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Research Article

Interferon-alpha induces durable cytogenetic remissions in about one-quarter of newly diagnosed patients with chronic myelogenous leukemia (CML). Even so, after short-term follow-up, previous studies have shown that residual leukemic cells can be detected by the polymerase chain reaction (PCR) in all of these individuals. The objectives of our study were therefore to obtain long-term follow-up data on residual disease in a cohort of complete responders and to determine if leukemic cells with clonogenic potential are present in patients despite the absence of relapse. We performed (a) serial analysis of blood and/or bone marrow for a reverse transcriptase PCR amplified BCR-ABL transcript at times well beyond the point that cytogenetic remission was first attained and (b) reverse transcriptase PCR of individually plucked myeloid and erythroid colonies for the presence of the same transcript. Seven CML patients who had previously attained complete cytogenetic remission while on interferon-alpha were investigated. Six of the seven patients were in complete cytogenetic remission at the time of analysis, whereas one patient had early evidence of cytogenetic relapse. With ongoing therapy, five patients with the longest follow-up eventually achieved PCR negativity at time periods of 27, 32, 36, 49, and 67 mo after a complete cytogenetic remission was first noted. Even so, residual disease was detected in progenitor cells derived from two patients, each of whom had […]

Persistence of Dormant Leukemic Progenitors during Interferon-induced Remission in Chronic Myelogenous Leukemia

Analysis by Polymerase Chain Reaction of Individual Colonies

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Abstract

Interferon-alpha induces durable cytogenetic remissions in about one-quarter of newly diagnosed patients with chronic myelogenous leukemia (CML). Even so, after short-term follow-up, previous studies have shown that residual leukemic cells can be detected by the polymerase chain reaction (PCR) in all of these individuals. The objectives of our study were therefore to obtain long-term follow-up data on residual disease in a cohort of complete responders and to determine if leukemic cells with clonogenic potential are present in patients despite the absence of relapse. We performed (*a*) serial analysis of blood and/or bone marrow for a reverse transcriptase PCR amplified *BCR-ABL* transcript at times well beyond the point that cytogenetic remission was first attained and (*b*) reverse transcriptase PCR of individually plucked myeloid and erythroid colonies for the presence of the same transcript. Seven CML patients who had previously attained complete cytogenetic remission while on interferon-alpha were investigated. Six of the seven patients were in complete cytogenetic remission at the time of analysis, whereas one patient had early evidence of cytogenetic relapse. With ongoing therapy, five patients with the longest follow-up eventually achieved PCR negativity at time periods of 27, 32, 36, 49, and 67 mo after a complete cytogenetic remission was first noted. Even so, residual disease was detected in progenitor cells derived from two patients, each of whom had been in continuous cytogenetic remission for ~ 2.5 and 3.5 yr, respectively. Progenitors expressing *BCR-ABL* transcripts were also detected in the patient with early cytogenetic relapse. These observations demonstrate that residual disease resides in colony-forming cells that should have the potential to repopulate the bone marrow. However, the presence of a minority of Ph-positive CML progenitor cells for a very long period of time is still compatible with durable remission, confirming that a situation of tumor dormancy may be induced in CML by interferon therapy. (*J. Clin. Invest.* 1994. 94:1383–1389.) Key words: chronic myelogenous leukemia • *BCR-ABL* • Philadelphia chromosome • polymerase chain reaction • interferon

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Introduction

The therapeutic objectives in chronic myelogenous leukemia (CML)[1] have changed from maintenance of normal counts in the chronic phase with minimal morbidity to an effort to suppress the malignant clone and restore normal hemopoiesis. Allogeneic bone marrow transplantation demonstrates consistent efficacy in meeting this objective and achieving complete and durable remissions in CML (1). However, with this treatment, the normal hemopoiesis is restored by donor bone marrow. Evidence for persistent, albeit suppressed normal progenitors in patients with CML comes from long-term bone marrow cultures, and clinically complete restoration of normal hemopoiesis can be obtained with intensive chemotherapeutic treatments (2, 3). However, the remissions induced by the latter are of short duration. Even so, monitoring remission by techniques such as karyotype analysis often fails to detect residual leukemic clones, which nevertheless subsequently fully repopulate the bone marrow. Therapeutic trials with alpha-interferon in CML began in 1981 and subsequently have demonstrated a high frequency of hematologic remission and consistent, though less frequent, development of cytogenetic remissions. These remissions are generally more durable than chemotherapy-induced remissions (4, 5).

The Philadelphia chromosome (Ph) is the hallmark of CML and provides a target for detection of residual disease. It represents a reciprocal translocation between the long arms of chromosomes 9 and 22 (t(9;22)(q34;q11)). At the molecular level, this translocation transposes the *ABL* gene from chromosome 9 and juxtaposes it to the disrupted 5′ end of the *BCR* gene on chromosome 22 (6). This abnormal configuration results in the creation of a chimeric gene (*BCR-ABL*) which encodes an abnormal protein designated $p210^{BCR\text{-}ABL}$. In the *BCR-ABL* transcript, either exon b2 or exon b3 of the major breakpoint cluster region (bcr) is coupled to *ABL* exon 2 (b_2-a_2 or b_3-a_2 junction).

