Increase of BCR-ABL Chimeric mRNA Expression in Tumor Cells of Patients With Chronic Myeloid Leukemia Precedes Disease Progression

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The translocation t(9;22) in chronic myeloid leukemia (CML) generates a bcr-abl fusion gene that codes for an aberrant chimeric mRNA. Cell lines established from CML patients in blast crisis show higher expression of this aberrant bcr-abl transcript than cells from patients in chronic phase of the disease. This observation provided the stimulus to investigate whether increased expression of the aberrant bcr-abl fusion transcript is critical to the progression of CML from chronic phase to blast crisis. We have monitored the bcr-abl mRNA expression in 25 patients by serial quantitative polymerase chain reaction analyses during a follow-up period of 12 to 156 months after diagnosis, with a median observation time of 28 months. In all patients who have shown disease progression to accelerated phase (n = 4) or blast crisis (n = 7), an increase in bcr-abl mRNA expression was detected up to 16 months before laboratory or clinical parameters showed phenotypic transformation of the malignant clone. To investigate whether the elevated levels of bcr-abl mRNA reflected an increase in the proportion of leukemic cells in the samples analyzed or primarily enhanced transcriptional activity of the bcr-abl fusion gene, we performed quantitative analyses of the bcr-abl gene at the DNA level and of the Ph chromosome at the cytogenetic level and compared these data with steady-state bcr-abl mRNA levels. We show that increased levels of the bcr-abl transcript did not reflect increased proportions of leukemic cells but elevated steady-state levels of the chimeric mRNA in the malignant cells before disease progression. Therefore, our data strongly suggest that an increase of the chimeric mRNA expression in the leukemic cells precedes the phenotypic transformation of the malignant clone.

C HRONIC MYELOID LEUKEMIA (CML) is a clonal disorder caused by neoplastic transformation of a pluripotent hematopoietic stem cell.1 The disease starts in a chronic phase (CP) characterized primarily by accumulation of granulocytes and their precursors in the bone marrow (BM) and peripheral blood (PB). This is followed by the acute phase (blast crisis; BC) in which the leukemic cells fail to differentiate and to respond to regulatory factors of myelopoiesis. In some patients, there is an intermediate stage referred to as accelerated phase (AC). The hallmark of the malignant cells is the Philadelphia chromosome (Ph), which is generated by a reciprocal translocation between chromosomes 9 and 22 fusing the c-abl oncogene from the q arm of chromosome 9 to the breakpoint cluster region (bcr) within the bcr gene on chromosome 22.2,3 The bcr-abl gene is transcribed into a bcr-abl-specific mRNA that is translated into a 210-kD protein possessing enhanced tyrosine kinase activity as compared with that of normal abl protein.4

The oncogenic potential of the bcr-abl fusion protein has been shown by its ability to transform hematopoietic progenitor cells in vitro. Furthermore, transgenic mice carrying bcr-abl constructs develop lymphoid tumors,5 and reconstitution of lethally irradiated mice with BM cells transfected with the gene encoding p210bcr-abl leads to the development of several fatal hematopoietic neoplasms. Taken together, these data provide evidence for bcr-abl gene participation in leukemogenesis.1-5-7

Analyses of fresh CML and normal hematopoietic BM cells show that p210bcr-abl, as well as the normal bcr and abl proteins, are expressed primarily in the early stages of myeloid maturation, and that levels of expression are reduced significantly as cells mature to polymorphonuclear leukocytes. The mechanisms by which the bcr-abl, bcr, and c-abl proteins are downregulated on differentiation of myeloid cells and cell lines are not known. This could occur at the transcriptional or posttranscriptional level.8 Additionally, recent studies have shown that K-562, a cell line established from a patient with CML in BC, shows amplification and enhanced expression of the c-abl oncogene.8-11 Other CML-BC cell lines including EM-2 and KCL-22 also show enhanced expression of the aberrant bcr-abl fusion transcript when compared with CP-CML cells.10

We have recently introduced a quantitative polymerase chain reaction (PCR) technique for the detection of the chimeric bcr-abl mRNA.12,13 This highly sensitive PCR technique facilitates the assessment of proliferation kinetics of residual neoplastic cells after BM transplantation, thus allowing early detection of an impending relapse.12-17 These observations provided the impetus to investigate whether increased expression of the aberrant bcr-abl fusion transcript is critical in the transition from CP to BC.

