BCR-ABL Protein Expression in Peripheral Blood Cells of Chronic Myelogenous Leukemia Patients Undergoing Therapy

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Chronic myelogenous leukemia (CML) is a myeloproliferative disorder associated with the Philadelphia chromosome (Ph') in more than 95% of these patients. The Ph' and the resulting BCR-ABL fused genes are markers for this type of leukemia. In CML, the product of the fused BCR-ABL gene is typically a protein of approximately 2,000 amino acids termed P210 BCR-ABL. We have developed an assay for the BCR-ABL protein involving Western blotting of circulating white blood cells (WBC) with an anti-ABL monoclonal antibody that can detect P210 BCR-ABL and P145 ABL in peripheral blood cells from chronic phase Ph'-positive leukemia patients. This assay was used to analyze the BCR-ABL protein content of circulating WBC from CML patients before and after various treatments. In parallel to changes in percentages of Ph'-positive blood cells as determined by cytogenetic analyses of bone marrow samples, BCR-ABL protein expression in blood cells decreased or increased as patients entered remission or underwent relapse. Of interest, six Ph'-negative CML patients were BCR-ABL protein-positive. All except one had a rearrangement in the major breakpoint cluster region and that patient expressed P185 BCR-ABL and not P210. Our results indicate that the BCR-ABL Western blotting assay has clinical applications for both diagnosis and prospective evaluation of Ph'-positive and Ph'-negative CML patients.

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CHRONIC MYELOGENOUS leukemia (CML) is characterized most frequently by its association with an abnormal chromosome 22, known as the Philadelphia chromosome (Ph'). It is estimated that at least 95% of CML cases possess the Ph'. In CML, this abnormal chromosome fuses a central portion of the BCR gene to the second exon of the ABL gene. The protein product produced by this fusion is termed P210 BCR-ABL. In one form the protein contains 927 amino acids encoded by the 5' portion of the BCR gene fused to 1096 amino acids encoded by the ABL gene. A second form fuses 902 amino acids of ABL fused to the same ABL sequences. These two forms of BCR-ABL protein together with alternate splicing patterns result from the fusion of one of two small central exons termed either b2 or b3 to the second ABL exon termed a2. RNA analyses indicate that CML patients can have either form or both, known as b2a2 or b3a2 junctions.

Another form of the BCR-ABL protein is detected typically in Ph'-positive acute lymphocytic leukemia (ALL). This form fuses the relatively long first exon of BCR (426 amino acids) to ABL exon 2 (a2). This protein termed P190 or P185 BCR-ABL has a higher specific protein kinase activity than P210 BCR-ABL. P185 BCR-ABL also has more neoplastic transformation activity than P210 as measured in cell culture systems. Consistent with its increased potency, P185 BCR-ABL expression is typically but not always detected in Ph'-positive ALL, which is a subgroup of ALL with a poor prognosis.

One important goal in CML is to develop methods to monitor the level of BCR-ABL proteins and their activity during treatment. A first attempt to do this was accomplished several years ago by using an in vitro kinase assay to measure the autophosphorylation activity of the activated ABL tyrosine protein kinase present in these BCR-ABL proteins. Unfortunately, although useful for detecting active BCR-ABL tyrosine kinase activity in terminal stage patients (blast crisis), this assay is not useful in monitoring patients in the early stage of this disease known as the chronic or benign stage. Lysis of mature blood cells harboring the BCR-ABL protein causes rapid destruction of this and other proteins. A Western blotting assay was developed that allows detection of BCR-ABL protein at early as well as late stages of CML. Using this assay, we have also been able to detect the P185 form of BCR-ABL protein in white blood cells (WBC) from a patient in chronic phase of CML. Cells from this patient lacked P210 BCR-ABL and lacked a DNA rearrangement in the major breakpoint cluster region (BCR) of the BCR gene. Furthermore, RNA extracted from this patient contained only transcripts with the BCR exon 1 fused to ABL exon 2.

We now report results of analyses of CML patients undergoing treatment, either chemotherapy or bone marrow transplantation. Of importance, six patients lacking a detectable Ph' at diagnosis were found to express BCR-ABL proteins in their peripheral WBC.

MATERIALS AND METHODS

Patient samples. Western blotting assays for all patient samples were performed in a BCR-ABL protein screening laboratory within the Department of Molecular Pathology at The University of Texas M.D. Anderson Cancer Center in Houston. Blood samples were obtained from patients as part of a program/project study sponsored by The National Cancer Institute (NCI). All patients signed an appropriate informed consent form. Peripheral blood samples yielding at least 2 × 10⁶ WBC were cryopreserved. Ph' percentages were obtained by cytogenetic analysis of bone marrow samples obtained on the same day as the blood sample thus allowing comparison between our results and Ph' chromosome percentage. Cytogenetic analyses

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were performed by the Division of Laboratory Medicine. CML and ALL were diagnosed according to standard criteria. Normal WBC were donated by volunteers from our laboratory.

