Polyclonal Hematopoiesis in Interferon-Induced Cytogenetic Remissions of Chronic Myelogenous Leukemia

By David Claxton, Albert Deisseroth, Moshe Talpaz, Christopher Reading, Hagop Kantarjian, Jose Trujillo, Sanford Stass, Grace Gooch, and Gary Spitzer

Interferon (IFN) therapy of early chronic myelogenous leukemia (CML) frequently produces partial or complete cytogenetic remission of the disease. Patients with complete cytogenetic remission often continue on therapy for several years with bone marrow showing only diploid (normal) metaphases. We studied hematopoiesis in five female patients with major cytogenetic remissions from CML during IFN therapy. Clonality analysis using the B23I PGK gene polymorphism showed that granulocytes were nonclonal in all patients during cytogenetic remission. BCR region studies showed rearrangement only in the one patient whose remission was incomplete at the time of sampling. Granulopoiesis is nonclonal in IFN-induced remissions of CML and may be derived from normal hematopoietic stem cells.

These studies show that cytogenetic remissions from the chronic phase of CML resulting from IFN and myelosuppressive therapy are polyclonal and thus may be derived from normal stem cells.

MATERIALS AND METHODS

Patients. All patients showed clinical findings typical of CML and at diagnosis had a predominance of Ph1-positive metaphases in unstimulated bone marrow. Female patients were selected for informativeness for the phosphoglycerate kinase (PGK) Bgl I and B23I polymorphic loci. IFN was administered as described on or according to institutional protocols.

Cytogenetic studies. The chromosome data was obtained from bone marrow aspirates from all of the patients in this study using routine cytogenetic methods. The samples were incubated overnight at 37°C in Ham's F-10 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (Intergen Armour Biochem, Purchase, NY). Harvesting of the cultures and slide preparations was made according to established procedures.

A minimum of 25 well-spread Giemsa-banded metaphases were analyzed from each culture. Karyotypes were prepared on at least two metaphases of each cell type using the International System for Human Cytogenetic Nomenclature (ISN 1985).

Bone marrow and bone DNA samples. Heparinized blood and, in some cases, bone marrow were obtained in the course of routine clinical sampling. Approval was obtained from the Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki. Blood samples were separated by density gradient centrifugation on Ficoll-Hypaque into mononuclear (interface) and granulocyte (pellet) fractions. Pellet fractions were examined by spreading a drop on a slide and staining with Wright's stain (Sigma, St Louis, MO). Reproducibly,

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greater than 85% of nucleated cells were granulocytes (in most cases greater than 90%). The pellet was resuspended in

\[ \text{NIHCl} \]

0.83% wt/vol and incubated at 37°C for 1 to 2 hours to lyse red cells. Genomic DNA was extracted by phenol followed by chloro-
form extractions. Bone marrow and, in some cases, whole blood was pelleted and DNA was extracted for blood fractions.

**T-cell culture.** T cells were expanded from peripheral blood mononuclear cells or bone marrow as follows. Cells (0.5 to 2 x 10^6) were cultured in wells of 24-well plates with starter medium (RPMI 1640) supplemented with phytohemagglutinin (PHA) 1% (M-form; GIBCO) and interleukin-2 (IL-2; recombinant human; Hoffman Laroche, Nutley, NJ) (100 U/mL). At 48 to 72 hours, the cultures were fed with growth medium (identical to starter medium but with PHA only 0.4%). Polyclonal T-cell lines were subsequently expanded by feeding at 24- to 72-hour intervals with growth medium. At days 14 to 21, the cells were counted in trypan blue 0.2%. Characteristically, cell numbers were 15- to 50-fold greater than at day 1 and viability was 80% to 96%. DNA was extracted as above.