PATIENTS AND METHODS

Patients. Twenty-five patients with CML, diagnosed between September 1981 and November 1992 at our institutions were followed for 12 to 156 months after diagnosis (Table 1). Their median age at diagnosis was 46 years (range, 2 to 65 years). Twenty-four patients were in CP and 1 (patient no. 12) was in AC of the disease. During the observation period, 11 patients progressed either to BC

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(n = 7) or AC of disease (n = 4). In the former 7 patients, blasts had a myeloid (n = 6) or lymphoid (n = 1) phenotype. At diagnosis, 24 patients were Ph-positive; 1 patient (no. 8) was Ph-negative but bcr-abl-positive. The majority of the patients (n = 21) received interferon-α (IFN-α) treatment; 1 patient (no. 19) received treatment with IFN-α and Ara-C; 1 patient (no. 8) received treatment with hydroxyurea (HU); and 2 patients (no. 15 and 16) were monitored during IFN-α therapy after relapse following BMT. The IFN-α therapy was administered at doses ranging between 9 million (Mio) U/wk to 70 Mio U/wk. If a patient developed World Health Organization grade 3 or 4 toxicity or persistent grade 2 toxicity or failed to enter a hematologic remission in response to IFN-α, HU was added, and the dose of IFN-α was reduced. Hematologic and cytogenetic responses were evaluated according to criteria established by Kantarjian et al. Complete hematologic remission (CHR) was defined as normalization of both PB leukocyte counts and splenic size; partial hematologic remission (PHR) was defined as a decrease in leukocyte counts by at least 50% and to less than 20 g/L or persistence of splenomegaly with normalization of the hematologic parameters; and no response (NR) was defined as less than partial hematologic remission. Cytogenetic response was defined as any reduction in Ph-positive metaphases. A complete cytogenetic response (CCR) was defined as a total elimination of Ph chromosome in the BM metaphases. A major cytogenetic response (MCR) was defined as suppression of Ph chromosome to levels between 5% and 34%; a minor cytogenetic response (mCR) was defined as suppression of Ph chromosome to levels between 35% and 95%.

Definitions of accelerated and blastic phases of CML (1). Blastic phase CML was defined as 30% or more blasts in the BM or PB or extramedullary disease with localized immature blasts. Accelerated phase CML was assumed when one or more of the following was present: peripheral blasts, 15% or more; peripheral blasts plus promyelocytes, 30% or more; thrombocytopenia less than 100 g/L unrelated to therapy; and/or cytogenetic clonal evolution.

Southern blot analysis. In all patients, Southern blot analysis was performed. High molecular weight genomic DNA was prepared according to standard methods; digested with restriction enzymes Bgl II, BamHI, and HindIII; electrophoresed through a 0.8% agarose gel; and blotted onto a nylon membrane. For hybridization, we used a 1.2-kb 3' bcr probe (Oncogene Science, Uniondale, NY), labeled with 32P-dCTP. Autoradiography was performed for 24 to 72 hours at ~70°C. In selected patients (n = 7), quantitative Southern blot was performed. Quantification of hybridization signals on Southern blots was performed by densitometric analysis of the autoradiographs on a Hirschman Gelscript densitometer (Hirschmann Gerätebau, Untershaching, Germany). On the autoradiographs, all bands within a lane were analyzed together. The relative intensity of each band is expressed as a fraction of the intensity of all bands in the lane.

In all patients, cytogenetic investigation has been performed. Chromosome preparations were made from BM at presentation and during the clinical course using standard techniques. A median of 20 metaphases (range, 5 to 100 metaphases) were analyzed in each BM sample. Cytogenetic analysis was performed as described previously.

