Heterogeneity in Lineage Derivation of Philadelphia-positive Acute Lymphoblastic Leukemia Expressing $p_{190}^{BCR-ABL}$ or $p_{210}^{BCR-ABL}$: Determination by Analysis of Individual Colonies with the Polymerase Chain Reaction

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ABSTRACT

The molecular hallmark of Philadelphia chromosome-positive acute lymphoblastic leukemia (ALL) is the expression of 1 of 2 alternate forms of the aberrant BCR-ABL protein—$p_{210}^{BCR-ABL}$ or $p_{190}^{BCR-ABL}$. The presence of BCR-ABL message provides a target for analyzing the lineage derivation of this disease. We, therefore, studied myeloid and erythroid progenitor involvement in Philadelphia chromosome-positive ALL. Bone marrow low-density cells from Philadelphia chromosome-positive ALL patients (5 with the $p_{190}^{BCR-ABL}$ and 2 with the $p_{210}^{BCR-ABL}$ anomaly) were cultured in the mixed colony culture assay. cDNA from individually plucked colony-forming unit-granulocyte-macrophage and burst-forming unit-erythroid colonies was then analyzed using the hybridization protection assay in conjunction with the polymerase chain reaction to detect BCR-ABL molecular aberrations. Colony-forming unit-granulocyte-macrophage and burst-forming unit-erythroid colonies from 1 of 5 patients rearranged the BCR-ABL from 1 of 2 $p_{210}^{BCR-ABL}$-positive patients and 1 of 2 $p_{210}^{BCR-ABL}$-positive patients expressed BCR-ABL transcripts, whereas colony-forming unit-granulocyte-macrophage and burst-forming unit-erythroid colonies from the other patients did not. Our study suggests that the origin of both $p_{190}^{BCR-ABL}$ and $p_{210}^{BCR-ABL}$-positive ALL is heterogeneous with involvement of either a pluripotent precursor or a lymphoid lineage-committed hematopoietic progenitor.

INTRODUCTION

Ph+ ALL is a malignant disorder in which treatment is only partially effective, and the prognosis is considerably worse than in ALL lacking the Philadelphia chromosome (1, 2). The relationship between this disease and Ph+ CML has been the subject of significant speculation (3), and, because of the poor prognosis and the presence of the Ph abnormality, the question of multipotential stem cell involvement in Ph+ ALL as in CML has arisen. Alternatively, the heterogeneous molecular aberrations found in Ph+ ALL may reflect the involvement of distinct target cells.

The Ph abnormality results from a reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9;22) (q34;q11)]. This anomaly transposes the C-ABL gene from chromosome 9 to chromosome 22. A new hybrid gene called BCR-ABL is thus created. Molecular studies of the breakpoint on chromosome 22 have, however, revealed 2 distinct patterns in Ph+ ALL. In about 50% of adults and 10% of children with Ph+ ALL, the break occurs in the central region of the BCR gene (the major bcr), and transcription of BCR-ABL results in a hybrid 8.5-kilobase mRNA that codes for a $M_{r}$ 210,000 protein ($p_{210}^{BCR-ABL}$; p210) (4–8). In all patients producing $p_{210}^{BCR-ABL}$, the genetic configuration of BCR-ABL is identical to that found in Ph+ CML, i.e., either exon b2 or exon b3 of the bcr is coupled to C-ABL exon 2 ($b_{2}-a_{2}$ or $b_{3}-a_{2}$ junction) (6). The first intron of the BCR gene is the site of the break on chromosome 22 in the other half of the adults and 90% of children with Ph+ ALL. A smaller 7.0-kilobase BCR-ABL fusion mRNA is produced and encodes a $M_{r}$ 190,000 protein ($p_{190}^{BCR-ABL}$; p190) (8–11). In these patients, the first exon of the BCR gene is spliced to the second exon of the C-ABL gene ($b_{2}-a_{1}$ junction).