**Cell lines.** The K562 (positive control), HL-60, and KG-1 cell lines (negative controls) were grown in RPMI medium containing 10% fetal bovine serum. K562 cells expressing P210 BCR-ABL were derived from a CML patient in blast crisis. KG-1 cells were derived from an acute myelogenous leukemia patient lacking BCR-ABL proteins.

**8E9 antibody.** The 8E9 antibody was originally isolated by Richardson et al. It is a mouse monoclonal antibody directed toward the SH2 region of the mouse gag-ABL protein.

**Processing of patient cells.** Peripheral blood was collected in heparanized tubes at room temperature. Blood was treated with a mixture of protease inhibitors immediately after receiving the specimens. Red blood cells were removed by two cycles of treatment with NH4Cl. Of interest, heparanized blood received from Ph+ positive and negative leukemia patients, but an example is shown in Fig 1. In this experiment, increasing numbers of K562 cells were added to KG-1 cells, totaling 1 x 10^5 K562 cells and 1 x 10^6 KG-1 cells. The samples were analyzed on 6.5% polyacrylamide gels, the gels were electroblotted at 4°C overnight and transferred to Immobilon P filters (Millipore, Bedford, MA).

**Western blotting procedure.** The BCR-ABL Western blotting test was performed as described, but modified to increase the sensitivity. The more sensitive test involved two fundamental changes. First the enhanced chemiluminescent (ECL) detection system was used in place of the [22] probe A detection system. Second, the backgrounds were dramatically reduced by substituting powdered milk for bovine serum albumin (BSA) in our blocking solutions. The detailed procedure is as follows: Western blotting was performed with an anti-ABL (8E9) monoclonal antibody as described previously. Briefly, frozen WBC were lysed in boiling sodium dodecyl sulfate (SDS) sample buffer for 5 to 7 minutes, and the lysate was clarified by centrifugation. Aliquots of the extracts corresponding to 10^6 cells were applied to each gel lane. Samples were electrophoresed through 6.5% polyacrylamide gels, the gels were electrophoretically transferred to Immobilon P filters (Millipore, Bedford, MA). The ECL Western blotting detection system was used to probe for BCR-ABL protein according to the manufacturer’s protocol (Amersham, Arlington Heights, IL). Filters were preblocked by washing with 10% non-fat milk (NFM) in Tris-buffered saline-Tween 20 (TBS-T) buffer (20 mmol/L Tris base, 137 mmol/L NaCl, 0.0038 N HCl) for 2 hours and then incubated with 1:15,000 to 20,000 dilution of 8E9 in 5% NFM TBS-T buffer overnight at room temperature. The filters were then incubated with a 1:3,000 to 3,500 dilution of horseradish peroxidase (HRP)-labeled sheep anti-mouse IgG (Amersham, Cat. No. NA 9310) for 2 hours. The filters were then incubated with various mixtures of cell extracts from K562 cells and KG-1 cells were analyzed. Each lane was loaded with cell extract from 10^6 cells. The amount of K562 + KG-1 cells used in each lane is as follows: lane 1, 1 x 10^6 K562 cells; lane 2, 5 x 10^5 K562 cells; lane 3, 2.5 x 10^6 KG-1 cells; lane 4, 1.2 x 10^7; lane 5, 6 x 10^6; lane 6, 3 x 10^7; lane 7, 1.5 x 10^8; lane 8, 7 x 10^7; lane 9, 2 x 10^8; lane 10, 1.7 x 10^7; lane 11, 8.5 x 10^6; lane 12, 1 x 10^7 KG-1 cells. The samples were analyzed on 6.5% gel, molecular weight marker was included. Exposure time: 8 minutes. BCR-ABL/ABL protein ratios were plotted versus the number of K562 cells ranging from 6 x 10^4 to 1 x 10^6 cells in a mixture with KG-1 cells, totaling 1 x 10^6 cells (B) and 0 to 6 x 10^5 K562 cells (C).