Over the last few years, the introduction of diagnostic techniques using reverse transcriptase polymerase chain reaction (RT-PCR) amplification of the *BCR-ABL* transcript has significantly enhanced our ability to detect a residual leukemic clone in CML patients in remission. Because of the sensitivity of the PCR technique and because the *BCR-ABL* mRNA is never found in normal cells, RT-PCR can distinguish one CML cell among $\geq 1 \times 10^5$ normal cells. Residual disease has been demonstrated

1. *Abbreviations used in this paper:* bcr, breakpoint cluster region; BFU-E, burst-forming unit–erythroid; CFU-GEMM, CFU–granulocyte, erythroid, monocyte, macrophage; CML, chronic myelogenous leukemia; HPA, hybridization protection assay; Ph, Philadelphia chromosome; RLU, relative light unit; RT-PCR, reverse transcriptase PCR.

in the majority of individuals with interferon-induced remissions and also in some patients after allogeneic bone marrow transplantation. However, this test does not identify the type of cells which remain Ph chromosome–positive (*BCR-ABL*–positive). Nor are their proliferative and clonogenic potentials understood. To further characterize the nature of remission induced by interferon, we initiated a study of *BCR-ABL* transcript expression in myeloid and erythroid progenitor cells from CML patients in remission.

Methods

Patients with a diagnosis of Ph-positive CML were the subjects of this study. Patients were required to have a documented Ph-positive abnormality by karyotype analysis (7) and a diagnosis of CML confirmed by bone marrow evaluation. All patients were treated with recombinant interferon-alpha, at a starting dose of 5×10^6 U/m^2 per day subcutaneously or intramuscularly. Approval for these studies was obtained from the Institutional Review Board and all patients gave informed consent. Workup of the patients at presentation included a history and physical examination, complete blood count with differential and platelet counts, leukocyte alkaline phosphatase score, bone marrow aspiration and biopsy for morphology, cytochemical and enzymatic stains, immunophenotyping, karyotypic analysis, and molecular studies as described below.

CFU-GEMM assay. A modification of a previously described colony-forming unit–granulocyte, erythroid, monocyte, macrophage (CFU-GEMM) assay was used (8, 9). In brief, 2×10^5 nucleated low-density bone marrow cells were cultured in 0.8% (vol/vol) methylcellulose with Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY), 30% FCS, 10 ng/ml recombinant GM-CSF, 15 ng/ml IL-3, 50 ng/ml stem cell factor (Immunex Corp., Seattle, WA), and 1.0 U/ml human erythropoietin (British Columbia Cancer Research Institute, Vancouver, Canada). 1 ml of the culture mixture was placed in 35-mm Petri dishes (Nunc, Inc., Naperville, IL) and incubated at 37°C with 5% CO_2 in air in a humidified atmosphere in duplicate. All cultures were evaluated after 14 d for the number of burst-forming unit–erythroid (BFU-E) colonies (defined as an aggregate of > 500 hemoglobinized cells or three or more erythroid subcolonies) and CFU-GM colonies (defined as a cluster of 40 or more granulocytes, macrophages, or both). Individual colonies were plucked from the cultures with a micropipette for further analysis. The plating efficiency of this assay is ~ 1:500–1:700 for samples from normal individuals, 1:1,000 for samples from untreated chronic phase CML patients, and 1:1,500 for samples from interferon-treated CML patients who are responders (reference 10 and Estrov, Z., unpublished data).

Cell line clonogenic assay. A CML erythroid blast crisis cell line (K562) was plated in duplicate in 35-mm Petri dishes (Nunc, Inc.) at a concentration of 2×10^4 cells/ml in 0.8% (vol/vol) methylcellulose in RPMI medium (Gibco Laboratories) with 10% FCS (FLOW) and incubated at 37°C in a humidified atmosphere of 5% CO_2 with air. Cultures were evaluated at day 6 using an inverted microscope. A cluster of > 40 cells was defined as a colony. Individual colonies were plucked from the cultures with a micropipette for further analysis.

Molecular studies. Bone marrow from Ph-positive CML patients was analyzed for production of mRNA encoding $p210^{BCR\text{-}ABL}$ with the use of RT-PCR and specific primers encompassing the RNA splice junctions to amplify cDNA. (There are two major splice junctions for the *BCR-ABL* mRNA which encodes the CML-associated $p210^{BCR\text{-}ABL}$. The junctions are designated b_2-a_2 or b_3-a_2.) The product was then detected with the use of the hybridization protection assay (HPA) as described previously (11, 12) and as detailed below. The K562 cell line used as a control has a b_3-a_2 junction and produces $p210^{BCR\text{-}ABL}$.