Current studies examining stem cell involvement in Ph+ ALL have been performed on only a very small number of patients (12–15). These investigations have suggested a multipotential stem cell origin in 4 of 5 adults and 2 of 2 children, because metaphases of individually removed CFU-erythroid, CFU-GM, and CFU-mixed, erythroid-granulocyte-macrophage were Ph+ (12, 13). However, the molecular subtypes of these leukemia cells were not evaluated. In a molecular-based study, Ph+ ALL with rearrangement within the 5.8-kilobase region of the bcr ($b_{2}-a_{2}$ junction or $b_{3}-a_{2}$ junction; $p_{210}$-type abnormalities) showed multilineage involvement in 2 of 3 patients (14). Currently, we have used a new technique—HPA in conjunction with the PCR (1, 16, 17)—to detect BCR-ABL cDNA in individually plucked colonies from seven Ph+ ALL patients. In all CFU-GM and BFU-E colonies from 4 of 5 patients, the BCR-ABL molecular aberration was not expressed. Similarly, 1 of the 2 p210+ patients lacked BCR-ABL+ colonies. However, one patient with each of $p_{190}^{BCR-ABL}$+ and $p_{210}^{BCR-ABL}$+ disease demonstrated BCR-ABL-expressing CFU-GM and BFU-E colonies. Our observations indicate that, in ALL, both the $p_{190}^{BCR-ABL}$ and the $p_{210}^{BCR-ABL}$ anomaly can be generated in either a lineage-committed or in a multipotent precursor cell.

MATERIALS AND METHODS

Patients with a diagnosis of Ph+ ALL were the subjects of this study. Patients were required to have: (a) a documented Ph abnormality by karyotype analysis (18); (b) a new onset of ALL presentation without a preceding antecedent hematological disorder or CML; and (c) 30% or more marrow blasts. Approval for these studies was obtained from the Institution Review Board. Workup of the patients at presentation included a history and physical examination; complete blood, differential, and platelet counts; SMA 12, including hepatic and renal functions; bone marrow aspiration and biopsy for morphology; cytochemical, and enzymatic stains (myeloperoxidase, chloracetate, nonspecific esterase, periodic acid-Schiff, Tdt); immunophenotyping (19, 20); karyotypic analysis (18); electron microscopy (21), and molecular studies as described below.

The classification of the acute leukemia was based on the French-American-British criteria and histochemical tests (22, 23). ALL was diagnosed if the blasts were morphologically lymphoid, myeloperoxidase-negative, and Tdt-positive and/or common acute lymphoblastic leukemia antigen (CD10)-positive. CFU-Granulocyte, Erythroid, Monocyte, Macrophage Assay. A modification of a previously described CFU-granulocyte, erythroid, monocyte, macrophage assay was used (24, 25). In brief, 2 x 106 nucleated low-density bone marrow cells were cultured in 0.8% (v:v) methylcellulose with Iscove’s modified Dulbecco’s medium (GIBCO, Grand Island, NY), 30% fetal calf serum, 12% horse serum, 5% newborn bovine serum, 1% L-glutamine, and 4 x 103 units/ml penicillin and 4 x 103 units/ml streptomycin. The classification of the acute leukemia was based on the French-American-British criteria and histochemical tests (22, 23). ALL was diagnosed if the blasts were morphologically lymphoid, myeloperoxidase-negative, and Tdt-positive and/or common acute lymphoblastic leukemia antigen (CD10)-positive.
0 ng/ml recombinant GM-colony-stimulating factor or 15 ng/ml IL-3, 50 ng/ml stem cell factor (Immunex Corp., Seattle, WA), and 1.0 units/ml human erythropoietin (British Columbia Cancer Research Institute, Vancouver, Canada). One ml of the culture mixture was placed in 35-mm Petri dishes (Nunc Inc., Naperville, IL) and incubated at 37°C with 5% CO₂ in air in a humidified atmosphere in duplicate. All cultures were evaluated after 14 days for BFU-E colonies (defined as an aggregate of more than 500 hemoglobinized cells or three or more erythroid subcolonies), and CFU-GM colonies (defined as a cluster of 40 or more granulocytes, macrophages, or both). Individual colonies were plucked from the cultures with a micropipette for a microscopic analysis.

Cell Line Clonogenic Assay (26). A CML erythroid blast crisis cell line (K562) and a Ph⁺ ALL cell line (ALL-1, kindly provided by Dr. G. Rovera, Wistar Institute) were plated in duplicate 35-mm dish sets (Nunc) at a concentration of 2 × 10⁴ cells/ml in 0.8% (v:v) methylcellulose in RPMI (GIBCO) with 10% fetal calf serum and incubated at 37°C in a humidified atmosphere of 5% CO₂ with air. Cultures were evaluated at day 6 using an inverted microscope. A cluster of more than 40 cells was defined as a colony.

Molecular Studies. Bone marrow from Ph⁺ ALL patients was analyzed for production of mRNA encoding p190BCR-ABL or p210BCR-ABL with the use of the PCR and specific primers encompassing the RNA splice junctions to amplify cDNA. The product was then detected with the use of HPA as described previously (16, 17) and as detailed below. Patients with an e₁-a₂ junction produce p190BCR-ABL (9-11); those with a b₂-a₂ or b₁-a₂ junction produce p210BCR-ABL (27). Individual colonies from K562 cells, a positive control for the normal ABL transcript (using primers encompassing ABL exons 1b and II) to ensure the presence of intact cDNA and a successful amplification procedure.

