>IFN</td>
<td>52 mo</td>
<td>BM</td>
<td>A, B</td>
<td>52 mo, T cells A</td>
</tr>
<tr>
<td>19</td>
<td>27</td>
<td>CML</td>
<td>B</td>
<td>IFN/Auto BMT</td>
<td>7 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 63+ m</td>
</tr>
<tr>
<td>20</td>
<td>50</td>
<td>CML</td>
<td>B</td>
<td>IFN/Auto BMT</td>
<td>12 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 20+ m</td>
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<tr>
<td>21</td>
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<td>B</td>
<td>IFN/Auto BMT</td>
<td>28 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 37+ m</td>
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<tr>
<td>22</td>
<td>27</td>
<td>Ph+ ALL</td>
<td>B</td>
<td>CHT</td>
<td>6 mo</td>
<td>BM</td>
<td>B</td>
<td>14 mo</td>
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<tr>
<td>23</td>
<td>41</td>
<td>Ph+ ALL</td>
<td>A</td>
<td>CHT</td>
<td>14 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 21+ m</td>
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</tr>
<tr>
<td>24</td>
<td>33</td>
<td>Ph+ ALL</td>
<td>B</td>
<td>CHT</td>
<td>14 mo</td>
<td>BM</td>
<td>A, B</td>
<td>CCR 34+ m</td>
</tr>
</tbody>
</table>

Abbreviations: PB, peripheral blood; CCR, continuous complete remission; rel, relapse.

* Also from BMT in patients no. 22, 23, and 24.
† Donor polymorphism.
nosis and remission. In 9 cases, additional remission samples were collected during the follow-up and in 2 cases at relapse. A T-cell fraction at a purity of 98% as shown by FACS analysis (Becton Dickinson, Mountain View, CA) was obtained from circulating blood cells in 2 CML pts (no. 18 and 21) and in 1 case of APL (no. 13) after immunomagnetic separation of Ficol isolated mononuclear cells coated with an anti-CD3 antibody under the recommendations specified by the manufacturer (Dynabeads; Unipath, Milan, Italy). A skin biopsy was performed in pt no. 13 in remission. In pt no. 21, a fibroblast culture was established from a BM specimen collected in remission. Briefly, 2 x 10^7 cells were seeded in flasks containing 8 mL of alpha medium and 20% fetal calf serum (FCS). Fibroblasts were grown to confluence, subcultured at intervals of 2 to 3 weeks, and then harvested and used for DNA extraction. Finally, DNA was also obtained from peripheral blood of the female BM donor of pt no. 24. Informed consent was obtained for all these procedures.

**BCR and PML/RAR-α genes rearrangements.** Analysis of the major BCR in CML and ALL pts and of the RAR-α and PML genes in APLs was performed as previously reported. X chromosome methylation analysis. Methylation patterns at the DXS255 polymorphic locus were analyzed in all cases at diagnosis and during remission phases after suppression at Southern blot of the BCR or PML/RAR-α rearrangements detected at diagnosis. DNAs were initially digested with BamHI. Two aliquots of the BamHI-digested DNA were separately digested with either Msp I or its methylation-sensitive isoschizomer Hpa II. After electrophoresis on 0.7% agarose gels, DNA was denatured and blotted onto nitrocellulose filters. Hybridization was performed with the M278 probe, which recognizes the DXS255 locus at cen-p11.2 on chromosome X. A partial restriction map of this locus with location of the M278 probe and variable copy number tandem repeat (VNTR) motif is shown in Fig 1. Filters were washed in 0.1% sodium dodecyl sulfate (SDS) and 2% SSC at room temperature for 15 minutes and subsequently at 65°C for 30 to 40 minutes and exposed for 48 to 72 hours at −80°C using intensifying screens.