After the splice junction of a patient was determined, individual colonies were analyzed. (In some of the samples, determination of the splice junction was performed on a frozen sample obtained earlier in the course of the patient's disease since the patient had been rendered *BCR-ABL*–negative as determined by RT-PCR at the time of colony analysis.

All samples were simultaneously coamplified for the normal *ABL* transcript (using primers encompassing *ABL* exons 1b and II) or for actin to ensure the presence of intact cDNA and a successful amplification procedure.

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

Bone marrow sample preparation for RT-PCR amplification. Total cellular RNA was extracted as described previously (13). RNA from K562 cells, a CML erythroid blast crisis cell line, and from patients with Ph-positive CML was used as positive controls for mRNA containing b_3-a_2 and b_2-a_2 junction, respectively. HL-60 (a Ph-negative myelocytic leukemic cell line) and normal human endometrial RNA were used as negative controls. 1 μg of total RNA from cell lines or 10% of the total RNA from $50–200 \times 10^6$ white blood cells from patient samples was used for amplification reactions.

cDNA preparation. Preparation of cDNA from RNA was done using the following procedure. 16 μl of reaction mixture was prepared: 5× reverse transcriptase buffer (Bethesda Research Laboratories, Gaithersburg, MD), 4 μl; RNAsin (40 U/μl) (Promega Corp., Madison, WI), 0.5 μl; 10 mM dNTPs (2 μl of each of dATP, dCTP, dGTP, and dTTP), 8 μl; H_2O (Rnase free), 1.5 μl; M-MLV reverse transcriptase (200 U/μl) (Bethesda Research Laboratories), 1 μl; and 3′ primer (10 pmol/μl), 1 μl. (For this reaction mixture, the RNAsin and M-MLV reverse transcriptase were added last.) The 16 μl of reaction mixture was added to 4 μl of total RNA or mRNA ($^1/_{10}$ of patients' total RNA or 1 μg for cell line). (Total volume of the RNA/reaction mixture is 20 μl.) The RNA/reaction mixture was then incubated at 37°C for 60 min.

Amplification of cDNA. The reaction mixture for RT-PCR contains: (*a*) 10× reaction buffer (Gene Amp Kit; Perkin-Elmer Cetus, Norwalk, CT), 8 μl; (*b*) 3′ primer 10 pmol/μl, 4 μl; (*c*) 5′ primer 10 pmol/μl, 5 μl; (*d*) Ampli-Taq enzyme (5 U/μl) (Perkin-Elmer Cetus), 0.5 μl; and (*e*) H_2O (Rnase free), 62.5 μl. After microcentrifugation, the 80 μl of RT-PCR reaction mixture was added to the 20-μl solution of cDNA obtained above, mixed well, microcentrifuged for 2 s, and overlaid with 80–100 μl of Nujol mineral oil (Perkin-Elmer Cetus) to prevent evaporation. The RT-PCR amplification was performed for 40 cycles as described previously (14).

Primers. The primers for the b_2-a_2 and b_3-a_2 junctions are 5′-GGAGCTGCAGATGCTGACCAAC-3′ (5′ primer homologous to sequences in *BCR*) and 5′-TCAGACCCTGAGGCTCAAAGTC-3′ (3′ primer homologous to sequences in *ABL*) (14). For amplification of the normal *ABL* transcript, a primer encompassing *ABL* exon II (14) (the 3′ *ABL* primer described above) and *ABL* exon Ib (a 20 mer beginning 65 bases upstream of the exon Ib–II junctions) (5′-CAAAGGAGCAGGGAAGAAGG-3′) were used (15). The actin primers were 5′-AACGGCTCCGGCATGTGCAAGGCCGGC-3′ (5′ actin primer) and 5′-TCAGTCAGGTCCCGGCCAGCCAGGTC-3′ (3′ actin primer) (16).

RT-PCR amplification of hematopoietic colonies. CFU-GM and BFU-E colonies from patient samples were plucked from the tissue culture dishes with a Pasteur micropipette as described previously (17). To validate our procedure for detection of *BCR-ABL* cDNA in hematopoietic colonies, our first experiments were performed on colonies from K562 cells. Subsequent experiments were done on CFU-GM and BFU-E colonies derived from two CML patients and two normal volunteers. Cells were cultured in duplicate and given to two technicians, who were blinded as to whether the plates were from a normal volunteer or from a CML patient. Each of the technicians then proceeded to independently

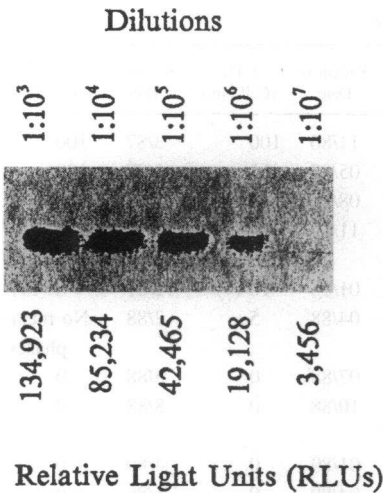

Figure 1. Comparison of sensitivity of Southern blotting and HPA for detection of RT-PCR amplified *BCR-ABL* transcripts in K562 cells (Ph-positive cell line). Detection by HPA is measured in RLU with < 5,000 RLU considered negative, > 10,000 RLU considered positive, and 5,000–10,000 RLU considered inevaluable.

pluck colonies, prepare RNA and cDNA, coamplify for *BCR-ABL* and normal *ABL*, and probe for *BCR-ABL* and normal *ABL* by the method outlined below. The results of these experiments demonstrated that our molecular methodology could consistently distinguish Ph-positive CML colonies versus colonies from normal individuals.

