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Advances in Brief

Detection of BCR-ABL Proteins in Blood Cells of Benign Phase Chronic Myelogenous Leukemia Patients[1]

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Abstract

More than 95% of patients with chronic myelogenous leukemia (CML) contain an abnormal chromosome termed the Philadelphia chromosome (Ph^1). Ph^1 and the resulting *BCR-ABL* fused genes are markers for this type of leukemia. The product of the fused *BCR-ABL* genes is a protein of about 2000 amino acids termed P210 BCR-ABL. Although the BCR-ABL protein can be routinely detected in blood cells from blast crisis CML patients by assaying for its activated tyrosine kinase activity, detection of P210 BCR-ABL in early stage CML patients (chronic phase) has not yet been possible (S. A. Maxwell *et al.*, Cancer Res., *47:* 1731, 1987). A procedure involving Western blotting with an anti-ABL monoclonal antibody was developed that allows detection of P210 BCR-ABL and P145 ABL in cells from chronic phase and blast crisis CML patients, but as expected only P145 ABL was found in normal white blood cells. Most chronic phase patients also contained one to two ABL proteins with a molecular weight of about 190,000. Interestingly, the ratio of BCR-ABL to ABL proteins increased in four blast crisis patients compared to 18 chronic phase patients. Also, one chronic phase patient analyzed on three separate occasions lacked P210 BCR-ABL and exhibited only the M_r 190,000 form. This assay should also be useful in other leukemias that express altered forms of the ABL protein.

Introduction

More than 95% of patients with CML[3] possess the Philadelphia chromosome (Ph^1), an abnormal chromosome within their leukemia cells that originates from the reciprocal translocation between chromosomes 9 and 22 (1, 2). This chromosomal exchange joins the 5′ two-thirds of the BCR gene (3) remaining on chromosome 22 to a large portion of the *ABL* gene (4, 5) translocated from chromosome 9 (6). The fused genes generate a hybrid mRNA with a continuous open reading (6) that encodes a BCR-ABL protein of more than 2000 amino acids (7), termed P210 BCR-ABL (8). Ph^1 is also present in some patients with ALL and acute myelogenous leukemia (9). Ph^1-positive ALL patients express either the typical P210 BCR-ABL protein or a smaller protein termed P185 BCR-ABL. The latter results from a more 5′ break in the *BCR* gene within a large intron between exons 1 and 2 (10). Both the CML and ALL forms of the BCR-ABL protein possess an activated tyrosine kinase activity (8, 11, 12). The assay for the BCR-ABL tyrosine kinase activity is widely used as a means to detect the BCR-ABL protein in cell lines derived from blast crisis CML patients. The BCR-ABL protein kinase assay has also been used to detect P210 BCR-ABL in uncultured cells from patients in blast crisis (13). Attempts to detect the BCR-ABL protein in chronic phase patients by this assay have been hindered by large numbers of mature cells in blood and bone marrow samples in these patients, which contain high concentrations of degradative enzymes (13). Extracts of mature granulocytes, from either normal or CML patients, rapidly destroy P210 kinase activity from K562 cell extracts (13). The degradative factors in mature WBC populations must be inhibited before the tyrosine kinase assay of BCR-ABL proteins can be used to monitor patients for the presence of leukemic cell clones. This inhibition has not yet been possible to achieve in our studies. We have, however, devised a method to detect BCR-ABL fusion proteins by a Western blotting method.

Materials and Methods

Cells. HL-60 (14), SMS-SB (15), and K562 (16) cells were grown in RPMI with 10% fetal calf serum. Normal WBC were drawn from volunteers through the leukapheresis unit directed by Dr. Jean Hester. Patient samples were obtained either from the leukapheresis unit (62%) or directly from the patient (38%). Generally, small amounts of blood samples (9–10 ml) were sufficient for assay. Patient samples were obtained through an approved institutional protocol which obtains informed consent of the patient.