**Molecular analysis.** For X-linked clonality analysis, DNA was digested as described by Vogelstein et al.21 Patients were screened for heterozygosity at the PGK locus with Bgl I digestion. Subse-

\[ \text{Bgl I} \]

quently, clonal analysis was performed using Bsr XI.21 For bcr region rearrangements, 10 µg of DNA was digested with Bgl II and Xba I with 8 U of enzyme/µg DNA for 6 hours in the manufacturer's recommended buffer. DNA was separated by electrophoresis on 0.5% agarose gels. Gels were agitated in 0.5N NaOH, 1.5 mol/L NaCl for 30 minutes, followed by 45 minutes in 0.5 mol/L Tris, pH 7, 3 mol/L NaCl. DNA was transferred to nylon (Hybond N; Amersham, Arlington Heights, IL) in 6X SSPE overnight. Membranes were vacuum-baked at 80°C for 2 hours before prehybridization in 6X SSPE, 10X Denhart's solution, 0.5% sodium dodecyl sulfate (SDS) and 50 µg/mL salmon sperm DNA (ssDNA) at 65°C for 3 to 5 hours. Probes were labeled by random priming (Amersham) and after 100°C denaturation were hybridized to membranes in 6X SSPE, 10% dextran sulfate, 0.5% SDS, and 50 µg/mL ssDNA at 65°C overnight. Final washes were in 0.1X SSPE and 0.5% SDS at 30 minutes at 60°C. The pSPT/PGK probe was generously provided by Dr Vogelstein. The universal bcr probe was purchased from Oncogene Sciences (Manhasset, NY).

**Densitometry.** A Joyce-Loebel (Oyster Bay, NY) Ephortec Chromacom 3 densitometer was used for quantitative assessment of hybridization signals on PGK clonality gels. The larger allele has been designated allele 1, and the smaller allele 2. Lanes of DNA before Hpa II digestion (but after Bst XI) we have designated "A," while those after Hpa II digestion are referred to as "H." Thus for any given sample's analysis there are four relevant bands and four corresponding densitometric signals A1, A2, H1, and H2. The A lane is helpful for addressing possible technical difficulties with the sample. The calculation A1/(A1 + A2) yields a figure that will be referred to as RIA1 (for relative intensity of band A1). This value is similar to the calculated value "A" discussed by Nash et al20 and should be close to or equal to 0.50. A similar calculation for the Hpa II-digested lanes H1/(H1 + H2) designated RMH1 (relative methylation of H1) provides quantitative assessment of allelic methylation.

**Interpretation of results.** Clonality studies were interpreted by previously published criteria21 and after the evaluation of densitometric studies (above). BCR rearrangements were identified when bands of mobility differing from the germline were seen hybridizing in lanes of completely digested DNA.

**RESULTS**

**Patient clinical characteristics.** Samples from five patients with Ph1-positive CML were studied during periods of partial cytogenetic remission (< 35% Ph1-positive bone marrow metaphases)24 or complete cytogenetic remission while on treatment with IFN. Patients 1, 2, 3, and 5 had all presented with typical Ph1-positive CML and were all treated with IFN beginning in first chronic phase within 6 months of diagnosis. Patient 4 differed in having had two episodes of lymphoid blast crisis treated with cytotoxic agents before 3.5 years of partial cytogenetic remission. During this remission, the patient received sequential IFN-α and γ and cyclic therapy with vincristine, predni-

\[ \text{prednisone} \]

sone, methotrexate, and 6-mercaptopurine. Salient clinical features and maximal cytogenetic responses are given for each patient in Table 1.

**Standardization of techniques.** Before analyzing the pa-

\[ \text{tient materials, we determined the sensitivity of Southern blotting procedures used in this study. The clonal analysis} \]

of VogeIstein et al21 was examined by mixing varying proportions of two DNA samples together before enzyme digestions. Sample A was known to have a PGK locus methylation skew towards allele 2 (ie, with allele 2 nearly completely methylated), while sample B was skewed to allele 1. Results are shown in Fig 1A. Admixture of one spec-

\[ \text{imen to the other in a 20:80 ratio appears clearly} \]

detectable. To verify the quantitative nature of the signals seen for individual mixes, densitometry was performed. Values for RIA1 and RMH1 are given for each DNA mixture in Table 2. RMH1 values are plotted relative to sample B in Fig 2. Regression analysis showed R^2 to be .93 with the corresponding regression line as shown. Both the quantitative densitometric analysis and visual inspection of the autoradiograph show the clear separation of unmixed samples (100% A and 100% B) from the corresponding 60% mixtures. Resolution of mixtures closer to one another is less reliable. The R^2 value of .93 shows, however, the overall close correlation between DNA mixture and the densitometric data.