Qualitative and quantitative PCR. The presence of bcr-abl-rearranged cells was determined by qualitative PCR. In 18 patients, a bcr3a2 (k-28 junction) and, in 7 patients, a bcr2a2 (L-6 junction) mRNA was detected. All patients remained PCR-positive throughout the entire observation time. The 25 patients presented, serial quantitative PCR (Q-PCR) analyses were initiated during CP in 24 patients. Of these patients, 10 were monitored in CP and during progression to AC and/or BC.
the remaining 14 patients stayed in CP during the observation period. One patient (no. 12) was analyzed during the progression from AC to BC. Q-PCR was performed from the time of diagnosis on in 5 cases. In 5 patients, serial Q-PCR analyses were initiated during CP of disease before the onset of IFN therapy, and in 13 cases, at a median time of 12 months into IFN therapy. In 1 patient (no. 4), PCR analysis was started after discontinuation of IFN therapy because of persistent WHO grade-II toxicity, and 1 patient (no. 8) was investigated during therapy with HU only. Overall, 150 serial PB samples derived from the patients were investigated. The mean interval between the collection of PB specimen was 4 months. The number of consecutive samples analyzed by PCR in each patient ranged from 2 to 15, with a median of 5 samples per patient. All samples were analyzed by Q-PCR for the presence and the steady-state levels of the aberrant bcr-ab1 fusion transcript.

Mononuclear cells were isolated from PB by standard techniques and were stored at -70°C. The methods of RNA extraction,26 reverse transcription, and both qualitative and quantitative two-step PCR were recently published.22,3,15

Our competitive PCR assays aimed at the assessment of relative changes in the bcr-ab1 steady-state levels in serial mononuclear cell samples, rather than the measurement of absolute quantities of the chimeric mRNA molecules in leukemic cells. In each patient, consecutive samples were analyzed in parallel under identical experimental conditions. Repeated Q-PCR analyses of patient samples showed a variability of the results within a range of one dilution step of the internal standard. Such a change in the position of the equivalence point in consecutive samples could, therefore, reflect inaccuracies inherent in the technique, rather than an increase or decrease in the bcr-ab1 expression. Thus, evaluation of changes in the bcr-ab1 mRNA steady-state levels was performed according to the following definitions: (1) Constant was defined as unchanged levels of the bcr-ab1 transcripts or changes to a maximum of one dilution step, ie, 3.3-fold. (2) Decreasing was defined as a 10-fold or greater reduction of the bcr-ab1 transcripts. (3) Increasing was defined as a 10-fold or greater elevation of the bcr-ab1 transcripts. (4) Fluctuating was defined as undulating steady-state levels of the bcr-ab1 transcripts between one and two dilution steps, ie, 3.3 to less than 10-fold.

For diagnosis of changes in the bcr-ab1 mRNA steady-state levels, alterations in the relative marker gene expression had to be confirmed by the analysis of at least one additional PB sample from the patient.

For quantification, a variant bcr-ab1 cDNA fragment of slightly different size was used as a standard that yielded a PCR product of detectably different length when coamplified with the bcr-ab1 fragment from a patient sample using the same set of primers. Depending on the type of chimeric bcr-ab1 message expressed by the cells to be analyzed, the competitor cDNA was derived from either the cell line K562 or BV173, each of which expresses one of the bcr-ab1 fusion products, bcr3abl2 or bcr2abl2, respectively. Before the PCR, the standard and the patient cDNA were quantitated by an ethidium-bromide fluorescence assay. Twenty-five nanograms of total patient cDNA were added to each of the serial semilogarithmic dilutions of the standard cDNA ranging from 0.5 μg to 500 fg. The reaction mix contained 100 μmol/L concentrations of each dNTP, 20 pmol of each primer, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.0 mmol/L MgCl2, 0.001% gelatin, and 1.5 U of Taq polymerase (Cetus) in a volume of 25 or 50 μL. Amplification was performed in a Biomed (Theres, Germany) thermocycler during one or, when necessary, two subsequent rounds of PCR for 40 and 35 cycles, respectively. The PCR steps included denaturation for 180 seconds at 93°C initially and 40 seconds in each subsequent cycle, annealing for 25 seconds at 57°C, and extension for 40 seconds at 72°C with a final extension step for an additional 7 minutes. The sequences of the external primers used in the first round of PCR were 5'-TTC AGA AgC TCC CTG-3' (sense), 5'-CTC CAC Tgg CCA CAA AT-3' (antisense), and the sequences of the internal nested primers for the second round were 5'-gTg AAA CTC CAg ACT gTC-3' (sense), 5'-CAA CgA AAA gTg Tgg ggT-3' (antisense). Twenty-microliter aliquots of the PCR products were electrophorosed on 2% NuSieve (FMC Corp, Rockland, ME)/1% NA (Pharmacia, Uppsala, Sweden) agarose gels stained with ethidium bromide. The gels were photographed with negative film (Polaroid 665; Polaroid, Boston, MA), and the intensity of the bands was evaluated by densitometry.

