Chronic Myelogenous Leukemia: Amplification of a Rearranged c-abl Oncogene in Both Chronic Phase and Blast Crisis

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The specific genetic events that distinguish the blast crisis from the chronic phase cells of chronic myelogenous leukemia (CML) are unknown. The most common karyotypic change that occurs as CML evolves from chronic phase to blast crisis is the development of multiple Philadelphia (Ph1) chromosomes, each of which is presumably harboring a translocated c-abl oncogene. We describe here a patient with CML who presented in lymphoid blast crisis with three Ph1 chromosomes/metaphase associated with an amplified, rearranged c-abl oncogene fragment and high levels of the aberrant 8-kilobase bcr-abl transcript. This rearranged c-abl fragment was amplified to a similar degree in both the patient's blast crisis cells and in his terminally differentiated granulocytes, but the level of the aberrant CML-specific bcr-abl transcript was some eight- to 16-fold higher in the blast crisis cells than in the granulocytes. This analysis indicates that genomic amplification of a translocated c-abl oncogene, although perhaps important in the evolution of CML, nevertheless cannot, by itself, be the sole genetic event giving rise to blast crisis.

C HRONIC MYELOGENOUS LEUKEMIA (CML) is a clonal disorder involving a pluripotent stem cell from which granulocytes, erythrocytes, platelets, monocytes, macrophages, and B lymphocytes arise.1 Over 90% of cases of CML exhibit the Philadelphia chromosome (Ph1)(22q −), which results from a reciprocal translocation involving the distal portions of the q arms of chromosomes 9 and 22 and which is the most specific chromosome abnormality associated with any human malignancy. A prominent feature of CML is the distinct clinical phases of the illness. The relatively benign chronic phase, characterized by hyperplasia of terminally differentiated granulocytes, inevitably progresses to the blast crisis phase in which immature blasts predominate and that usually proves lethal.

Recent observations have provided considerable insight into the molecular genetics of the Ph1 chromosome formation in CML. The c-abl oncogene is defined by virtue of its homology to v-abl, the transforming sequences of the Ablson murine leukemia virus.2 In CML, this oncogene is translocated from its usual position on the distal end of the q arm of chromosome 9 to a relatively restricted region on the Ph1 termed the breakpoint cluster region (bcr).3,4 As a result of this translocation, a hybrid bcr-abl transcript is generated.5,6 This abnormally large transcript (8.2 kilobase [kb]) probably codes for the abnormally large abl-related protein product (P210) that has been demonstrated in CML cells.3,8 Although the physiological function of the c-abl oncogene is unknown, these findings indicate that abnormalities in the structure and expression of this oncogene may be critical to the pathogenesis of CML.

Several lines of evidence indicate that enhanced expression of the altered c-abl oncogene may be important in the progression of CML from chronic phase to blast crisis. We have previously demonstrated that this oncogene is amplified some four- to eightfold in the K-562 CML blast crisis cell line.7 The development of multiple Ph1 chromosomes, each of which is presumably harboring a translocated c-abl oncogene, is the most frequent karyotypic abnormality that occurs as CML evolves from the chronic phase to blast crisis.8,9 In addition, enhanced expression of the abnormal 8.2-kb bcr-abl hybrid transcript is noted in CML blast crisis cells v chronic phase cells.11 Thus, one genetic event leading to enhanced expression of the translocated c-abl oncogene during progression of CML from the chronic phase to blast crisis may be an increase in the copy number of the abnormal bcr-abl locus.

Is the enhanced expression of the aberrant 8.2-kb bcr-abl transcript due solely to the presence of the multiple Ph1 chromosomes in certain CML cells? Is the presence of multiple Ph1 chromosomes the main genetic change distinguishing CML chronic phase from blast crisis? We have been able to approach these questions by using a molecular genetic analysis of blast crisis and chronic phase cells from a patient with CML who presented in lymphoid blast crisis with multiple Ph1 chromosomes/metaphase associated with amplification of a rearranged c-abl oncogene. Although there was a marked difference in the level of expression of the aberrant bcr-abl transcript in the blast crisis v the chronic phase cells, both of these cell types in this patient exhibited similar amplification of the rearranged c-abl fragment, which suggests that both the blast crisis and chronic phase cells harbored multiple Ph1 chromosomes.