**RESULTS**

**Increased sensitivity of the ECL detection system.** After a number of unsuccessful attempts to improve the sensitivity of the test by various methods, we found that the ECL system could improve the sensitivity more than 100-fold. A typical example is shown in Fig 1A. In this experiment, increasing concentrations of K562 cells were added to KG-1 cells so
that in all cases the total number of cells was kept constant at 10^9. The results show that approximately 2,000 to 3,000 K562 cells gave a detectable BCR-ABL signal in a mixture of 10^9 KG-1 cells (i.e., 0.3%) (Fig 1A, lanes 9 and 10). About 3,000 K562 cells could also be detected in a mixture of 10 million KG-1 cells (results not shown). A plot of the BCR-ABL protein response versus the number of K562 cells showed that approximately 2,000 to 3,000 K562 cells could also be detected in a mixture of 10 million KG-1 cells (i.e., band intensity). The ratios of BCR-ABL to ABL proteins were plotted versus the K562 cell number.

Similar results were obtained with mixtures of WBC from CML chronic phase patients that were 100% Ph' positive when mixed with normal WBC (Fig 2). These results clearly showed that with cells from one patient (J.S.), as few as 1.8 × 10^3 scored positive for BCR-ABL protein expression (Fig 2, lane 10). In other studies, there was a positive correlation between the ratio of BCR-ABL to ABL proteins in the peripheral blood sample and the percentage of Ph' positive cells in the bone marrow (manuscript in prep).

**BCR-ABL protein expression in individual patient samples.** Using the more sensitive assay, Western blot analysis has been performed on more than 250 blood samples from Ph' positive CML patients at various stages of treatment, many of which had cytogenetic analyses performed on bone marrow cells on the same day. Typically, the relative intensity of the BCR-ABL protein bands compared with the ABL protein band increased or decreased as the Ph' percentage in the marrow increased or decreased. As an example, we show Western blotting data on peripheral WBC together with cytogenetic results of nine such patients as they underwent therapy. Figure 3A shows blots of a patient (patient M.J.) who was treated with interferon and homoharringtonine simultaneously. PCR analyses indicated that M.J. had a b3/a2 BCR-ABL junction. After a brief cytogenetic remission in May of 1992, the patient relapsed because the therapy was stopped due to severe anemia. The patient's peripheral blood cells showed a marked increase in the BCR-ABL protein, seen as early as November of 1992. Patient J.J. (b3/a2) underwent 24 months of homoharringtonine/interferon treatment. Cytogenetic analyses on marrow indicated that diploid cells were predominant in this patient (Nov 1993). The blood sample analyses indicate a low level of BCR-ABL protein throughout the course of treatment, consistent with the cytogenetic results (Fig 3A).

Blood samples from two other patients were analyzed during the course of their chemotherapy. Figure 3B shows Western blot results from patient J.R. (b3/a2) who was treated with interferon alone. The patient entered into remission as of January of 1992, as indicated by cytogenetic studies where the bone marrow contained 40% Ph' positive cells (6 of 15 cells analyzed). BCR-ABL protein expression decreased dramatically at that time and remained undetectable on two other occasions in 1993. Figure 3B also shows the results of patient S.F., who was resistant to homoharringtonine treatment but after changing to interferon, responded well based on BCR-ABL protein analysis in March of 1993. At that time the bone marrow showed 30% of the cells to be Ph'-positive. In September of 1993, the patient lacked detectable BCR-ABL in peripheral blood cells and contained one Ph'-positive metaphase in 20 analyzed.