We took precautions to prevent contamination as recommended by Kwok and Higushi.21 To assess the quality of the mRNA, all samples were analyzed on denaturing formamide agarose gels or amplified by PCR using primers for abl, β2-microglobulin or actin c-DNA. Samples that did not amplify with these primers were excluded from analysis. Sample collection, PCR sample preparation, and analysis were performed in different rooms. Only positive displacement pipettes were used. Different sets of pipettes were used for mRNA preparation and analysis of the amplified products. All primers and buffers were aliquoted. All samples were handled with disposable gloves that were changed frequently. All pipette tips and Eppendorf tubes were autoclaved before use. All experiments were run with at least one negative and one positive control. For positive controls, the CML cell lines K562 and BV173 were used. For negative controls, we analyzed leukocytes from healthy individuals and from the Ph-positive acute lymphoblastic leukemia cell line SUP B 15 in which the breakpoint on chromosome 22 lies outside of the 5.8-kb major breakpoint cluster region. To ensure the specificity and sensitivity of our PCR procedure, hybridization was performed using oligonucleotide probes recognizing the abl (abl internal probe) and bcr (bcr internal probe) sequences internal to the second-step nested primers. The level of sensitivity achieved was 1:10,000 after the first round and 1:1,000,000 after the second round of PCR.15

Statistical analysis. For statistical evaluation, Fisher's exact test was used (Stat Xact; Version 2.04; Cytel Software Corp, Boston, MA). All P values reported are two-sided.

RESULTS

We addressed the question whether the progression of disease from CP to AC or BC is associated with enhanced transcription of the fused bcr-ab1 mRNA and showed that the increase of steady-state levels of the bcr-ab1 fusion mRNA in the leukemic cells preceded the phenotypic transformation of the malignant clone. Results are summarized in Tables 2 and 3.

Patients progressing to AC of the disease and/or BC (n = 11). The median duration of CP in these patients was 51 months (range, 15 to 95 months). The first increase of bcr-ab1 transcripts was seen after a median CP duration of 45 months (range, 13 to 82 months). We observed an increase of bcr-ab1-specific mRNA steady-state levels in all patients who later progressed to BC (7 of 7) or to AC of disease (4 of 4). In 4 of these patients, data on bcr-ab1 levels pretreatment and posttreatment with IFN were available, and none of them showed a decrease in bcr-ab1 levels during treatment with IFN. Elevation of the bcr-ab1 transcripts occurred at a median time of 6 months (range, 0 to 16 months) before hematologic signs of disease progression according to the definition of AC (Table 2). Hence, at the time of first detection of increased bcr-ab1-specific mRNA, none of the criteria for AC1 were present in most of the patients (10 of 11). In 1 patient only, careful examination of PB smears indicated...

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the presence of more than 1% blasts at the time of increased bcr-abl mRNA (Table 2).

To investigate whether the elevated levels of bcr-abl mRNA reflected an increase in the proportion of leukemic cells in the samples analyzed or primarily an increase of steady-state levels of the bcr-abl fusion mRNA in the leukemic cells, Q-PCR data were compared with results of quantitative Southern blotting, cytogenetic analysis, and differential blood count (Fig 1a-d and Fig 2a-c).

In almost all patients within this group (n = 10), cytogenetic analysis showed constant presence of 100% Ph-positive metaphases throughout the observation period. Moreover, quantitative Southern blot analysis indicated a constant amount of bcr-abl-rearranged cells or a minor increase of the bcr-abl-rearranged band as compared with that of the germline band (<30%). Contemporaneous analyses of PB smears showed that changes in the bcr-abl expression occurred without changes in the proportion between mature and less mature mononuclear cells. By contrast, Q-PCR analysis indicated impending disease progression by showing a greater than 10-fold increase of the bcr-abl-specific mRNA (Fig 1, Table 2).