MATERIALS AND METHODS

Case Summary

The patient was a 42-year-old man who presented with a 1-month history of weight loss, malaise, fatigue, and headaches. His physical exam revealed pallor and splenomegaly without adenopathy. A complete blood cell count (CBC) revealed a hemotocrit value (Hct) of 29, platelet count of 32,000, and a WBC count of 430,000 with...
54% blasts, 3% lymphocytes, 4% monocytes, 3% nucleated RBCs, 10% myelocytes, 3% metamyelocytes (metas), 7% bands, and 16% segmented neutrophils (segs). A bone marrow aspirate and biopsy sample revealed a marrow packed with over 90% blasts that were terminal transferase-positive. Karyotypic examination revealed 52 XXY, +5, +11, +20, +3 Ph in virtually all unstimulated metaphases. In contrast, phytohemagglutinin (PHA)-stimulated metaphases displayed a 46 XY karyotype. Therapy was initiated with oral hydroxyurea followed by two 3-week courses of vincristine, prednisone, and Adriamycin. Six weeks following initiation of therapy, the patient was noted to be in partial remission with an Hct of 28, platelet count of 56,000, and a WBC count of 7,000 with 3% blasts, 8% lymphocytes, 8% monocytes, 5% nucleated RBCs, 11% myelocytes, 12% metas, 15% bands, and 38% segs. The patient refused further chemotherapy and was discharged. Two months later he returned with a peripheral WBC count of 110,000 consisting of greater than 90% lymphoblasts. Therapy with oral hydroxyurea was re instituted.

**Cell Separation**

At initial presentation the peripheral blood was diluted 1:1 with plain RPMI, layered over isolymph (Gallard/Schlesinger, Carle Place, NY), and spun at 1,800 rpm for 20 minutes. The interface cells were washed several times in RPMI and upon Wright staining were found to consist of over 85% blasts, with the remaining cells being predominantly myelocytes and metamyelocytes. The RBC-granulocyte isolymph pellet was brought to 25 mL with plain RPMI and mixed with 25 mL 3% dextran (T-500) in phosphate-buffered saline. The RBCs were allowed to sediment for 1 to 1.5 hours, and the supernate was harvested and the cells pelleted. The contaminating RBCs were lysed several times in 0.2% NaCl. The pelleted cells were then washed several times in plain RPMI and consisted of greater than 95% mature granulocytes.

After the chemotherapy-induced partial remission, peripheral blood was subjected to 3% dextran sedimentation followed by lysis of RBCs as described above. These cells consisted of approximately 70% metamyelocytes, bands, and segs, with the remaining cells consisting of mature lymphocytes and monocytes. There were fewer than 5% blasts in this cell population.

At the time of relapse, mononuclear cells were isolated following isomvph gradient centrifugation of peripheral blood. This fraction consisted of greater than 95% blasts.

**DNA and RNA Extractions**

DNA extractions from the various leukocyte populations were performed by digesting nuclei with protease K followed by phenol/chloroform extraction and ethanol precipitation as previously described. RNA was extracted by homogenizing the cells in guanidine thiocyanate followed by ultracentrifugation through a cesium chloride cushion as described by Chirgwin et al. Poly A + RNA was selected by using oligo-dT cellulose.

**Southern, Northern, and Dot Blot Hybridizations**

Restriction endonuclease digestion of genomic DNA was performed following manufacturer specifications. Southern and Northern blots were made following standard procedures. RNA dot blot analysis on the total cellular RNA was performed as previously described. All blots were hybridized to nick-translated probes and subsequently washed under exactly the same conditions described previously.