Figure 4 shows BCR-ABL Western blotting results of five patients undergoing bone marrow transplantation receiving...
Fig 3. Changes in BCR-ABL protein expression in peripheral blood cells following chemotherapy. BCR-ABL Western blotting analyses and cytogenetic tests were performed on blood and bone marrow samples, respectively, from four CML patients at various stages of treatment with chemotherapy. Western blot analyses were performed on gels in which positive and negative control cell lines were analyzed at the same time along with molecular weight markers. Dates of BCR-ABL Western blotting are shown at the bottom of gel lane; results (Ph%) and dates of bone marrow cytogenetic tests also are shown at the bottom of each lane, if performed. (A) Patient M.J. initially showed partial cytogenetic remission (5/26/92) and then relapsed as is evident in the 11/12/92 sample. Patient J.J. shows decreasing Ph' percentage from 96% to 10% after undergoing about 20 months HHT/IFN therapy. (B) Patient J.R. was treated with interferon. Patient S.F. was initially treated with Homoharringtonine and later with interferon.
cells from a healthy donor. Patient T.S. (b2/a2) (Fig 4A) had high levels of BCR-ABL proteins in his blood cells before the transplant. After transplant, patient T.S. contained no detectable BCR-ABL protein as analyzed on six occasions through May of 1993, but was consistently b2/a2 positive after transplant except on May 15, 1992 and May 28, 1993, which were negative by PCR. There was an excellent correlation with cytogenetic studies, as the patient was 80% Ph1-positive before transplant (16 of 20 cells positive for the Ph1), but lacked the Ph1 after transplant. Patient K.H. (b2/a2) (Fig 4B), although lacking detectable BCR-ABL protein and containing only diploid cells in the marrow shortly after receiving an allotransplant, was weakly positive for BCR-ABL protein in peripheral blood cells at about 7 months posttransplant. Of importance, cytogenetic analysis failed to detect a Ph1-positive cell in the bone marrow of this patient analyzed on the same day. Of interest, patient K.H. had the b2/a2 junction on June 30, 1993. Patient D.S. (b2/a2) received an allogeneic bone marrow transplant in April of 1993. Before that, patient D.S. was strongly BCR-ABL pro-
protein positive and 100% Ph't-positive (Fig 4B). After the transplant, the patient lacked detectable BCR-ABL protein in blood cells and no detectable Ph't in marrow cells. Patient A.H. (b2/a2) (Fig 4C) underwent an allotransplant in October of 1990. Western blot analyses on three occasions in 1991 did not detect BCR-ABL protein expression in circulating blood cells, although the patient was b2/a2 positive on September 13, 1991. After 17 months, this patient relapsed as indicated in the Western blot of March of 1992. This was confirmed by PCR as the patient was b2/a2 positive. Subsequent analyses through June of 1993 showed a high level of BCR-ABL protein in blood cells and 100% Ph't-positive marrow cells in samples analyzed on the same day. Patient S.H. (b2/a2) (Fig 4C) showed a relapse following an allotransplant some 30 months earlier. This patient had the b2/a2 junction before and after transplant. BCR-ABL proteins were present in high amounts in his peripheral blood cells at that point and bone marrow cytogenetic analyses showed 18 of 19 cells to be Ph't-positive. In summary, all nine patients showed a strong correlation between cytogenetic findings within marrow cells and the level BCR-ABL protein expression in peripheral blood cells. The use of P145 ABL as an internal control clearly showed a diminution of the intensity of the BCR-ABL bands compared with the P145 ABL band as the percentage of Ph't-positive cells decreased. Moreover, as patients underwent cytogenetic relapse, the intensity of BCR-ABL bands showed a corresponding increase relative to the P145 ABL band.

Our results show that we can detect the BCR-ABL protein both in peripheral blood samples and bone marrow samples (Fig 5, patient 8, P & B). Also, our Western blot assay not only detected P210 BCR-ABL in Ph't-positive CML patients, but also detected the BCR-ABL protein in Ph't-positive ALL, either P210 BCR-ABL (patient no. 7) or P185/P190 BCR-ABL (patient no. 9).

Using the more sensitive assay, we also analyzed clinical samples from patients with various other medical problems (Fig 5). Analysis of a large number of blood samples (more than 95 at this point) have not yet detected a false positive, as BCR-ABL proteins were not detected in these samples. Similarly, in more than 231 CML patients (357 blood samples) that were 100% Ph't-positive, we have not had a false negative (manuscript in preparation).

**BCR-ABL protein detection in Ph't-negative patients.** We also analyzed blood samples from six patients that were found to lack a detectable Ph't at diagnosis of disease (originally Ph't-negative) (Fig 6A-C). Western blotting results detected P210 BCR-ABL in five of these patients. Patients J.N. (b2/a2), T.D. (b3/a2), G.S., and J.L. (b2/a2) were BCR-ABL protein-positive in more than one blood sample taken on different dates (Fig 6A and B). Of interest, blood cells from patient J.M. lacked P210 BCR-ABL expression but contained P185 BCR-ABL (Fig 6C). To verify the presence of a rearranged BCR gene, Southern blotting using a bcr probe was performed on these six patients. The results indicated that five of these BCR-ABL protein-positive patients had a rearrangement in the major bcr site and one patient (J.M.) lacked detectable major bcr rearrangement (germ line) (Table 1). The finding that patient J.M. expressed P185 and not P210 BCR-ABL is consistent with the lack of rearrangement in the major bcr region. Presumably, this patient has a break in the first intron of the bcr gene. A seventh patient (P.H.) also diagnosed as Ph't-negative, bcr-positive, was not informative because her blood cells gave only a faint P145 ABL signal (not shown). These findings emphasize the medical utility of the BCR-ABL Western blotting test in Ph't-associated leukemias. Our results indicate that it is more reliable for diagnosing these leukemias than either classical cytogenetics or the widely used bcr Southern blotting assay.