We then proceeded to use the identical methodology to analyze Ph-positive CML colonies from patients in hematologic and cytogenetic remission on interferon. Colonies were plucked using a sterile Pasteur pipette and transferred into vials containing 100 μl RNAzol B solution to lyse the cells. 10 μl of chloroform was added to the homogenate, vortexed for 15 s, kept on ice for 5 min, and then centrifuged at 12,000 *g* at 4°C for 15 min. RNA from the upper aqueous phase was removed with a pipette and transferred into 0.6-μl vials (Perkin-Elmer Cetus). An equal volume of isopropanol was added, followed by gentle mixing, and the solution was left at 4°C for 15 min to allow precipitation. The solution was then centrifuged at 12,000 *g* at 4°C for 15 min. Supernatant was removed, and RNA pellets were washed with 200 μl of 75% ethanol and then spun down (7,500 *g* at 4°C for 8 min). RNA pellets were then dried on speed vacuum for 8–10 min and dissolved in 4 μl of sterile double-distilled water. RNA samples were then incubated at 60°C for 5 min. cDNA was then prepared from RNA and subsequently amplified for 40 cycles as described above and in the paper by Kawasaki et al. (14).

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

HPA. We have described the clinical application of a rapid method for detecting amplified *BCR-ABL* cDNA (11, 12, 18) that uses acridinium ester–labeled probes (Gen-Probe, Inc., San Diego, CA) with high chemoluminescent properties. In the presence of hydrolysis buffer, the rate of hydrolysis of free probe is much faster than that of hybridized probe, and separation of hybridized probe on a solid support, as in Southern blotting, is unnecessary. This method is performed in solution and allows for reliable detection of transcripts within 30 min after amplification. HPA results correlated with those of Southern blotting of amplified product in all 60 samples from patients with Ph-positive CML, acute leukemia, Ph-negative leukemia, or normal volunteers (12, 18).

Acridinium ester–labeled oligonucleotides complementary to the *BCR-ABL* junction sequences were synthesized by Gen-Probe, Inc. (11, 12). The chemical labeling of the DNA probes with acridinium ester was achieved by reacting alkylamine linker-arms, which were introduced during DNA synthesis, and an *N*-hydrozysuccinimide ester or a methyl acridinium phenyl ester. The b_2-a_2 probe is a 28 mer with 22 bases from bcr exon 2; the b_3-a_2 probe is a 25 mer with 11 bases from bcr exon 3. These probes detect transcripts encoding $p210^{BCR\text{-}ABL}$. The probe for detection of the normal *ABL* transcript is a 24 mer spanning the *ABL* exon Ib–II junction with 14 bases in exon II. A chemiluminescence reading expressed in relative light units (RLU) indicates whether a sample is positive or negative (11, 12).

Southern blotting of RT-PCR amplified product. In some cases, whole cell samples were analyzed using a conventional Southern blot technique in addition to the hybridization protection assay. In these cases, *BCR-ABL* was coamplified with actin rather than with normal *ABL*. 1 μl of 10× stop buffer was added to 10 μl of the RT-PCR amplified sample (see above) and run on a minigel (3% Nusieve agarose and 1% Seakem agarose; FMC Corp. BioProducts, Rockland, ME) at

Table I. Patient Characteristics and Clinical Course

Case	Age Sex	Date of diagnosis	Date IFN-α started	Date complete hematologic remission achieved	Date complete cytogenetic remission first achieved (date IFN stopped)	RT-PCR results			Percentage of Ph+ cells at time of colony analysis	Last follow-up	
						Date*	Blood	Bone marrow		Date	Percentage of Ph+ cells
1	46 M	2/87	11/87	1/88	11/88 (10/92)	7/91	Neg	Neg	0	6/93	0
2	42 M	6/88	7/88	10/88	4/89	6/91	Neg	Not done	0	3/93	95
3	38 M	2/82	3/82	1/83	12/85 (6/89)	7/91	Neg	Not done	0	5/93	0
4	40 M	10/88	11/88	6/89	3/90	10.91	Not done	Not done	20	5/93	0
5	30 M	3/90	7/90	10/90	7/91	10/91	Not done	Pos	0	5/93	40
6	33 M	11/86	2/87	5/87	7/88 (10/92)	9/91	Not done	Neg	0	5/93	0
7	33 M	2/87	4/87	6/87	11/87 (3/92)	09/92	Not done	Neg	0	4/93	0

* Coincides with time of colony analysis. In patient 4, who had a partial cytogenetic relapse (20% Ph-positive metaphases), an unfractionated blood or bone marrow was not available at the time of colony analysis.