ABL Antibody. A monoclonal antibody prepared against an ABL protein expressed in bacteria was used in these studies. BALB/c mice were immunized with a v-ABL fusion protein purified from bacteria (17). Three hundred hybridomas were screened for the production of anti-ABL using microtiter wells coated with the purified v-ABL immunogen. Hybridoma clone 8E9 was selected because it produced an anti-ABL antibody with the highest apparent affinity in enzyme-linked immunosorbent assay and immunoblotting tests. The 8E9 antibody is of the murine IgG1 subtype. Large scale antibody preparation was performed from ascites fluid by ammonium sulfate precipitation followed by DEAE-cellulose chromatography. The 8E9 antibody recognizes an epitope in the B box of the SH2 domain of the c-ABL and the v-ABL proteins.[4] Although 8E9 was produced using mouse ABL protein as an immunogen, it reacts with ABL proteins from human and monkey cells efficiently. The antibody was adjusted to a concentration of 5.7 mg/ml in phosphate-buffered saline.

Preparation of WBC. WBC (from blood, bone marrow, or processed previously by leukapheresis) were processed in buffers containing protease inhibitors including Trasylol (100 KIU/ml), 3 mM phenylmethylsulfonyl fluoride and 5 mM benzamidine. Granulocyte and lymphocyte fractions were isolated by gradient centrifugation at room temperature using Mono-Poly Resolving Medium (Flow Laboratories, McLean, VA). Contaminating RBC were removed by two cycles of hypotonic shock in 0.20% NaCl (18). Except for five samples (PS18, PS25, PS27-PS29), all patient sample cells were treated with 5 mM diisopropylfluorophosphate for 30 min on ice (18). However, the use of diisopropylfluorophosphate does not appear to be required for P210

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[3] The abbreviations used are: CML, chronic myelogenous leukemia; ALL, acute lymphocytic leukemia; SDS, sodium dodecyl sulfate; TN buffer, 50 mM Tris-HCl, 150 mM NaCl, 0.2% Nonidet P-40, and 0.02% sodium azide, pH 7.5.

[4] J. Y. J. Wang, unpublished observations.

BCR-ABL detection in fresh cells, but it is necessary for long term storage of cell samples.

Western Blotting. For Western blotting, cells were lysed in boiling SDS sample buffer composed of 1% SDS and 10% 2-mercaptoethanol containing Tris buffer (pH 8), EDTA, bromphenol blue, and glycerol. After boiling for 5–7 min, the lysate was clarified by centrifugation for 30–60 min in the Beckman L8-M ultracentrifuge at 40,000 rpm in a Beckman 50 Ti angle rotor at 25°C. Lysates from 10^6-10^7 cells were applied to 6.5% gels of polyacrylamide. After electrophoresis, the gel was electroblotted at 4°C in 192 mM glycine, 25 mM Tris, pH 7.5, and 10% methanol for 2 h at 2 amps onto 0.2-μm cellulose nitrate filters, or in later experiments Immobilon P (Millipore, Bedford, MA). Filters were prepared for blotting by washing in 3% bovine serum albumin for 6 h at 4°C. After reaction with a 1:3000 dilution of 8E9 antibody overnight at 4°C and subsequent washing with TN buffer four times for 15 min (the last wash was performed without Nonidet P-40), the antibody bound to protein bands was detected by ^{125}I-protein A (Amersham Co., Arlington Heights, IL) mixed with rabbit anti-mouse IgG (1 μg/10 μCi of radioactive protein A) for 1 h. A total of 10 μCi of ^{125}I-protein A/anti-IgG was diluted in 10 ml of TN buffer containing 3% bovine serum albumin. This solution was used no more than three times for detection of ABL-related proteins on two 5- x 3-inch filters in small trays. Filters were then washed 6 times, the first 5 times with TN buffer containing 0.2% Nonidet P-40 detergent and the final wash in only TN buffer, and air dried before exposure to X-ray film (XAR-5).