**Molecular Probes**

S' abl. A molecular probe from the S' end of the c-abl oncogene was cloned from a genomic library constructed from the K-562 CML blast crisis cell line. DNA from this cell line was partially digested with Sau 3A and fractionated by sucrose density gradient centrifugation, and the 15- to 23-kb DNA fragments were isolated. This DNA was ligated with Bam-digested EMBL 3 phage vector DNA, and the ligation mixture was packaged in vitro. Approximately 500,000 recombinant clones were screened with a nick-translated 550-bp HindII-Smal fragment derived from the pAB1 sub plasmid, which contains a v-abl insert. This 550-bp fragment represents the most S' sequences of v-abl. Following screening, ten positive clones were isolated and purified. One of these clones contained a 17-kb genomic insert that exhibited a restriction map similar to that of the previously published S' end of c-abl, and from this clone a 0.3-kb HindIII-EcoR, fragment free of human repetitive sequences was isolated and used as a molecular probe.

Genomic bcr probe. A 1.4-kb BglII-Sst human genomic fragment representing sequences within the bcr gene that are immediately S' to the bcr itself was isolated from a normal genomic library.

**RESULTS**

**Cell Harvesting and Separation**

We obtained peripheral blood cells from this patient at three different times during the course of his illness including (1) at initial presentation, at which time he had a WBC count of 430,000 with 54% blasts; (2) during a brief chemotherapy-induced partial "remission" when his peripheral count was 7,000 with >70% terminally differentiating granulocytes and <5% blasts; and (3) during relapse of blast crisis when his peripheral count rose to greater than 100,000 with over 90% blasts. At initial presentation, we separated the immature blast cells from the mature terminally differentiated granulocytes by isolymph gradient centrifugation as described in Materials and Methods. DNA and RNA were extracted from these cells as well as from the mature granulocytes isolated at the time of partial remission and from the immature blasts isolated at the time of relapse.

**Lymphoid Nature of the Blast Crisis Cells**

Many cases of CML blast crisis are B lymphoid in origin and exhibit immunoglobulin gene rearrangements. This patient's blast cells were positive for terminal transferase, thus suggesting their lymphoid origin. This was confirmed by Southern blot analysis of the blast cell DNA, which revealed several extra nongermline bands hybridizing to a heavy-chain immunoglobulin joining region probe (JH) (Fig 1). The
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**Fig 1.** Rearrangement of the immunoglobulin heavy-chain locus in the CML blasts. Genomic DNA was digested with \textit{BglII}, electrophoresed in 1.0% agarose, transferred to nitrocellulose, and hybridized to a 32P-labeled \textit{J_H} probe specific for the immunoglobulin heavy-chain joining region. Samples include CML blasts isolated at presentation (lane 1); initial presentation granulocytes (lane 2); blasts isolated at relapse (lane 3); HL-60 cells, which exhibit the germline pattern (lane 4); and partial remission CML cells (lane 5). Rearranged fragments in the CML blasts are indicated by arrows.

An identical restriction pattern was seen with this probe in DNA from both the blasts at initial presentation and at relapse (Fig 1). In contrast, predominantly the germline pattern was seen in the granulocyte populations isolated at initial presentation and during the chemotherapy-induced partial remission (Fig 1). Therefore these blots not only reveal the B-lymphoid nature of the patient’s blasts but also indicate that the separated granulocyte fraction is not contaminated with any significant number of these blasts.

**RNA Analysis**

RNA from the patient’s blast and initial presentation granulocyte populations were subjected to Northern and dot blot analysis. The patient’s blast cells exhibited the abnormal 8.2-kb \textit{bcr-abl} hybrid transcript that has been previously described in Ph⁺ chromosome-positive CML cells\textsuperscript{5,6,11} (Fig 2). The relative amount of this transcript is quite high and is comparable with that found in the K-562 cell line, which exhibits a four- to eightfold amplification of the \textit{c-abl} oncogene (Fig 2). Although we did not obtain enough granulocyte RNA to perform Northern blots, we did note by RNA dot blot analysis a marked decrease of at least eightfold in the level of \textit{abl}-related transcripts in the granulocyte population compared with the blasts (Fig 3). In addition, a similar eightfold decrease in the level of \textit{bcr}-related transcripts was noted in the granulocyte population compared to the blasts. These results indicate that the level of \textit{bcr-abl} hybrid transcripts is significantly higher in the blasts than in the granulocyte population in this patient. It is of interest that hybridization of this dot blot to a 3' \textit{bcr} probe, which measures levels of \textit{bcr} transcripts from normal chromosome 22 but does not detect \textit{bcr-abl} hybrid transcripts, reveals a similar decrease in levels of normal \textit{bcr} transcripts in the granulocyte populations compared to the blasts (Fig 3). As a control, we hybridized these same samples to an actin probe that revealed similar levels of actin-related transcripts in both (Fig 3).