Table II. Cytogenetic Results Presented as the Percentage of Cells Positive for the Ph Chromosome

Patient 1 Date	% Ph+ (Cell No.)*	Patient 2 Date	% Ph+ (Cell No.)	Patient 3 Date	% Ph+ (Cell No.)	Patient 4 Date	% Ph+ (Cell No.)	Patient 5 Date	% Ph+ (Cell No.)	Patient 6 Date	% Ph+ (Cell No.)	Patient 7 Date	% Ph+ (Cell No.)
11/87	100	06/88	100	03/82	100	11/88	100	07/90	95	11/86	100	3/87	100
02/88	90	10/88	76	04/82	100	06/89	88 (14)	10/90	97	05/87	65	5/87	100
05/88	85	04/89	0 (9)	07/92	100	09/89	10 (11)	01/91	65	08/87	55	7/87	60
08/88	17	07/89	0 (4)	10/82	90	12/89	No metaphases	07/91	0 (8)	11/87	15	9/87	70 (7)
11/88	0 (11)	11/89	0 (15)	12/82	95	03/90	0 (7)	10/91	0‡	01/88	16 (7)	11/87	0 (19)
02/89	0	02/90	0 (4)	02/83	100	06/90	0 (6)	02/92	0	04/88	5	2/88	No metaphases
05/89	0	05/90	0	04/83	90	09/90	0 (11)	07/92	10	07/88	0	5/88	0
09/89	0 (8)	08/90	No metaphases	08/83	72 (11)	12/90	0 (2)	05/93	40	10/88	0	8/88	0
01/90	0	12/90	10	10/83	70	04/91	5			01/89	0	4/89	0
05/90	0	03/91	0	05/84	50 (10)	07/91	30			04/89	0	5/89	0
07/90	0 (10)	06/91	0 (5)‡	08/84	No metaphases	10/91	20‡			07/89	0 (3)	8/89	0
11/90	0 (6)	09/91	8	12/84	10	01/92	20			11/89	No metaphases	1/89	0 (15)
02/91	0	01/92	45	04/85	10	04/92	0 (8)			04/90	0 (3)	3/90	0
06/91	0 (6)‡	12/92	20	09/85	5	08/92	0 (20)			07/90	0 (14)	7/90	0
10/91	0	03/93	95	12/85	0	11/92	0 (3)			10/90	0 (7)	11/90	0
02/92	0			01/87	4	02/93	10			01/91	0 (6)	3/91	0
08/92	0			11/87	0 (3)	05/93	0			04/91	0	7/91	0
02/93	0			05/88	0					08/91	0‡	12/91	0
06/93	0			01/89	0					04/92	0	3/92	0
				06/89	0 (15)					07/92	0 (6)	9/92‡	0
				02/90	0					10/92	0 (10)	4/93	0
				09/90	0					02/93	0 (16)		
				07/91	0‡					05/93	0 (12)		
				04/92	0								
				05/93	0								

* Typically, ≥ 20 metaphases were analyzed. Where a smaller number of metaphases were available for analysis, the number is stated in parentheses. ‡ Numbers represent cytogenetic results within 1 mo of colony analysis.

40 V for 3.5 h in 1× Tris/borate/EDTA (TBE) running buffer. Transfer was to a GeneScreen Plus membrane (New England Nuclear, Boston, MA). The filter was then baked at 80°C for 2 h. Oligonucleotide probes recognizing the junctional sequences (14) were 5′ end-labeled with ^{32}P and hybridized overnight using hybridization buffer with (for b_3-a_2) or without (for b_2-a_2) formamide. The filters were washed as recommended by the manufacturer and exposed to Kodak XAR film for 6–36 h. The b_2-a_2 and b_3-a_2 probes detect amplification products 125 and 200 bp long, respectively. Actin was visualized by ethidium bromide staining of gels.

Results

Sensitivity of the HPA/RT-PCR technique. Using serial dilutions of K562 cells, we first demonstrated that the sensitivity of the HPA technique was equal to that of Southern blotting in detection of RT-PCR amplified products. Starting with 1 μg of total RNA, 10-fold serial dilutions were performed with RNA from normal endometrial cells. After 40 cycles of amplification, the *BCR-ABL* transcript could be detected at a dilution of $1:10^6$ cells by either Southern blotting (19) or by HPA (Fig. 1). (This is equal to $^1/_{100}$ of the RNA present in one cell [at 10 pg/cell] as $^1/_{10}$ of the final amplification product was used for Southern blots or HPA.) With fresh CML cells, HPA (and Southern blotting) could detect RT-PCR amplified *BCR-ABL* transcripts at $\leq 1:10^5$ dilutions (data not shown). (Our previous experience with the use of HPA on RT-PCR amplified cDNA derived from over 200 bone marrow or blood samples indicates that all Ph-negative samples show < 5,000 RLU, and we therefore consider values below 5,000 as background. Ph-positive samples all demonstrated > 10,000 RLU and most exhibited > 25,000 RLU. [We consider samples which show between 5,000 and 10,000 RLU as inevaluable.])