**BCR region rearrangement was similarly studied by mixing varying proportions of a sample known to consist of bcr rearranged cells (CML marrow in untreated chronic phase) with a normal granulocyte DNA. Figure 1B shows that our procedure detects an addition of 20% rearranged DNA to the normal sample.**

**Table 1. Clinical Data Including Bone Marrow Cytogenetic Changes**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Date of First IFN</th>
<th>IFN Treatment</th>
<th>Metaphases Pre-IFN</th>
<th>Metaphases on IFN*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3/83</td>
<td>huIFN</td>
<td>96% Ph1</td>
<td>0% Ph1-3/86</td>
</tr>
<tr>
<td>2</td>
<td>5/84</td>
<td>α-IFN</td>
<td>100% Ph1</td>
<td>0% Ph1-11/85</td>
</tr>
<tr>
<td>3</td>
<td>3/86</td>
<td>α-IFN</td>
<td>100% Ph1</td>
<td>0% Ph1-1/89</td>
</tr>
<tr>
<td>4†</td>
<td>7/87†</td>
<td>α-IFN</td>
<td>30% Ph1</td>
<td>10% Ph1-3/89</td>
</tr>
<tr>
<td>5</td>
<td>9/87†</td>
<td>α-IFN</td>
<td>92% Ph1</td>
<td>0%-6/90</td>
</tr>
</tbody>
</table>

Abbreviations: α-IFN, recombinant α- or α1-IFN; αγ-IFN, sequentially administered recombinant α- and γ-IFN; huIFN, human leukocyte IFN.22

*Maximal Ph1 suppression with date of sampling.

†This patient's prior course was complicated by two episodes of lymphoid blast crisis treated with cytotoxic chemotherapy. During the period of IFN therapy the patient received cyclic therapy with methotrexate, 6-mercaptopurine, vincristine, and prednisone.

‡The patient's pre-IFN bone marrow cytogenetics were from a sample obtained after remission induction for lymphoid blast crisis.
Fig 1. Mixing experiments for standardization of techniques. (A) Clonal analysis. Varying mixtures of sample A, DNA specimen known to have PGK allele 1 methylated (and allele 2 unmethylated) with sample B (known to have the opposite methylation pattern). Allele sizes are shown at left in kilobases. (B) BCR rearrangements. Bgl II digestion of varying mixtures of sample C, a normal human DNA with sample D, known to be composed of Ph1 positive, bcr rearranged cells. The size of the germline band is indicated in kilobases at left.

Clonal analysis. As shown in Fig 3, granulocytes were nonclonal during IFN-induced cytogenetic remission in all five patients. In each case, two separate specimens obtained during IFN treatment and at least one T-cell line are assessed. Densitometric quantitation of the autoradiographic images is given in Table 3. For patient 2, a
pretreatment bone marrow sample is shown (Fig 3B, lanes 1 and 2). Clonal hematopoiesis is clearly shown at that point (ie, allele 1 appears unmethylated despite the DNA overloading). This finding is confirmed by densitometry as RMH1 is 0 for this early specimen. In this individual, as in patients 1 and 3, remission samples show a skewed distribution of methylation of alleles with a majority of allele 2 (the lower allele) being methylated. In all cases, however, autologous T cells show methylation similar to the remission samples, suggesting that the methylation skew is present in constitutional (normal) tissues. As seen in Table 3, densitometry confirms that for any given patient RMH1 values of all remission samples and T cells are within a close range. The degree of variation is expected given the results of densitometry performed on the standardization mixtures. It must be noted that our technique is not precise enough to exclude a 20% to 30% clonal population coexisting with the nonclonal cells.

BCR region studies. Figure 4 shows these results. As expected, the pretreatment sample available for patient 2 shows bcr rearrangement. Samples taken during IFN-induced remission show only germline bands for this patient and patients 1, 3, and 5. Only patient 4 is shown to have any bcr region rearrangement in samples obtained during IFN-induced remission. This finding is consistent with the fact that only a partial cytogenetic remission was observed in the period of sample collection and that Ph1-positive (bcr rearranged) cells were present in these samples.