**c-abl Rearrangement and Amplification in Both Blasts and Granulocytes**

We hybridized Southern blots of restriction digests of DNA from patient blasts and initial presentation granulocyte fractions with a 300-bp \textit{R_V}-\textit{HindIII} \textit{c-abl} probe (probe Ab1). The indicated quantities of RNA were dotted onto nitrocellulose and hybridized sequentially to the indicated probes. Between successive hybridizations, probes were removed from the blot by the addition of boiling water followed by cooling to room temperature with constant rocking. Exposure time was 20 hours for the \textit{v-abl}, \textit{bcr}, and 3' \textit{bcr} probes and 4 hours for the actin probe. The 2-kb \textit{bcr} cDNA probe recognizes both normal \textit{bcr} transcripts as well as \textit{bcr-abl} fusion transcripts. The 700-bp 3' \textit{bcr} probe recognizes only normal \textit{bcr} transcripts.

**Fig 2.** Enhanced expression of the aberrant 8.2-kb \textit{bcr-abl} transcript in the CML blasts. Poly A+ RNA (5 \textmu g/line) was electrophoresed in a formaldehyde gel, transferred to nitrocellulose, and hybridized to a \textit{v-abl} probe. The normal 7.4- and 6.6-kb transcripts as well as the aberrant 8.2-kb transcripts are noted. RNA samples are from K-562 (lane 1), HL-60 cells (lane 2), and the patient CML blasts (lane 3).

**Fig 3.** Enhanced expression of both \textit{abl} and \textit{bcr}-related transcripts in the CML blasts (row A) v initial presentation CML granulocytes (row B). The indicated quantities of RNA were dotted onto nitrocellulose and hybridized sequentially to the indicated probes. Between successive hybridizations, probes were removed from the blot by the addition of boiling water followed by cooling to room temperature with constant rocking. Exposure time was 20 hours for the \textit{v-abl}, \textit{bcr}, and 3' \textit{bcr} probes and 4 hours for the actin probe. The 2-kb \textit{bcr} cDNA probe recognizes both normal \textit{bcr} transcripts as well as \textit{bcr-abl} fusion transcripts. The 700-bp 3' \textit{bcr} probe recognizes only normal \textit{bcr} transcripts.
B) located within a 12-kb BglII fragment on chromosome 9 at the 5' end of c-abl (Fig 4). This probe detected an extra fragment in both the blast and granulocyte DNA digests, thus indicating that the break on chromosome 9 in this patient lies within this 12-kb BglII fragment and that both the blast and granulocyte subpopulations harbor a similar chromosome 9 breakpoint (Fig 4). In both the blast and granulocyte fractions, this probe hybridizes more intensely to the rearranged BglII fragment than to the germline fragment, which suggests that the rearranged c-abl fragment is amplified in this patient. We also noted a rearranged and amplified KpnI fragment in genomic digests of blast and granulocyte DNA hybridized to a chromosome 22 probe (probe A) located just 5' to the breakpoint cluster region on