**Interpretation of methylation analysis.** Because of the presence of a VNTR sequence flanked by 3' and 5' Msp I sites (Fig 1), maternal and paternal X chromosomes are distinguishable at DXS255 on BamHI/Msp I digestion after hybridization with the M278 probe. Whereas Msp I also cuts its methylated CCGG recognition sequence, the isoschizomer Hpa II cuts this latter only if unmethylated. Based on the random nature of X chromosome inactivation during early embryogenesis, in the presence of a monochromosomal cell population only one of the parental chromosome is methylated and, hence, only one of the parental bands is cut by Hpa II, whereas in polyclonal cells, both parental alleles are in active and inactive states; therefore, both bands are partially cut by Hpa II. BamHI predigestion was performed, as suggested by others, to better resolve higher molecular weight fragments arising in Hpa II digests. To facilitate comparison between diagnostic and remission features, we have conventionally indicated as A and B the higher and lower molecular weight fragments visualized after BamHI/Msp I digestion, corresponding to unmethylated fragments detectable on BamHI/Hpa II lanes (Table 1 and Figs 2 through 4). Autoradiographic signals were quantitated using an Ultrospec XL laser densitometer (LKB). The percentage of A and B bands detected on BamHI/Hpa II digestions was determined and expressed as a ratio.

**RESULTS**

The main clinical and molecular features at diagnosis and remission are shown in Table 1. The results may be summarized as follows.

(1) Methylation analysis at diagnosis showed the presence of a single digested (unmethylated) A or B BamHI/Hpa II fragment in all cases, suggesting clonal origin of the cell population. The same DNA specimens showed specific bands of rearrangements of the BCR or RAR-α/PML genes whose intensity was always identical to that of the germline allele.

(2) Disappearance of the gene rearrangements at remission was associated in all but 3 cases (nos. 13, 21, and 24; Table 1), with an approximate equal proportion of unmethylated A and B alleles, which suggested the restoration of polyclonal hematopoiesis. However, the ratio of A/B bands, as shown by densitometric scanning, varied in some cases from the expected 50:50 proportion, ranging from 1.65 to 0.61 considering all polyclonal cases (in which two A/B bands were visible at remission). Figure 2 shows Southern blot results at diagnosis and remission in 2 representative cases.

(3) In pts analyzed sequentially, polyclonal A/B ratios of DXS255 unmethylated alleles were always maintained during remission in conjunction with suppression of specific gene rearrangements. By contrast, relapses (cases no. 12 and 22, Table 1) were characterized by reappearance of the abnormal BCR or PML/RAR-α fragments and of the same unmethylated DXS255 allele as was present at diagnosis. In no instance did a clonal methylation pattern precede the appearance of specific rearrangements in these cases.

(4) Three of the 24 pts showed the same methylation pattern in remission as that observed at diagnosis, despite Southern blot suppression of the specific leukemic marker (nos. 13, 21, and 24). In the APL pt no. 13, the single unmethylated fragment seen at diagnosis could be detected in two sequential BM samples collected in remission, as well as in DNA from circulating T cells and from cells from a skin biopsy. Pt no. 21 (with CML) showed the same methylation picture at diagnosis and after treatment in four sequential BM specimens collected at 6, 14, 17 and 19 months after autologous BMT. In this pt, the same DXS255 unmethylated fragment was also detected in DNA from peripheral blood (PB) T lymphocytes and BM fibroblasts (Fig 3).
POLYCLONAL HEMATOPOIESIS IN LEUKEMIA REMISSION

Fig 2. Detection of DXS255 methylation patterns and specific rearrangements in a CML patient (left side) and an APL patient (right) at diagnosis and remission. Hybridization with the M27β probe shows in lanes 1 (BamHI plus Msp I digestion) heterozygosity, and in lanes 2 (BamHI plus Hpa II digestion) the assessment of methylation status. A single allele is unmethylated in both cases in diagnostic BM DNA, whereas two BamHI/Hpa II bands of similar intensity are visible in remission DNA. The highest molecular weight bands in lanes 2 arise from digestion at 3' and 5' BamHI sites to DXS255, whereas the additional more faint bands visible in same lanes probably result from digestion at 3' BamHI site and 5' Msp I/Hpa II site, if unmethylated. DNA configuration of the BCR (CML case, left) and PML (APL) gene loci on the same diagnostic and remission DNA is shown below. Allelic abnormal fragments (arrows) are detected at diagnosis, whereas only germline bands are visible in remission controls. Bgl II and HindIII digestions were performed for BCR and RH15 hybridizations, respectively. (For more details on the PML locus gene map and location of the RH15 probe see references 8 and 11.)

In the case of pt 24, who had Ph'+ ALL and underwent allogeneic BMT in first CR, a polyclonal A/B ratio was observed after chemotherapy before transplantation, whereas a single new unmethylated allele of donor origin was documented in two sequential analyses following BMT from a female sibling. This latter picture was compared with the DXS255 methylation status of blood cells from the donor, which showed an identical pattern (Fig 4). These 3 pts (nos. 13, 21, and 24) are presently in continuous unmaintained remission at 20, 22, and 34 months from diagnosis with cytogenetic and Southern blot suppression of their specific leukemic markers.