For example, in patient no. 15, Q-PCR showed an increase of bcr-abl mRNA expression by 2 logs while the patient was still in complete hematologic remission and no changes in therapy had been performed. Furthermore, careful examination of PB smears showed no increase in the absolute or relative number of immature mononuclear cells. Three months later, cytogenetic analysis showed signs of clonal evolution, and Southern blot indicated a minor increase of bcr-abl-rearranged DNA. Seven months later, the patient showed increasing numbers of white blood cells in the PB and thrombocytopenia unrelated to therapy. In patient no. 8, who had a Ph-negative, bcr-abl-positive CML, Q-PCR showed an increase of the rearranged mRNA, whereas Southern blot analysis showed constant amounts of bcr-abl-positive cells without any change in the proportion of immature cells. Progression to AC of the disease was observed 16 months thereafter (Figs 1a and c and Fig 2a).

**Patients remaining in CP (n = 14).** The median duration of CP in this group of patients was 49 months (range, 12 to 156 months). A total of 10 patients achieved a CHR, 2 patients achieved a PHR, and 2 patients showed NR to IFN therapy.

The majority of patients in CP showed decreased (n = 5) or constant steady-state levels (n = 7) of bcr-abl-specific mRNA in the leukemic cells (Table 3). In 2 patients (no. 18 and 25), fluctuating levels of bcr-abl mRNA have been detected without clinical or laboratory signs of AC during

### Table 2. Clinical and Laboratory Data in Patients Progressing to AC or BC

<table>
<thead>
<tr>
<th>No.</th>
<th>UPN</th>
<th>Treatment</th>
<th>Initial Response to Therapy</th>
<th>T1 (mo)</th>
<th>Clinical Status</th>
<th>Hem Status</th>
<th>WBC (µL)</th>
<th>Blasts (%)</th>
<th>PMC (%)</th>
<th>Pct Count (µL)</th>
<th>Ph (%)</th>
<th>Quantitative Southern Blot</th>
<th>Spleen</th>
<th>T2 (mo)</th>
</tr>
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<tbody>
<tr>
<td>21</td>
<td>CHR</td>
<td>18</td>
<td>CP</td>
<td>CHR</td>
<td>CHR</td>
<td>CH</td>
<td>4,090</td>
<td>0</td>
<td>0</td>
<td>166,000</td>
<td>100</td>
<td>—</td>
<td>Normal</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>CHR</td>
<td>8</td>
<td>CP</td>
<td>CHR</td>
<td>CHR</td>
<td>CH</td>
<td>6,000</td>
<td>0</td>
<td>0</td>
<td>127,000</td>
<td>100</td>
<td>Increase</td>
<td>Normal</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>CHR</td>
<td>43</td>
<td>CP</td>
<td>PHR</td>
<td>CHR</td>
<td>CH</td>
<td>2,300</td>
<td>0</td>
<td>0</td>
<td>175,000</td>
<td>0</td>
<td>Constant</td>
<td>Enlarged</td>
<td>8</td>
</tr>
<tr>
<td>16</td>
<td>CHR</td>
<td>8</td>
<td>CP</td>
<td>PPH</td>
<td>CHR</td>
<td>CH</td>
<td>9,250</td>
<td>1</td>
<td>0</td>
<td>790,000</td>
<td>100</td>
<td>Normal</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>CHR</td>
<td>61</td>
<td>CP</td>
<td>PPH</td>
<td>CHR</td>
<td>CH</td>
<td>8,750</td>
<td>1</td>
<td>0</td>
<td>695,000</td>
<td>100</td>
<td>—</td>
<td>Enlarged</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>PPR</td>
<td>13</td>
<td>CP</td>
<td>PPH</td>
<td>CHR</td>
<td>CH</td>
<td>5,900</td>
<td>1</td>
<td>0</td>
<td>557,000</td>
<td>100</td>
<td>Normal</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>PPR</td>
<td>99</td>
<td>CP</td>
<td>PPH</td>
<td>CHR</td>
<td>CH</td>
<td>5,220</td>
<td>0</td>
<td>0</td>
<td>134,000</td>
<td>100</td>
<td>—</td>
<td>Enlarged</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>PPR</td>
<td>82</td>
<td>CP</td>
<td>NR</td>
<td>CHR</td>
<td>CH</td>
<td>8,400</td>
<td>1</td>
<td>1</td>
<td>43,000</td>
<td>100</td>
<td>—</td>
<td>Enlarged</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>NR</td>
<td>52</td>
<td>CP</td>
<td>NR</td>
<td>CHR</td>
<td>CH</td>
<td>31,000</td>
<td>1</td>
<td>1</td>
<td>694,000</td>
<td>Ph</td>
<td>Constant</td>
<td>Enlarged</td>
<td>16</td>
</tr>
<tr>
<td>9</td>
<td>NR</td>
<td>62</td>
<td>CP</td>
<td>NR</td>
<td>CHR</td>
<td>CH</td>
<td>32,000</td>
<td>7</td>
<td>3</td>
<td>131,000</td>
<td>100</td>
<td>—</td>
<td>Enlarged</td>
<td>13</td>
</tr>
<tr>
<td>12</td>
<td>CHR</td>
<td>45</td>
<td>AC</td>
<td>NR</td>
<td>CHR</td>
<td>CH</td>
<td>79,000</td>
<td>1</td>
<td>8</td>
<td>32,000</td>
<td>100</td>
<td>—</td>
<td>Enlarged</td>
<td>0</td>
</tr>
</tbody>
</table>