Results

Detection of P210 BCR-ABL and P145 ABL in Cell Lines. Cells expressing P210 BCR-ABL (K562 cells), those lacking the BCR-ABL protein (HL-60 and SMS-SB cells), and normal WBC were lysed in boiling SDS sample buffer at a concentration of 10^6-10^7 cells/60 μl and subjected to Western blotting. The ABL monoclonal antibody detected two major bands in K562 cells in about equal amounts with the mobilities of P210 BCR-ABL and P145 ABL (Fig. 1). These results were very reproducible (Fig. 2, *Lane 1*). Ph^1-negative HL-60 and SMS-SB cells contained a major band of P145 ABL and lacked the BCR-ABL protein (Fig. 2, *Lanes 2* and *3*).

Normal WBC also contained only P145 ABL, as expected (Fig. 2*A*, *Lanes 4* and *5*; Fig. 2*B*, *Lane 4*). As a control, blots were exposed to the ^{125}I-protein A-rabbit anti-mouse IgG in the absence of the anti-ABL monoclonal antibody. No bands were detected in any of the extracts tested (results not shown). In contrast to normal cells, cells from chronic phase patients contained a major band of P210 BCR-ABL in addition to P145 ABL (Fig. 2).

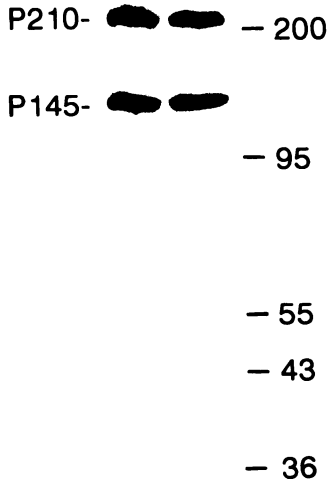

Fig. 1. Detection of P210 BCR-ABL and P145 ABL by Western blotting of extracts of K562 cells. In each lane, 10^7 K562 cells were lysed in sample buffer and processed by immunoblotting with an anti-ABL monoclonal antibody termed 8E9 (see "Materials and Methods"). Bands were detected by exposure to ^{125}I-protein A. Exposure time, 1 day.