Detection of RT-PCR amplified BCR-ABL transcripts in plucked colonies. To test the ability of the assay to detect the *BCR-ABL* message in individually plucked colonies, 10 colonies from the K562 cell line were analyzed. All were positive for the *BCR-ABL* message (b_3-a_2 junction). (As expected, the b_2-a_2 junction was negative.) The median RLU for the b_3-a_2 junction was 25,939 (range 15,555–55,948 RLU); the median RLU for the b_2-a_2 junction was 2,262 (range 984–4,782 RLU). Colonies from HL-60, a Ph-negative leukemic cell line, which does not express the *BCR-ABL* transcript, demonstrated < 5,000 RLU when analyzed with either the b_2-a_2 or the b_3-a_2 *BCR-ABL* probes. Subsequently, we analyzed 20 colonies from normal

individuals and all demonstrated < 5,000 RLU, whereas 20 colonies from 2 CML patients had > 10,000 RLU/colony when analyzed with the aforementioned probes. Individual CML patients and normal volunteers were analyzed in pairs, and the laboratory technician was blinded to the diagnosis associated with the samples.

RT-PCR analysis of interferon-treated CML patients in cytogenetic remission for BCR-ABL. The characteristics of the cytogenetic responses in the seven studied patients are outlined in Table I. All of these patients were followed at M. D. Anderson Cancer Center for 3–11 yr (median 6 yr). Their first complete cytogenetic remissions were recorded 3–67 mo (median 30 mo) before the molecular analysis. All remissions were induced by interferon therapy and maintained with interferon. These patients were followed by cytogenetic analysis after the molecular analysis was conducted, and follow-up cytogenetic data were available 7–24 mo (median 21 mo) after the molecular analysis. Detailed cytogenetic follow-up of these patients is provided in Table II. These data demonstrate that these patients had a variable duration of uninterrupted complete cytogenetic remission before colony analysis. This was of short duration (3 mo) for patients 2 and 5 and of long duration (32, 44, 36, and 58 mo) for patients 1, 3, 6, and 7, respectively. Patient 4 had attained a complete cytogenetic response according to several examinations but had relapsed with 20% Ph-positive metaphases at the time of colony analysis. This patient was subsequently again rendered into complete cytogenetic remission with continuation of interferon treatment.

Using RT-PCR followed by HPA, we analyzed *BCR-ABL* expression in cDNA derived from individually plucked colonies derived from these seven interferon-alpha–treated CML patients (Fig. 2). Simultaneously obtained unfractionated blood or bone marrow samples were available in the six patients in complete cytogenetic remission (Table I, cases 1–3 and 5–7) but not in the patient in cytogenetic relapse. Five of the six patients were negative for *BCR-ABL* expression by HPA/RT-PCR. (Duplicate samples were analyzed in all patients and were consistently concordant. In addition, in the patients in whom HPA/RT-PCR indicated *BCR-ABL* negativity, a third sample was analyzed by RT-PCR followed by standard Southern blotting rather than by HPA. Results remained negative in each patient. Simultaneous coamplification for actin was positive.)

In all seven patients, unfractionated blood and/or bone marrow samples were available from earlier in the course of the disease. HPA/RT-PCR analysis demonstrated that *BCR-ABL* expression could be detected in all of these patients at an earlier time in their therapy, i.e., 3–46 mo after achieving complete cytogenetic remission. Of interest, one patient (case 4) showed negative RT-PCR results in an unfractionated blood sample but positive results in an unfractionated bone marrow sample at initial analysis 6 mo after achieving cytogenetic remission.

All colonies from four of our seven patients were negative for *BCR-ABL* transcripts (patients 2, 5, 6, and 7; Table III). The remaining patients (Table III, patients 1, 3, and 4) had detectable *BCR-ABL* transcript in some of the colonies. Several residual positive colonies were detected in two of four patients with long-standing, uninterrupted complete cytogenetic remission (Table III, patients 1 and 3). In addition, all colonies studied were positive in the patient with evidence of minor cytogenetic relapse (20% Ph-positive cells) at the time of the study (patient 4) (Tables II and III), although complete cytogenetic remission was later reinduced in this patient with interferon therapy.