To avoid false-positive results with the PCR technique due to potential contamination, the following precautions were undertaken: (a) the thermal cycler was kept in a separate laboratory from the room where cell collection and RNA processing, and cDNA synthesis were performed; (b) no amplified samples were brought back into the room where RNA processing was performed; (c) at least one negative control was run for each experiment; (d) bone marrow samples from each patient were run at least two different times; and (e) colonies from each patient were plucked, prepared, and amplified by 2 technicians, who worked totally independently.

Bone Marrow Sample Preparation and PCR Amplification. We used PCR to establish the splice junction in each patient analyzed. Total cellular RNA was extracted and cDNA prepared as described previously (28). RNA from K562 cells, a CML erythroid blast crisis cell line, and patients with Ph⁺ CML were used as positive controls for cDNA containing b₁-a₂ and b₂-a₂ transcripts. The ALL-1 cell line was used as a positive control for the e₁-a₂ mRNA transcript (9, 10, 29), HL-60 (a Ph⁺ myelocytic leukemia cell line) and normal human endometrial RNA were used as negative controls. One μg of total RNA from cell lines or 10% of the total RNA from 50 to 200 × 10⁶ WBC from patient samples was used for amplification reactions. The PCR amplification was performed for 40 cycles using previously described primers encompassing the b₂-a₂, b₁-a₂, and e₁-a₂ junctions (30). For amplifications of the normal ABL transcript, a primer encompassing ABL exon II (31) and ABL exon I (32) were run in 2 dilutions (20) of PCR amplification of hematopoietic colonies. CFU-GM and BFU-E colonies from patient samples were plucked from the tissue culture dishes with a Pasteur micropipette as described previously (31). To validate our procedure, colonies derived from 2 CML patients and 2 normal volunteers. Cells were cultured in duplicate and given to 2 technicians who were blinded as to whether the samples were from a CML patient or a normal volunteer. Each of the technicians then proceeded to independently pluck colonies, prepare RNA and cDNA, coamplify for BCR-ABL and normal ABL, and probe for BCR-ABL and normal ABL by the method outlined below. The results of these experiments demonstrated that our molecular methodology could consistently distinguish CML Ph⁺ colonies versus normal colonies from normal individuals.

We then proceeded to use the identical methodology to analyze Ph⁺ ALL colonies. Plucked colonies were transferred into vials containing 100 μl RNAzol B solution; 10 μl of chloroform (0.1 volume of RNAzol) were added to the homogenate, vortexed for 15 s, kept at 4°C for 5 min, and centrifuged at 12,000 × g at 4°C for 15 min. RNA from the upper aqueous phase was transferred into 0.6-ml vials (Perkin Elmer Co.), an equal volume of isopropanol was added, and the vial was gently mixed and left at 4°C for 15 min to allow precipitation and then centrifuged at 12,000 × g at 4°C for 15 min. Supernatant was removed, RNA pellets washed with 200 μl of 75% ethanol and then spun down (7500 × g at 4°C for 8 min). RNA pellets were then dried on speed vacuum for 8–10 min and dissolved in 4 μl of sterile double distilled water. RNA samples were then incubated at 60°C for 5 min. cDNA was then prepared from RNA and subsequently amplified for 40 cycles. The amplification method and primers have been described previously (30). Positive and negative controls were performed for each experiment.

As mentioned above, to avoid false-negative results, all samples and colonies were simultaneously amplified for the normal ABL message. Only colonies in which normal ABL could be detected after amplification were considered evaluable.

HPA. We have described the clinical application of a rapid method for detecting amplified BCR-ABL cDNA (8, 16, 17), which uses acidinium ester-labeled probes (Gen-Probe, Inc., San Diego, CA) with high chemiluminescent properties. In the presence of hydrolysis buffer, the rate of hydrolysis of free probe is much faster than that of hybridized probe, and separation of hybridized probe on a solid support as in Southern blotting is unnecessary. This method is performed in solution and allows for reliable detection of transcripts within 30 min after amplification. HPA results correlated with those of Southern blotting of amplified product in all 60 samples from patients with Ph⁺ CML, acute leukemia, Ph⁻ leukemia, or normal volunteers (17).