See Materials and Methods section for definitions of AC and BC.

Abbreviations: UPN, unique patient number; T1, time from diagnosis to first bcr-abl mRNA increase; Hem Status, hematologic status; WBC, white blood cell count; PMC, polymorphonuclear cells; Pct Count, platelet count; —, not done; T2, time from first bcr-abl mRNA increase to AC or BC; CHR, complete hematologic remission; PPH, partial hematologic remission; NR, no response.

### Table 3. Results in Patients Who Remained in CP Throughout the Observation Period

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Initial Response to Therapy</th>
<th>Hematol</th>
<th>%Ph</th>
<th>O-S Blot</th>
<th>Q-PCR</th>
<th>Current Status</th>
</tr>
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<tr>
<td>23</td>
<td>CHR</td>
<td>55</td>
<td>Decrease</td>
<td>Decr</td>
<td>CP, alive 29 mo</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>CHR</td>
<td>0</td>
<td>Decrease</td>
<td>Decr</td>
<td>CP, alive 25 mo</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>CHR</td>
<td>ND</td>
<td>Decrease</td>
<td>Decr</td>
<td>CP, died 12 mo*</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>CHR</td>
<td>20</td>
<td>—</td>
<td>Decr</td>
<td>CP, alive 159 mo</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>CHR</td>
<td>100</td>
<td>—</td>
<td>Decr</td>
<td>CP, alive 83 mo</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>CHR</td>
<td>0</td>
<td>—</td>
<td>Const</td>
<td>CP, alive 46 mo</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>CHR</td>
<td>90</td>
<td>—</td>
<td>Const</td>
<td>CP, alive 64 mo</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>CHR</td>
<td>77</td>
<td>—</td>
<td>Const</td>
<td>CP, alive 74 mo</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CHR</td>
<td>100</td>
<td>—</td>
<td>Const</td>
<td>CP, alive 38 mo</td>
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<tr>
<td>7</td>
<td>PPR</td>
<td>100</td>
<td>—</td>
<td>Const</td>
<td>CP, alive 141 mo</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>NR</td>
<td>—</td>
<td>Increase</td>
<td>Const</td>
<td>CP, alive 80 mo</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>CHR-PRH</td>
<td>80</td>
<td>—</td>
<td>Fluct</td>
<td>CP, alive 20 mo</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>NR</td>
<td>100</td>
<td>—</td>
<td>Fluct</td>
<td>CP, alive 42 mo</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Hematol, hematologic response; O-S Blot, quantitative Southern blot; Decr, decrease of the bcr-abl transcripts; Const, constant levels of the bcr-abl transcripts; Fluct, fluctuating levels of the bcr-abl transcripts; mo, months after diagnosis; —, not done.