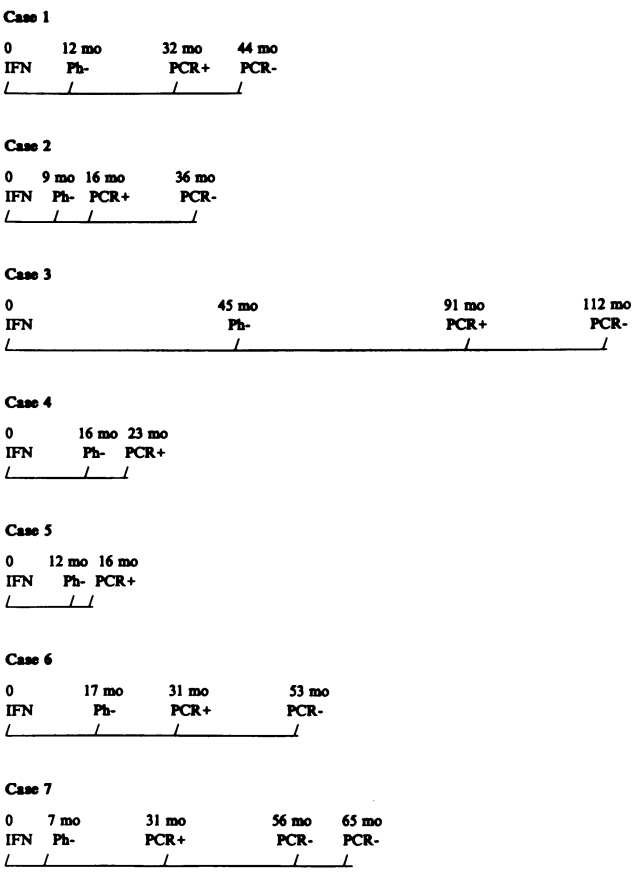

Figure 2. Time course of clinical, cytogenetic, and molecular events in our seven CML patients. Time point 0 denotes the date that interferon-alpha (*IFN*) was started. Ph- denotes the point at which complete cytogenetic remission was first achieved. PCR+ denotes a time point at which RT-PCR analysis of *BCR-ABL* transcripts was performed on unfractionated blood and/or bone marrow and found to be positive. PCR- denotes a time point at which RT-PCR analysis of *BCR-ABL* transcripts was performed on unfractionated blood and/or bone marrow and found to be negative.

In two patients (cases 1 and 3), the b_2-a_2 and b_3-a_2 splice junctions were detected in separate colonies (i.e., in patient 1, three colonies were positive for a b_3-a_2 junction and one colony was positive for a b_2-a_2 junction). In one patient (case 4), all colonies contained both b_2-a_2 and b_3-a_2 junctions.

Discussion

Cytogenetic responses in CML have been observed with chemotherapy and with interferon therapy. However, only with the latter are remissions sustained for long periods (4, 5). In a recent analysis, we noted that 45 of 49 patients remain in ongoing remission (albeit not necessarily complete) for periods ranging from 4 to 10 yr (20). However, it is also apparent that cytogenetic analysis is insufficiently sensitive to detect small numbers of residual Ph-positive cells in a bone marrow sample studied during remission primarily because the small sample size analyzed by cytogenetic testing (between 25 and 50 cells) provides a measure of sensitivity ranging only between 2 and

*Table III. Results of HPA/RT-PCR Colony Assays in Seven CML Patients with Complete or Partial Cytogenetic Response**

Patient	Date that complete cytogenetic remission first achieved	Date *BCR-ABL* junction detected by RT-PCR	Date of colony testing	RT-PCR on blood or bone marrow‡	Number of colonies positive for *BCR-ABL*/total number of colonies§	Comment‖
1	11/8	7/90 b_2-a_2 and b_3-a_2	07/91	Neg	4/10¶	One colony was positive for b_2-a_2 junction and three colonies were positive for b_3-a_2
2	04/89	11/89 b_2-a_2	06/91	Neg	0/22¶	
3	12/85	9/89 b_2-a_2 and b_3-a_2	07/91	Neg	3/10¶	Two colonies were positive for b_2-a_2 junction and one colony was positive for b_3-a_2
4	03/90	10/90 b_2-a_2 and b_3-a_2	10/91	Not done	10/10¶	All colonies were positive for b_2-a_2 and b_3-a_2 junctions
5	07/91	10/91 b_3-a_2	10/91	Pos	0/20¶	
6	07/88	11/89 b_2-a_2	09/91	Neg	0/20¶	
7	11/87	11/89 b_2-a_2 and b_3-a_2	09/92	Neg	0/20¶	

*All patients except case 4 had a complete cytogenetic response. ‡ RT-PCR was done on unfractionated blood (cases 1–3) and/or bone marrow (cases 1, and 5–7) collected at the same time as the bone marrow sample for colony testing. In case 4, who had 20% Ph+ cells (minor cytogenetic relapse) at the time of colony analysis, simultaneously obtained unfractionated samples were not available but RT-PCR had been tested on samples collected at a previous time (10/90). § Only colonies that were positive for control (normal *ABL*) transcripts on RT-PCR were considered evaluable. In patients 1–6, half the colonies were CFU-GM and half were BFU-E. In patient 7, there were 15 CFU-GM and 5 BFU-E colonies. ¶ All colonies that were considered *BCR-ABL* negative showed <5,000 RLU as determined by HPA. In patient 1, the 4 positive colonies showed 12,251, 16,644, 18,075, and 26,578 RLU, respectively. In patient 3, the 3 positive colonies showed 12,497, 75,124, and 130,072 RLU, respectively. In patient 4, the 10 positive colonies showed between 21,696 and 543,537 RLU. ‖ The *BCR-ABL*–positive colonies in patient 1 included two CFU-GM and two BFU-E; in patient 3, two CFU-GM and one BFU-E; in patient 4, five CFU-GM and five BFU-E.