DISCUSSION

Most current strategies used in the treatment of human leukemia are based on the assumption that normal hematopoietic stem cells, although temporarily suppressed by the tumor, still survive in the BM. The reemergence of morphologically normal hematopoietic cells during remission supports this view. Nevertheless, based on X chromosome inactivation studies, several investigators have questioned the nature of this remission, suggesting that in some cases the persistence of clonal hematopoiesis implies the existence of a preleukemic clone surviving treatment. This clone might antedate, in the pathogenetic process, the occurrence of identifiable gene rearrangements.

In this study, we analyzed methylation patterns at the X linked DXS255 locus in conjunction with PML/RAR-α and BCR gene configuration to assess the nature of hematopoietic reconstitution at remission in APL and Ph'' leukemias. Suppression (at Southern blot) of the clone carrying the specific rearrangement was accompanied by restoration of polyclonal hematopoiesis in 21 of 24 cases, following different treatment approaches (conventional chemotherapy, ATRA, α-2b interferon, and BMT). Claxton et al have reported similar observations in five CML pts who obtained Ph' suppression after interferon therapy. The ap-
Fig 3. Methylation assessment and gene rearrangement in pt no. 21, affected by CML. A single unmethylated A fragment, after BamHI plus Hpa II digestion (lanes 2) is detected by M27β probe on diagnostic and remission BM, blood T cells, and cultured BM fibroblasts. A rearranged BCR band present at diagnosis (arrow) and germline configuration of remission BM are shown below.

Parent clonal pattern in our remaining 3 cases is likely to be attributable to skewed lyonization with preferential methylation of a single allele. Such a phenomenon has recently been described to occur in a significant proportion of normal females. However, we note that the extent of skewed methylation observed in our cases is unusually high if compared with skewing ratios found by these authors. Therefore, another possibility is that an aberrant methylation of the DXS255 region was present in our 3 cases. Unfortunately, because of the lack of heterozygosity at the phosphoglycerate kinase (PGK) and hypoxantine-guanine phosphoribosyl transferase (HPRT) loci, we were unable to have other informative data in these 3 pts.

In our study, we found that pt no. 13 had an identical pattern in remission marrow, in T cells, and in cells from a skin biopsy; in pt no. 21, cultured BM fibroblasts and T lymphocytes showed the same methylation pattern as that detected in the BM at presentation and in several BM specimens at remission (Fig 3). In the third pt (no. 24), the single unmethylated allele found after BMT was identical to that found in PB cells from her healthy donor (Fig 4). Whatever the reasons for the features observed (skewed lyonization or aberrant methylation), we note that comparison with non-malignant tissues is mandatory whenever apparently clonal methylation features are observed. The fact that these 3 pts are free of disease more than 20 months after achievement of CR further supports our interpretation of apparent clonality. After our submission of the present report, Gale et al reported similar data in a consistent series of AML pts. After detection in some cases of skewed lyonization in both T-cells and granulocytes at remission, these authors note that preponderance of a single clone is rather unusual in hematopoietic reconstitution and emphasize the difficulty of using X chromosome inactivation studies for the assessment of clonality. Finally, Gale et al did not observe a distinct prognostic outcome in pts with skewed lyonization with respect to the median overall survival of AML pts. Although these data and our own support claims against the evidence of frequent clonal remission in leukemia, some instances of true clonal hematopoietic reconstitution might have occurred within the cases of previously reported series, particularly when constitutive X-inactivation patterns were also analyzed. However, the clinical significance of this rare event is still obscure, and the possibility also exists that one or only a few normal progenitors spared by chemotherapy had reconstituted hematopoiesis. Finally, it is worth noting that, with the exception of the few CML cases described by Claxton et al, none of the previous studies reported the analysis of leukemias characterized by specific markers.

In conclusion, our data indicate that clonal hematopoietic reconstitution rarely, if ever, occurs in leukemia pts.
while they persist in cytogenetic remission with suppression of specific tumor markers. Moreover, as shown in cases studied sequentially before and during relapse, it appears unlikely that the expansion of a preleukemic and apparently normal clone may precede the reappearance of specific gene rearrangements.

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REFERENCES

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