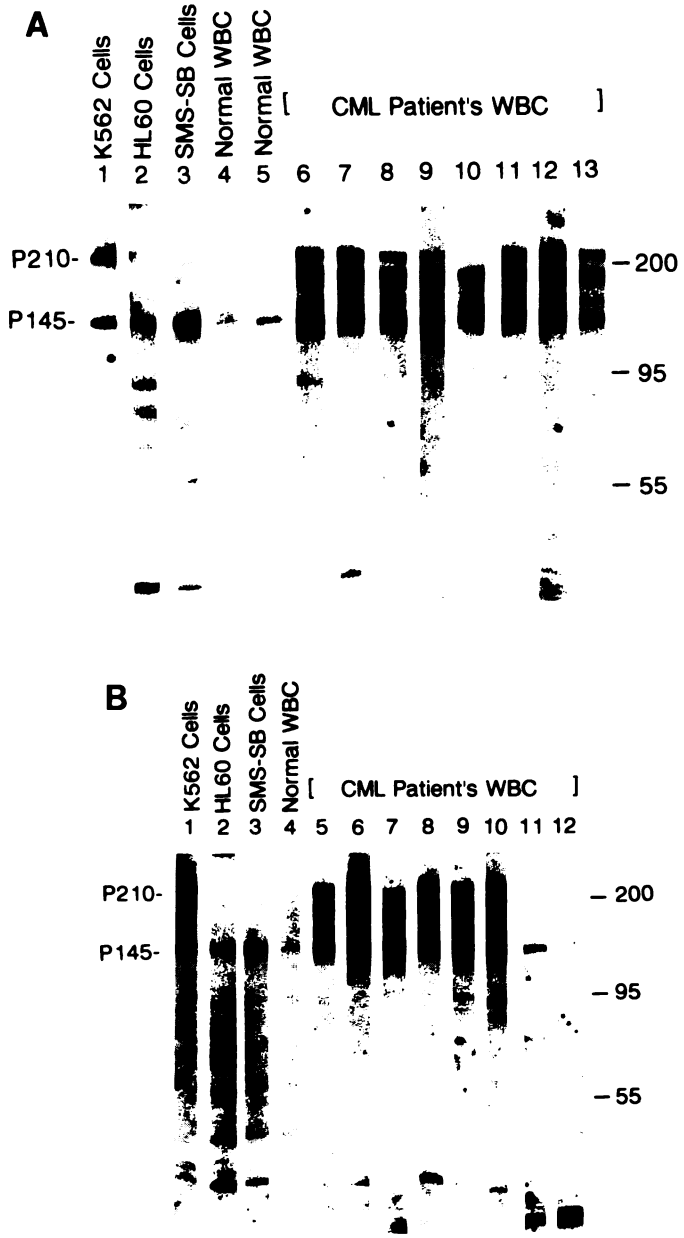

Fig. 2. Detection of P210 BCR-ABL in chronic phase Ph^1-positive CML patients by Western blotting. *A*, K562 cells (*Lane 1*); HL-60 cells (*Lane 2*); SMS-SB cells (*Lane 3*); normal WBC (*Lanes 4, 5*); patient sample 1 (PS1) (*Lane 6*); PS2 (*Lane 7*); PS3 (*Lane 8*); PS4 (*Lane 9*); PS5 (*Lane 10*); PS8 (bone marrow blast crisis phase CML, peripheral blood cells lacked significant blast cells) (*Lane 11*); PS9 (*Lane 12*); PS10 (*Lane 13*). *B*, K562 (*Lane 1*); HL-60 (*Lane 2*); SMS-SB (*Lane 3*); normal WBC (*Lane 4*); PS11 (*Lane 5*); PS12 (blast crisis CML) (*Lane 6*); PS13 (*Lane 7*); PS17 (*Lane 8*); PS18 (*Lane 9*); PS19 (*Lane 10*); PS28 Ph^1-negative ALL (blast crisis) (*Lane 11*); PS29 (*Lane 12*). Patient clinical data are shown in Table 1. All samples were analyzed on gels containing positive and negative controls, and molecular weight markers were always included. Each lane was loaded with extract from 10^7 cells except for K562 which was from 10^6 cells. Exposure times, 3 days.

Detection of the BCR-ABL Protein in Blood Cells of Chronic Phase CML Patients. More than 25 blood samples from 19 chronic phase CML patients were collected to determine the

Table 1 *Clinical characteristics of CML patient samples*

Patient samples	Age/sex	Phase of CML	% Ph¹	% BC[a]	WBC ($\times 10^3/\mu l$)	Sample source	Gene products		P210 + P190	Pretreatment
							P210	P145	P145	
1	43/M	C	100	0	113.8	P, L	+	+	1.10	
2	56/F	A	100	15	28.0	P, L	+	+	1.38	+
3	28/M	C	100	2	95.6	P, L	+	+	1.35	−
4	39/M	C	100	35[b]	NI	BM	+	+	1.55	+
5[c]	50/M	C	100	5	160.6	P, L	+ (P190)	+	0.92	−
6[c]	50/M	C	100	0	34.4	P	+ (P190)	+	3.98	+
7[c]	50/M	C	100	3	19.2	P	+ (P190)	+	1.27	+
8	38/M	B	100	35[b]	7.5	P	+	+	2.99	+
9	22/M	C	100	5	118.8	P, L	+	+	1.90	+
10	44/M	C	100	0	260.0	P, L	+	+	1.84	+
11	33/M	C	100	1	216.0	P, L	+	+	2.05	−
12	57/M	B	100	68	38.8	P	+	+	2.93	+
13	77/M	C	92	0	15.7	P, L	+	+	1.09	−
14	31/M	C	100	0	57.9	P, L	+	+	2.25	+
15	35/M	C	100	1	67.6	P, L	+	+	1.13	+
16	37/M	C	96	0	30.0	P, L	+	+	1.11	+
17	25/M	C	100	2	88.2	P, L	+	+	1.50	+
18	56/F	C	100	0	28.5	P	+	+	1.79	+
19[d]	64/M	C	100	4	77.2	P, L	+	+	1.20	+
20[d]	64/M	C	100	1	22.1	P	+	+	1.26	+
21	37/F	C	100	1	19.6	P, L	+	+	3.03	+
22	68/M	C	100	0	72.5	P, L	+	+	1.92	−
23	48/M	C	100	0	10.7	P, L	+	+	0.46	+
24	40/F	C	100	0	87.4	P, L	+	+	0.68	+
25	46/M	B (lymphoid)	100	32.8	95.0	P	+	+	3.14	+
26	37/M	C	100	0	20.9	P, L	+	+	1.24	+
27	54/F	B	100	88	11.2	P	+	+	6.40	+
28	29/M	ALL (blast)	0	95	185.4	P	−	+		+
29	68/M	C	100	0	10.9	P	−	+		+