* Patient died of septicemia after BM transplantation.
BCR-ABL INCREASE PRECEDES DISEASE PROGRESSION

Fig 1. (a and b) Quantitative monitoring of the bcr-abl mRNA expression by competitive PCR during CP and AC of disease in CML patients. Two exemplary patients (a, patient no. 11; b, patient no. 8) are displayed showing increasing bcr-abl mRNA levels in serial samples derived from PB. The arrows indicate points of equivalent signal intensity between serially diluted internal standard cDNA representing known concentrations of bcr-abl transcript and the patient cDNA tested. Because of the competitive nature of the PCR technique used, the shift of the equivalence point (arrow) to the right shows an increase of the bcr-abl mRNA level in the specimen analyzed. The amount of competitor cDNA in each reaction is indicated by the numbers 1 to 7. The serial dilutions of the internal standard were semilogarithmic, the dilution factor being the square root of 10 (ie, every second step represents a 10-fold dilution). Lane 1, 10 pg/μL standard cDNA; lane 3, 100 pg/μL standard cDNA; lane 5, 1 ng/μL standard cDNA; lane 7, 10 ng/μL standard cDNA. As indicated by the position of the arrows, continuously increasing relative quantities of the bcr-abl transcript (a, lanes B, C, and D) were detected in patient no. 11 during CP of the disease 8 months before (lane B) diagnosis of an extramedullary BC. In patient no. 8, increasing bcr-abl mRNA levels were detected (b, lanes B and C) during CP 16 months before (lane B) diagnosis of AC. (c and d) Southern blot analyses in patients no. 8 and 11. To investigate whether the elevated levels of bcr-abl mRNA reflected an increase in the proportion of leukemic cells in the samples analyzed (comparison of the PCR results with the differential blood cell count is shown in Table 2 and Fig 2) or reflected primarily enhanced transcriptional activity of the bcr-abl fusion gene, we performed quantitative analyses of the bcr-abl gene at the DNA level. Aliquots of the same cell samples used for RNA extraction were used for DNA preparation. Autoradiographs used for densitometric analyses in patients no. 8 and 11 are shown in (c) and (d). The upper band (G) represents the unarranged bcr allele and the lower band (R) represents the rearranged bcr allele. Lanes A to D in (c) and lanes A to C in (d) represent time points corresponding to those shown in (a) and (b), respectively. The densitometric values (ratio of the rearranged bcr gene to the normal bcr gene) remained constant throughout the entire observation period in both patients. G, germline band; R, rearranged bcr band.
follow-up periods of 13 and 20 months, respectively. A comparison of Q-PCR results with cytogenetic analyses, Southern blot data, and hematologic response to therapy is shown in Table 3.

Of 10 patients in CHR, 6 showed a cytogenetic response to IFN-α therapy, 2 patients showed a CCR, 1 showed an MCR, and 3 showed an mCR. In an additional patient (no. 25), an mCR was detected in CHR; this patient is currently in PHR and shows fluctuating levels of the chimeric mRNA. Quantitative Southern blot analysis showed a decreased intensity of the rearranged bcr-abl-specific band as compared with that of the germline band in 3 of these 7 patients. In 1 patient (no. 14) who showed NR to IFN therapy, Q-Southern blot analysis showed an increase of the rearranged bcr-abl-specific band. The remaining patients had constant Southern blot, cytogenetic and hematologic findings throughout the observation period (Table 3).

All patients with CHR showed decreased (n = 5) or constant (n = 5) levels of the bcr-abl transcripts throughout the observation period. Therefore, our data show a highly significant association between clinical course of disease and steady-state levels of the bcr-abl transcript (P < .0001).