**DISCUSSION**

The results presented here indicate that the level of BCR-ABL protein expression in peripheral WBC mimics the changes in percentages of Ph't-positive cells in bone marrow cell populations. An improved assay was developed that can detect BCR-ABL protein expression in mixed populations of normal and leukemic cells. BCR-ABL expression can be detected in mixtures of BCR-ABL negative cells containing...
Fig 6. Detection of BCR-ABL protein in peripheral blood cells from Ph' negative CML patients. (A) Patient J.N., patient T.D., and patient G.S.; (B) patient J.L.; (C) patient J.B. and patient J.M.; samples were analyzed by BCR-ABL Western blotting. Patient J.M. was assayed by the [\(^{125}\)I] procedure.\(^9\) As in Figs 3 and 4, appropriate positive and negative controls as well as molecular weight markers were run on the same gel in each case.

As few as 2,000 to 3,000 K562 (Fig 1A). Moreover, the intensity of the P210 BCR-ABL band is proportional to the number of BCR-ABL cells in the mixture even at very low levels (Fig 1B and C). In studies carried out with 100% Ph'-positive chronic phase CML patients, BCR-ABL protein expression can be detected in mixtures of WBC that contain as few as 0.2% to 0.4% BCR-ABL expressing leukemic cells (approximately 20,000 to 40,000 leukemic cells in a mixture of 10 million cells) (Fig 2). Thus, in addition to its medical utility for diagnosis, the BCR-ABL protein test performed on peripheral blood provides a convenient way to monitor CML patients as they enter remission or as they undergo relapse.
However, it is clear that even with the improved BCR-ABL test, mixtures of 10^7 circulating WBC containing significantly less than 10^7 cells leukemic cells will score negative. Moreover, in CML patients who are in remission and have less than 20% Ph'-positive bone marrow cells, BCR-ABL protein detection in circulating blood cells may in some cases fall below the level of detection. Of interest, patients that lack detectable Ph'-positive cells in bone marrow cytogenetic assays may in some cases score positive for BCR-ABL protein expression in blood cells (Fig 4B, patient K.H.). Thus, cytogenetic bone marrow analyses and BCR-ABL protein expression in circulating blood cells may not always agree in CML patients with less than 20% Ph'-positive bone marrow cells (manuscript in preparation). Nevertheless, the assay should be quite useful to physicians, as reduction of leukemic cells by more than two logs can be discerned by assay of peripheral WBC from the patient. Although further studies are needed to determine the percentage of false-positives, our experience with more than 350 patients with various types of leukemias, lymphomas, and other diseases shows a perfect correlation between diagnosis of CML and BCR-ABL protein expression (manuscript in preparation).

Studies of nine CML patients at various stages of treatment and remission and/or relapse showed a clear correlation between the patients cytogenetic analyses for the Ph' in marrow cells and BCR-ABL protein levels in circulating WBC. Examples of these types of analyses are shown in Figs 3 and 4. The BCR-ABL Western blot test was also shown to detect both forms of BCR-ABL proteins, either P210 or P185 BCR-ABL, in Ph'-positive ALL patients (Fig 5). A study is underway to establish firmly the test's usefulness for diagnosing Ph'-positive ALL (H. Kantarjian, J.Q. Guo, and Arlinghaus, in prep).

Also of importance, analysis of blood samples from six patients with a history of being Ph'-negative revealed that they were BCR-ABL protein-positive despite being Ph'-negative (Table 1, Fig 6). One of these patients lacking the Ph' was also bcr negative, whereas the other five were bcr positive. Of interest, this patient expressed P185 BCR-ABL and not P210 BCR-ABL, suggesting the presence of a BCR exon 1: abl 2 junction. A similar phenotype has been previously reported by us in a chronic phase CML patient. Further studies are in progress on cells from this Ph'-negative patient to identify the type of junction.

Considering the 100% correlation with the presence of the Ph' in newly diagnosed CML patients and the lack of false-positives in patients with other diseases (manuscript in preparation), the BCR-ABL Western blotting assay has value as a relatively easy means of assessing whether or not patients with leukostasis and/or myeloproliferative syndrome have Ph'-positive leukemia.

The sensitivity of the BCR-ABL Western blotting assay does not compare with that of the polymerase chain reaction (PCR) methodology, as indicated by comparing PCR results with BCR-ABL Western blotting data on patients T.S., A.H., and S.H. (Fig 4). However, the detection of the BCR-ABL gene product in peripheral blood cells has importance based on two considerations. First, it indicates that the patient's cells are expressing the BCR-ABL gene product (which may or may not be kinase active). Second, detection of leukemic cells in the peripheral blood indicates that the leukemic clone is actively dividing. Further, studies are needed to determine whether low levels of detectable BCR-ABL protein in circulating blood cells has clinical prognostic significance in patients who are in remission.

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