[a] BC, blast cells; A, accelerated phase of CML; B, blast crisis phase of CML; BM, bone marrow; C, chronic phase of CML; L, leukapheresis; P, peripheral blood sample; NI, no information.
[b] Blast cells in bone marrow.
[c] Three samples from the same patient were taken on different dates.
[d] Two samples from another patient were taken on different dates.

utility of the Western blotting assay for detection of P210 BCR-ABL in early stages of the disease. Western blots from representative patient samples are shown in Fig. 2. The analysis detected bands with the mobility of P210 BCR-ABL and P145 ABL in most of the samples tested (Fig. 2*A, lanes 6–10, 12,* and *13*; Fig. 2*B, lanes 5, 7–10,* and *12*). Variable amounts of lower molecular weight proteins were usually detected in all samples regardless of their origin.

Table 1 summarizes the samples studied to date and lists clinical data for each patient. Nineteen patients were classified as in chronic phase, one in accelerated phase, four in blast crisis, and one as having Ph¹-negative ALL. No significant correlations were apparent regarding whether therapy (pretreatment) was administered to the patient, and the age, sex, or WBC of the patient. Most of the samples were processed by leukapheresis (Table 1, L) prior to subsequent purification to completely remove RBC. For analysis of blood samples taken directly from patients, typically less than 10 ml of blood was required to do blotting analyses. Except for one patient who lacked detectable BCR-ABL (Table 1, PS29; Fig. 2*B, Lane 12*) and one who contained a P190 ABL band (Fig. 2*A, Lane 10*; Table 1, PS5-PS7), all other chronic phase patients contained P210 BCR-ABL, and most contained one to two bands with a molecular weight of about 190,000 (P190 ABL). It should be emphasized that the Ph¹-negative ALL patient lacked a BCR-ABL protein thereby further validating this method (Fig. 2*B, Lane 11*; Table 1, PS28).

The structure of the P190 ABL protein is unknown. Patient samples 5, 6 and 7 (Table 1) drawn from the same patient on three separate occasions lacked detectable P210 BCR-ABL and contained only P190 ABL (Fig. 2*A, Lane 10*). A single band, P145, was also detected in these samples. Normal WBC preparations lacked P190 ABL and contained only P145 ABL as expected (Fig. 2).

Blast crisis patients (Fig. 2*A, Lane 11;* Fig. 2*B, Lane 6*) were also found to contain P210 BCR-ABL and P145 ABL; they contained P190 ABL as well. It was of obvious interest to compare the band intensities of P210/P190 BCR-ABL to P145 ABL from patients in chronic phase and those in blast crisis (Fig. 2*A, Lane 11*; Fig. 2*B, Lane 6*). We compared blots from 4 blast crisis and 19 chronic phase patients by scanning the autoradiograms with a laser scanning densitometer (Zeineh Co., Fullerton, CA). The ratio of P210 plus P190 to P145 was larger than 2.5 (range, 2.9 to 6.4) in 4 blast crisis patients and in K562 cells (derived from a blast crisis patient) whereas the ratio ranged from 0.5 to 2.3 in 18 chronic phase patients (Table 1). Of interest, PS21 had a ratio of 3 despite being in chronic phase. In all of these patients, the percentage of cells possessing the Ph¹ was between 92 and 100% indicating that WBC uniformly possessed the *BCR-ABL* gene. This latter point is important because our results have shown that prepared mixtures of Ph¹-positive and -negative cells show a linear correlation between the ratio of P210 BCR-ABL to P145 ABL and the percentage of Ph¹-positive cells.[5] Although more blast crisis samples must be analyzed in order to draw firm conclusions, these results suggest that P210 BCR-ABL expression is increased in leukemic cells from blast crisis patients compared to those in chronic phase.