**DISCUSSION**

In this study, we have shown that an increase of bcr-abl-specific mRNA that precedes disease progression reflects elevated steady-state levels of the fusion message in the leukemic cells of CML patients. A possible interpretation of our observation is that IFN downregulates transcription of bcr-abl mRNA. Therefore, the increase in bcr-abl mRNA observed could indicate an acquired partial resistance to IFN. The elevation of bcr-abl mRNA could indicate that these patients are prone to transformation, rather than the increase in bcr-abl being causally related to transformation. To investigate this hypothesis, patients with elevated versus nonelevated levels of bcr-abl mRNA were compared. Both groups had identical median PCR observation periods of 29 months and received equal doses of IFN, with a median of 35 Mio IU/wk. Moreover, responders and nonresponders to IFN therapy had received equal doses of IFN. At the time of first detection of increase in bcr-abl mRNA, 6 patients were either in CHR (n = 2) or PHR (n = 4) in response to IFN therapy. In 4 of these patients, data on bcr-abl levels pretreatment and posttreatment with IFN were available, and none of them showed a decrease in bcr-abl levels during treatment with IFN. Furthermore, in 2 patients not receiving IFN, an increase in bcr-abl mRNA was observed during CP, 10 and 16 months before disease progression. Thus, our data indicate that increase of bcr-abl mRNA precedes hematologic resistance to antileukemic therapy, which ultimately results in disease progression.

Observations by Wetzler et al\(^5,6\) indicate that expression
of the bcr-ab1 protein in CML cells is inversely related to maturation. Therefore, another possible explanation for our findings could be that Q-PCR data reflect the proportion of less to more mature mononuclear cells in the preparation. However, contemporaneous analyses of PB smears showed that changes in bcr-ab1 expression occurred without substantial changes in the proportion between mature and less mature mononuclear cells. Moreover, elevation of bcr-ab1 transcripts preceded increase of immature cells by a median time span of 6 months. So far, the mechanisms by which the bcr-ab1 protein is downregulated during differentiation of myeloid cells and cell lines are not known. McWhirter et al. have found that components of the cytoskeletal framework may be the principal substrates of the bcr-ab1 kinase. Phosphorylation of these substrates may block transduction of the growth-inhibitory signal, thus allowing deregulated growth of the Ph-positive stem cells.

Quantitative Southern blot analyses of the rearranged bcr-ab1 gene, cytogenetic evaluation of the Ph chromosome, and thorough examination of PB smears showed no correlation between increase of chimeric mRNA and increase of leukemic cells or less differentiated myeloid cells in the PB. Thus, our observations can be explained by increased amounts of the bcr-ab1 mRNA in the leukemic cells rather than an increase of leukemic or immature cells in the PB of the patients analyzed. This observation also was made in patients who were in CHR and showed constant percentages of Ph-positive metaphases and bcr-ab1-rearranged cells.

By contrast, all patients (n = 14) who remained in CP of disease showed decreased, constant, or fluctuating expression of the chimeric mRNA. These findings indicate a highly significant association between Q-PCR results and clinical course of disease in CML patients (P < .0001). According to our data, the Q-PCR technique facilitates early assessment of the course of disease, because, in the majority of patients, an increase of the chimeric mRNA is detectable while the patient is still in CP without any signs of clinical or hematologic disease progression. Therefore, the increase of bcr-ab1-specific mRNA in leukemic cells detected in consecutive samples may be interpreted as molecular acceleration of disease, provided that no changes in therapy were made.

We have shown that increasing levels of bcr-ab1 mRNA can be detected in patients showing constant amounts of bcr-ab1 fusion DNA in consecutive samples, without changes in the proportion of less to more mature mononuclear cells before progression of disease. Increased transcription of the bcr-ab1 message and possibly the resulting higher expression of p210^Ph^{-}/^Ph^{+} protein may lead to impaired maturation and, thus, to increased numbers of phenotypically immature cells in the PB. The occurrence of diverse clonal evolutions suggests that a variety of molecular mechanisms may be involved in the transition to terminal BC. Our data indicate that increased expression of bcr-ab1 transcripts could be one of these mechanisms.

Based on our data, we conclude that an increase of bcr-ab1 mRNA precedes BC. Molecular acceleration appears to be an early event during progression of CML to acute phase of disease. The cause of the increased mRNA expression is not known. However, it is likely that additional genetic changes associated with or independent from bcr-ab1 regulation are involved in the molecular acceleration.

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Increase of bcr-abl chimeric mRNA expression in tumor cells of patients with chronic myeloid leukemia precedes disease progression

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