Fig 4. Amplification and rearrangement of abl and bcr sequences in CML blasts and granulocytes. Genetic maps of the bcr region on chromosome 22 (A), the 5' c-abl region on chromosome 9 (B) and the 9:22 Ph' breakpoint region on the Ph' chromosome in this particular patient (C) are shown. These maps were derived from data from references 4, 5, and 17 and from our own mapping data. The approximate breakpoints on chromosomes 22 and 9 in this patient have been previously mapped and are indicated by arrows. Probe A is a 1.4-kb BglII-SstI chromosome 22 fragment located immediately 5' to the breakpoint cluster region (BCR) and hybridizes to a 19-kb KpnI germline fragment as indicated in A. Probe B is a 0.3-kb Kgl-HindIII fragment located in the 5' c-abl region of chromosome 22 that hybridizes to a 12-kb BglII germline fragment as indicated in B. c-abl exons are denoted by Δ. These probes were hybridized to Southern blots of genomic digests of CML DNA from patient blasts (lanes 1 and 3) and from initial presentation patient granulocytes (lanes 2 and 4). Lanes 1 and 2 are BglII digests; lanes 3 and 4 are KpnI digests. In both blasts and granulocytes probe B identifies the 12-kb germline BglII fragment as well as a rearranged, amplified 7.5-kb fragment as indicated (lanes 1 and 2). Probe A identifies the 19-kb KpnI germline fragment as well as a rearranged, amplified 7.8-kb fragment as indicated (lanes 3 and 4). These rearranged fragments are 9:22 junction fragments as indicated in C. Densitometric scanning reveals a ratio of rearranged:germline fragment of approximately 6:1 using probe B (lanes 1 and 2) and 10:1 using probe A (lanes 3 and 4) in both the blast and granulocyte DNA digests.
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The recent identification of specific genes that are involved in Ph$^+$ chromosome formation in CML now allows the identification of cells harboring this marker chromosome by Southern blot hybridization of restriction digestes of genomic DNA.$^{3,4}$ The major advantage of this technique is that metaphases from immature, proliferating cells are no longer essential for analysis, since the assay can be performed with any cellular DNA at any particular level of cell differentiation. We utilized this approach to analyze various cell populations of a patient who presented with CML in blast crisis. Several somewhat unusual aspects of this patient’s illness made the analysis particularly useful. At presentation there were a significant number of terminally differentiating granulocytes in his peripheral blood in addition to the lymphoid blast crisis cells, which allowed for ready separation of distinct CML cell subpopulations. Moreover, Southern blot analysis readily distinguished these lymphoid blast crisis cells from the terminally differentiating granulocytes, since only the blasts exhibited a rearrangement of the immunoglobulin heavy-chain $J_H$ locus. In addition, a karyotype analysis of bone marrow blasts in this patient revealed three Ph$^+$ chromosomes/metaphase. The breakpoint on chromosome 9 that contributed to the Ph$^+$ formation in the leukemic cells was relatively close to the $v$-\textit{abl} homologous region such that Southern blots revealed a rearranged \textit{c-abl} fragment utilizing a $5'$ \textit{c-abl} probe. (Only 15% to 20% of CML patients exhibit a breakpoint this close, with most CML chromosome 9 breakpoints occurring much further $5'$ to the $v$-\textit{abl} homologous region.)$^{4,22}$ This rearranged $5'$ \textit{c-abl} fragment was amplified in this patient’s blast cells, most likely reflecting the presence of the multiple Ph$^+$ chromosomes/metaphase. Thus using this $5'$ \textit{c-abl} probe, we were not only able to determine the presence of the Ph$^+$ in various cell populations but could also, by comparing the intensity of hybridization of the amplified, rearranged fragment to the germline fragment, estimate the number of Ph$^+$ chromosomes that each isolated cell population harbors.