One Ph¹-positive CML patient in the chronic phase lacked detectable BCR-ABL proteins but contained P145 ABL (Fig. 2*B, Lane 12*; Table 1, PS29). Additional blood samples from this patient are being sought to confirm this result. Several additional patient samples not described in this study were

[5] J. Guo and R. Arlinghaus, unpublished results.

found to lack both P210 BCR-ABL and P145 ABL. Since normal blood samples routinely express the normal ABL protein, the lack of P145 ABL in any assay is indicative of protein degradation during sample processing. In our studies to date, this has been an infrequent occurrence.

Discussion

A procedure involving Western blotting was developed that allows detection of BCR-ABL proteins in blood samples from patients in the chronic phase of Ph[1]-positive CML. This assay should also be useful in detecting other abnormal size ABL proteins, such as those expressed in some Ph[1]-ALL and acute myelogenous leukemia patients. Quantitative measurements of known mixtures of K562 cells and Ph[1]-negative cells demonstrated that the ratio of the BCR-ABL protein to the normal ABL protein can be used to estimate the percentage of K562 cells within mixtures of Ph[1]-negative SMS-SB cells.[6] Application of this test to patients progressing from chronic phase through the accelerated phase and into blast crisis may allow a determination of whether the level of P210 BCR relative to P145 ABL changes throughout these transitions. Our initial findings indicate that P210 BCR-ABL expression is significantly higher in leukemic cells derived from blast crisis patients than from chronic phase patients.

Our findings indicate that many chronic phase CML patients have one to two proteins with a molecular weight of about 190,000 in addition to P210 BCR-ABL. It is likely that these are BCR-ABL proteins and, based upon previous experience, they are likely to be degradation products of P210 BCR-ABL (8, 12) or may represent some altered form of the BCR-ABL protein. Of interest, one chronic phase patient, whose blood cells were analyzed on three separate occasions, lacked detectable P210 and contained only the M_r 190,000 abnormal size ABL protein. These samples contained P145 ABL of the correct size (see Fig. 2*A, Lane 10*). Although the structure of the M_r 190,000 protein observed in this one patient is unknown, removal of amino- or carboxyl-terminal sequences by nonspecific protein degradation seems unlikely because P145 ABL was not similarly affected in three different samples collected at different times. Importantly, Southern blotting indicated that this patient lacked rearrangement in the breakpoint cluster region.[7] In this regard, two Ph[1]-positive CML patients in chronic phase studied by Selleri *et al.* (19) lacked central *BCR* sequences; one of these patients had an ALL type mRNA which would encode a M_r 185,000–190,000 protein. The form of the BCR-ABL protein (P185 BCR-ABL) found in some Ph[1]-positive ALL patients is encoded by a RNA that joins the first *BCR* exon to the second exon of *ABL* (10). In this regard, two Ph[1]-positive ALL patients which contain *BCR-ABL* transcripts that encode a P190 BCR-ABL protein with yet a different structure were recently reported. The structure of these latter RNAs joins either the first exon of *BCR* or the 2′ *bcr* exon to *ABL* exon 3 (20). It will be of interest to determine whether the M_r 190,000 protein observed in our studies has some physiological significance with respect to severity or progression of the disease.

[6] Unpublished results.

[7] S. Stass, Division of Laboratory Medicine, personal communication.

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