Acridinium ester-labeled oligonucleotides complementary to the BCR-ABL junction sequences were synthesized by Gen-Probe (16, 17). The chemical labeling of the DNA probes with acridinium ester was achieved by reacting alkylamine linker-arms, which were introduced during DNA synthesis, and an N-hydroxysuccinimide ester or a methyl acridinium phenyl ester. The b₂-a₂ probe is a 28 mer with 22 bases from b₂ exon 2; the b₁-a₂ probe is a 25 mer with 11 bases from b₁ exon 3. These probes detect transcripts encoding p190BCR-ABL. The e₁-a₂ probe, a 26 mer with 17 bases from the first exon of the BCR gene, detects transcripts coding for p190. The probe for detection of the normal ABL transcript is a 24 mer spanning the ABL exon IB-II junction with 14 bases in exon II. A chemiluminescence reading, expressed in RLUs, indicates whether a sample is positive or negative (17).

DNA Analysis for JH Rearrangement. Ten μg of DNA were digested with restriction endonucleases (HindIII and EcoRI) in conditions recommended by the supplier (Boehringer Mannheim, Indianapolis, IN), electrophoresed on 0.7% agarose gel, blotted, and hybridized with a JH probe (28). The probe was labeled by oligo-primer extension to a specific activity of 2.5 × 10⁷ dpm/μg of DNA. After hybridization, filters were washed once for 15 min at 60°C, twice for 15 min each at room temperature, and once for 60 min at 52°C with a solution of 0.1× standard saline-citrate (1× standard saline-citrate = 0.15 mol/liter sodium chloride + 0.015 mol/liter sodium citrate) and 0.1% sodium dodecyl sulfate, dried, and autoradiographed.

RESULTS
We studied marrow samples from 7 Ph⁺ ALL patients. There were 2 men and 5 women, and their ages ranged from 9 to 71 (Table 1). Our control studies were performed on 2 cell lines and cells from normal donors and CML patients. The blasts derived from Patient 5 were morphologically typical for L2 ALL, had immunoglobulin heavy chain (JH) rearrangement, and expressed B-cell markers, but were also peroxidase positive (TdT was not performed) and would therefore be considered by some investigators as mixed-lineage leukemia (20).

PCR-amplified cDNA from colonies derived from the Ph⁺ ALL-1 cell line revealed an e₁-a₂ BCR-ABL junction with HPA detecting high chemiluminescence counts for each colony (Table 2). Similarly, 55 colonies from K562 cells or Ph⁺ CML patients always showed >10,000 (and usually >25,000) RLUs with the b₂-a₂ or b₁-a₂ BCR-ABL or the Ib-II normal ABL probes (data not shown). Representative results of experiments performed on colonies from a CML patient and a normal volunteer are presented in Table 3. Twenty additional colo
From the given text, we see that it discusses the detection of BCR-ABL transcripts in leukemia samples. The text mentions the use of hybridization protection assay to detect these transcripts. It also refers to the results of a clonogenic assay and the use of Southern blotting to confirm the presence of BCR-ABL transcripts. The text further explains the lineage derivation of Ph+ ALL and the differences between Ph+ acute leukemia and Ph+ CML. The table (Table 1) provides clinical data on ALL patients, including age, sex, FAB classification, cytogenetics, BCR-ABL type, and survival. The table (Table 2) contains BCR-ABL junctions in ALL-1 cell line, including patient numbers, BCR-ABL type, and survival times.

**DISCUSSION**

In the current study, we have applied a hybridization protection assay to detect PCR-amplified BCR-ABL cDNA from individual CFU-GM and BFU-E colonies. This technique provides a simpler and more rapid alternative to the more standard approach of resolving PCR products on agarose gels and confirmation of identity by Southern blotting. We found that CFU-GM and BFU-E colonies obtained from 4 of 5 Ph+ ALL patients who were p190+ did not show molecular evidence of involvement in the malignant process. Our observations are consistent with the notion that in some individuals with p190BCR-ABL+, the disease is lineage-restricted and does not involve the committed myeloid or erythroid precursors (i.e., CFU-GM and BFU-E) or a pluripotent stem cell. Several alternate explanations are conceivable, though unlikely. For instance, it is possible that the maturation of the leukemic myeloid precursors is "arrested" before the stage of CFU-GM or BFU-E, or that the leukemic process may have rerearrangement without expressing BCR-ABL transcripts. However, expression was found in committed progenitors such as those found in our clonogenic assay and is therefore unlikely to confound our results. Taken together, these observations suggest that both p210- and p190+ diseases are heterogeneous and can originate in either a lymphoid lineage-committed or a pluripotent precursor.