We isolated blast crisis cells from this patient at the time of initial presentation and at the time of relapse. We also isolated terminally differentiated granulocytes at initial presentation and during a chemotherapy-induced partial “remission” when the peripheral blood blast cell population fell below 5%. The morphologically immature blasts displayed a rearrangement of the immunoglobulin heavy-chain locus that indicated a B lymphoid blast crisis, whereas the mature granulocytes exhibited a germline immunoglobulin heavy-chain pattern and were therefore representative of chronic phase cells. If one of the major genetic events that distinguishes these lymphoid blast crisis cells from the terminally differentiated chronic phase granulocytes is the development of multiple Ph$^+$ chromosomes with concomitant amplification of the rearranged \textit{c-abl} fragment, then one would predict that the terminally differentiated granulocytes would not display amplification of the rearranged \textit{c-abl} fragment (ie, precursors of these granulocytes display a single Ph$^+$). However, our Southern blots show that this is clearly not the case, since this rearranged \textit{c-abl} fragment is amplified to a similar degree in both the blast and granulocyte cell populations. Indeed, these Southern blots indicate that the terminally differentiated granulocytes also harbor multiple Ph$^+$ chromosomes. Therefore, the development of multiple Ph$^+$ chromosomes per se cannot be the sole genetic event that leads to the blast crisis phenotype, and one would predict that there are other genetic factors besides multiple Ph$^+$ chromosomes that distinguish the blast crisis from the chronic phase cells.

One potential problem in interpreting our Southern blots is that the enhanced hybridization of the \textit{bcr} and \textit{abl} probes to the rearranged fragments may be secondary to preferential transfer from the agarose gel to nitrocellulose of the smaller, rearranged fragments compared with the larger, germline fragments. Indeed since the rearranged \textit{bcr} and \textit{abl} fragments most likely are present on each individual Ph$^+$ chromosome, one would predict a ratio of intensity of hybridization of rearranged:germline fragment of 3:1 with these probes (ie, three Ph$^+$ chromosomes/one normal chromosome 9 or 22/metaphase). However, our densitometric scanning indicates a hybridization intensity ratio of 6:1 with the $5'$ \textit{c-abl} probe and 10:1 with the \textit{bcr} genomic probe (Fig 4), which suggests that there indeed has been preferential transfer of the smaller rearranged fragments in these blots. However, the important observation is that the hybridization intensity ratios of rearranged-germline fragments using both \textit{abl} and \textit{bcr} probes are virtually identical in the blasts (which by karyotypic analysis harbor three Ph$^+$ chromosomes/metaphase) and the chronic phase granulocytes. Thus even though preferential transfer of the smaller rearranged fragments is probably present in these blots, this does not interfere with our conclusion that the chronic phase granulocytes are similar to the blast crisis cells in harboring multiple Ph$^+$ chromosomes.

Although we noted amplified, rearranged \textit{c-abl} sequences in both the lymphoid blast crisis cells and the chronic phase granulocytes in this patient, the blast crisis cells had an eight- to 16-fold greater level of \textit{bcr-abl}-related transcripts than the chronic phase granulocytes (Fig 3). Thus, the presence of amplified \textit{c-abl} sequences per se within the genome of certain CML cells does not necessarily lead to enhanced expression of these sequences. What controls the expression of the aberrant \textit{bcr-abl} transcript in CML? The \textit{c-abl} oncogene in CML is translocated into the \textit{bcr} gene on the Ph$^+$, and transcription of this oncogene may then come under control of those factors that normally control transcription of the intact \textit{bcr} gene on the normal chromosome 22. Consistent with this hypothesis is our finding that there is a decrease in the normal chromosome 22 \textit{bcr} transcripts in the CML granulocytes $v$ blast crisis cells that is almost
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exactall proportional to the decrease in the level of Ph' chromosome bcr-abl-related transcripts in these cells (Fig 3). Indeed, one hypothesis to explain the progression of CML to blast crisis is that a subset of Ph'-positive CML cells harboring trans-activating factors specific for the normal bcr gene might also develop enhanced expression of the aberrant bcr-abl transcript and presumably gain a selective growth advantage over other Ph'-positive CML cells that do not harbor such specific factors. This model would explain how some CML cell populations (for instance, the granulocytes from this patient) would not display high levels of the bcr-abl transcript even though they harbor multiple Ph' chromo-

somess. An alternative hypothesis to explain the evolution of CML is that a further genetic event or mutation, perhaps involving another oncogene in addition to the Ph' transloca-

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