DISCUSSION

As our data show that conversion from clonal to polyclonal hematopoiesis occurs with IFN-induced replacement of Ph1-positive hematopoiesis by diploid cells, they give no support to the suggestion of a two-step pathogenesis of CML. Indeed, if a clonal Ph1-negative process antedates the development of the Ph1-positive hematopoiesis,20 then IFN must result in the suppression of most of this earliest clonal population. It must be noted that a population (up to approximately 30%) of clonal cells could be missed by our analysis. For such a population to exist in our patients it
POLYCLONAL REMISSIONS OF CML

Table 3. Densitometric Quantitation of Clonality Analysis for Patients 1 Through 5

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>Date</th>
<th>RIA1</th>
<th>RMH1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>PB</td>
<td>1/90</td>
<td>0.52</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>1/90</td>
<td>0.53</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>10/90</td>
<td>0.57</td>
<td>0.26</td>
</tr>
<tr>
<td>Patient 2</td>
<td>BM</td>
<td>9/84</td>
<td>0.59</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>PB</td>
<td>8/86</td>
<td>0.53</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>1/90</td>
<td>0.51</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>1/90</td>
<td>0.56</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>1/90</td>
<td>0.54</td>
<td>0.29</td>
</tr>
<tr>
<td>Patient 3</td>
<td>PB</td>
<td>1/87</td>
<td>0.54</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>11/87</td>
<td>0.56</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>11/89</td>
<td>0.56</td>
<td>0.18</td>
</tr>
<tr>
<td>Patient 4</td>
<td>GR</td>
<td>10/88</td>
<td>0.57</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>6/89</td>
<td>0.59</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>11/89</td>
<td>0.63</td>
<td>0.36</td>
</tr>
<tr>
<td>Patient 5</td>
<td>GR</td>
<td>1/90</td>
<td>0.48</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>GR</td>
<td>3/90</td>
<td>0.43</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>11/89</td>
<td>0.51</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Abbreviations: PB, peripheral blood; GR, granulocytes; BM, bone marrow; T, T cells.

would have to be less IFN sensitive than its suppressed Ph1-positive progeny.

Four of the five patients we have studied exhibited complete or near complete suppression of Ph1-positive progenitors. The cytogenetic studies in these four patients performed at or near the time of blood sampling showed no or very few abnormal metaphases. Examination of the bcr region for rearrangement has shown only germline bands in both Bgl II and Xba I digests. Under these conditions, greater than 95% of rearrangements of this region are detected. As these studies would have detected a 20% admixture of bcr rearranged (Ph1-positive) cells, our bcr studies in these four patients are in agreement with the cytogenetic findings. In all these patients granulopoiesis during IFN therapy appears nonclonal at the resolution of our technique.

In patient 4, the cytogenetic remission was less complete than that of our other patients. This patient also differed from the others in that she had sustained two episodes of blast crisis and had received considerable cytotoxic chemotherapy. Two samples, one of whole blood and the other of granulocytes, show a clear nonclonal pattern in this patient. These same samples contain bcr-rearranged sequences. Thus, at the times of sampling, this patient’s hematopoiesis arose from a mixture of normal nonclonal progenitors and bcr-rearranged (Ph1-positive) progenitors. T cells expanded from this individual (and all the other patients studied) showed no evidence of bcr rearrangement. This finding is consistent with previous reports of Ph1-negative T-cell lines expanded from Ph1-positive CML patients.

Previous studies have suggested that the vast majority of circulating T cells are diploid and nonclonal in CML. Both the visual and densitometric assessment of our clonal studies strongly suggests a similar progenitor pool for T cells and granulopoiesis during IFN therapy in our patients. Nonetheless, we are not able to exclude the possibility that the control T-cell preparations used in this study were partially clonal (ie, derived in part from a clonal Ph1-negative progenitor). Were this to be the case and even in the absence of a normal “control” tissue, the presence of a “nonclonal” pattern (two partially methylated alleles) in clonal studies of granulocytes indicates that granulopoiesis is at least partially nonclonal.

Our data show nonclonal granulopoiesis in IFN-induced cytogenetic remissions of CML. This observation confirms the presence of residual and apparently normal hematopoietic progenitors in this disorder and their ability to stably reconstitute the bone marrow. The finding of hematopoietic reconstitution with diploid cells has provoked interest in IFN treatment for CML. We have shown that these diploid cells are nonclonal and may thus be derived from normal stem cells.

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Fig 4. Bgl II-digested DNA probed for bcr. Xba I digestion gave similar results (not shown). (A through E) patients 1 through 5. The germline band seen in each lane is 4 kb. Rearrangements are seen in patients 2 (Fig 2A lane 1) and 4 (Fig 2B lanes 1 and 2).
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