Differentiating de novo Ph+ acute leukemia from Ph+ CML in blast crisis is difficult because the blastic stage of CML is morphologically indistinguishable from acute leukemia. In addition, CML patients can be asymptomatic during the chronic phase of the disease. It is therefore possible that some cases of Ph+ acute leukemia may be misclassified as de novo Ph+ acute leukemia.

**Table 1 Clinical data on ALL patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/sex</th>
<th>FAB classification</th>
<th>Cytogenetics</th>
<th>BCR-ABL type</th>
<th>Survival (mo.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59/F</td>
<td>L1</td>
<td>+ 46,XX,inv7, t(9q+;22q+)</td>
<td>p190+ e1-a2 junction</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>46/F</td>
<td>L2</td>
<td>+ 45,XX-7, t(9q+;22q-)</td>
<td>p190+ e1-a2 junction</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>15/M</td>
<td>L1</td>
<td>Metaphases with 42-45 chromosomes and t(9q+;22q+)</td>
<td>p190+ e1-a2 junction</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>60/F</td>
<td>L1</td>
<td>+ 45,XX-7, t(9q+;22q-)</td>
<td>p190+ e1-a2 junction</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>9/F</td>
<td>L2</td>
<td>Hyperdiploid with 54-90 chromosomes and t(9q+;22q+)</td>
<td>p190+ e1-a2 junction</td>
<td>20+</td>
</tr>
<tr>
<td>6</td>
<td>71/M</td>
<td>L1</td>
<td>+ 46,XY(t(9q+;22q))</td>
<td>p210+ b2-a2 and b3-a2 junctions</td>
<td>20+</td>
</tr>
<tr>
<td>7</td>
<td>13/F</td>
<td>L1</td>
<td>+ 44,XX-7,9,t(9q+;22q+)</td>
<td>p190+ b2-a2 and b3-a2 junctions</td>
<td>20+</td>
</tr>
</tbody>
</table>

**Table 2 BCR-ABL junctions in ALL-1 cell line (Ph+, p190BCR-ABL+)**

<table>
<thead>
<tr>
<th>Junction</th>
<th>(p210) (RLUs)</th>
<th>(p190) (RLUs)</th>
<th>Normal ABL (RLUs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>b2-a2</td>
<td>4,926 (neg)</td>
<td>4,861 (neg)</td>
<td>568,934 (pos)</td>
</tr>
<tr>
<td>e1-a2</td>
<td>99,967 (pos)</td>
<td>789,456 (pos)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3 BCR-ABL junctions in a CML patient and a normal volunteer**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Colony type</th>
<th>Plucked colonies</th>
<th>(p210) (RLUs)</th>
<th>(p190) (RLUs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML patient</td>
<td>CFU-GM</td>
<td>2,542 (neg)</td>
<td>222,020 (pos)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>2,963 (neg)</td>
<td>52,223 (pos)</td>
<td></td>
</tr>
<tr>
<td>BFU-E</td>
<td>4,310 (neg)</td>
<td>298,225 (pos)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal volunteer</td>
<td>CFU-GM</td>
<td>2,375 (neg)</td>
<td>1,970 (neg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BFU-E</td>
<td>2,384 (neg)</td>
<td>2,001 (neg)</td>
<td></td>
</tr>
</tbody>
</table>

* R. Kurzrock, unpublished observations.
are actually CML blast crisis in a patient who eluded diagnosis before transformation. Because p210BCR-ABL occurs in the vast majority of CML patients, and because p190BCR-ABL is found in Ph+ acute leukemia but not generally in CML, several investigators have postulated that p190BCR-ABL+ acute leukemia represents a de novo leukemic process, whereas p210BCR-ABL acute leukemia represents CML blast crisis. Yet, in examining a series of 32 patients with Ph+ ALL, we found that the clinical manifestations, response to therapy, and morphological features of p210+ ALL did not differ from those of p190+ ALL (8). Our current observations suggesting that p190-positivity is often not associated with a multilineage disorder are consistent with the concept that most patients bearing this molecular abnormality do not have CML in blast crisis; rather, they may have a true ALL. Further, the finding that the disease in p210+ ALL patients may originate either in a multipotent stem cell (as in CML) or in a lineage-committed progenitor (as in most cases of ALL) suggests the possibility that a subset of p210+ acute leukemia actually represents CML blast crisis. It may therefore be worthwhile to examine a larger number of individuals to ascertain whether the multilineage form of p210+ acute leukemia behaves differently from the lineage-committed form or from p190+ disease.

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