4%. Furthermore, this test is carried out on cells in metaphase and does not necessarily reflect on the larger pool of leukemic, nondividing cells.

From studies using RT-PCR amplification of the *BCR-ABL* mRNA, it has become apparent that most patients with interferon-induced complete cytogenetic remission have residual disease (19), though, as shown in the current study (Fig. 2), some of these individuals may eventually convert from PCR-positive to PCR-negative with ongoing prolonged treatment. However, it is not clear from such tests whether the residual population of leukemic cells detected by RT-PCR maintains clonogenic potential or if only mature nondividing cells, such as mature lymphocytes, are expressing this transcript. In our current investigation of individually plucked colonies, we show that clonogenic *BCR-ABL*–positive cells remain in patients with cytogenetic remission and even in some patients whose blood or bone marrow is *BCR-ABL* negative by routine RT-PCR. It is therefore apparent that residual leukemic progenitors persist and may predispose these patients to a risk of disease relapse. As mentioned, in two of our patients (cases 1 and 3), evidence for residual disease was detected only by the study of myeloid and erythroid colonies, as RT-PCR on unfractionated blood and/or bone marrow was negative. This observation suggests that analysis of myeloid or erythroid progenitors may allow detection of residual disease not otherwise discernible. A possible explanation for this phenomenon may be an ongoing interferon-mediated suppressive effect in vivo, which interferes with the maturation and expansion of the leukemic progenitors which are, however, expanding ex-vivo under the influence of growth factors in the clonogenic assay. Further support to this notion also comes from the data on patient 4, who exhibited a minor cytogenetic relapse (20% Ph-positive metaphases) but nevertheless demonstrated *BCR-ABL* positivity in all colonies studied. Alternatively, this phenomenon may also be explained by a putative in vivo suppressive effect on *BCR-ABL* transcript expression in these cells.

Our assays failed, however, to detect recurrent disease in one of the patients who subsequently demonstrated cytogenetic relapse (case 2). There are several reasons that may be accountable. It is conceivable that some Ph-positive colonies do not express the *BCR-ABL* transcript (21). Alternatively, the number of colonies that were studied may have limited our ability to discern residual disease in all patients. The same reasons may explain the lack of *BCR-ABL*–positive colonies in patient 5, even though RT-PCR results on unfractionated bone marrow were positive. A more extensive study of myeloid and erythroid colonies using fluorescent in situ hybridization and appropriate probes might be helpful.

Our results also raise a question regarding the sensitivity of the *BCR-ABL*–based RT-PCR assay in peripheral blood samples. In one patient (case 4) in whom unfractionated blood and bone marrow were analyzed soon after achieving cytogenetic remission, RT-PCR results were positive for *BCR-ABL* transcripts in the bone marrow but negative in the blood. This patient subsequently had a minor cytogenetic relapse (though complete cytogenetic remission was again reinduced with ongoing therapy). Explanations for this observation may include unequal distribution of residual leukemic progenitors in the blood and bone marrow (22) or a decrease in *BCR-ABL* expression in mature cells. Interferon-induced complete cytogenetic

remission is invariably associated with normalization of peripheral blood and the absence of immature myeloid progenitors in the peripheral blood. In this regard, we demonstrated recently that the expression of the BCR-ABL protein ($p210^{BCR\text{-}ABL}$) is downregulated with myeloid maturation (23). Thus, the shift in cell population may result in false-negative *BCR-ABL*–based RT-PCR assays in the peripheral blood. This may perhaps help to account for some of the discrepancies in the number of CML patients identified as having residual disease in studies which have examined *BCR-ABL* expression by RT-PCR after bone marrow transplantation (24, 25).

An additional finding that was noted as a byproduct of our studies was that alternative splicing of *BCR-ABL* (b_2-a_2 versus b_3-a_2 splice junctions) may appear in the same colony as in patient 4 in whom all colonies had both transcripts, or alternatively, in different colonies as in patients 1 and 3 in whom some colonies were positive for one junction and others were positive for the other junction. The latter suggests the possibility of the emergence of a new clone or an altered version of the original clone.

Finally, evidence for residual disease was demonstrated in two of the patients (patients 1 and 3) with sustained durable complete cytogenetic remission, in one of whom treatment with interferon-alpha had already been discontinued for a period of 2 yr without any evidence of cytogenetic relapse. The presence of leukemic progenitors without evidence of either cytogenetic or clinical disease relapse raises the possibility of therapy-induced tumor dormancy. This condition lends itself to further studies, which may examine cellular immune-mediated control of residual leukemia or changes in the malignant potential of such clones.

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