British Journal of Haematology, 2001 112, 749–759

Expression of bcr–abl mRNA in individual chronic myelogenous leukaemia cells as determined by *in situ* amplification

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Received 23 February 2000; accepted for publication 10 September 2000

Summary. We present the results of a novel method developed for evaluation of *in situ* amplification, a molecular genetic method at the cellular level. Reverse transcription polymerase chain reaction (RT-PCR) was used to study bcr–abl transcript levels in individual cells from patients with chronic myelogenous leukaemia (CML). After hybridizing a fluorochrome-labelled probe to the cell-bound RT-PCR product, bcr–abl mRNA-positive cells were determined using image analysis. A dilution series of bcr–abl-positive BV173 into normal cells showed a good correlation between expected and actual values. In 25 CML samples, the percentage of *in situ* PCR-positive cells showed an excellent correlation with cytogenetic results ($r = 0{\cdot}94, P < 0{\cdot}0001$), interphase fluorescence *in situ* hybridization (FISH) ($r = 0{\cdot}95$, $P = 0{\cdot}001$) and hypermetaphase FISH ($r = 0{\cdot}81$, $P < 0{\cdot}001$). The fluorescence intensity was higher in residual CML cells after interferon (IFN) treatment than in newly diagnosed patients ($P = 0{\cdot}004$), and was highest in late-stage CML resistant to IFN therapy and lowest in CML blast crisis ($P = 0{\cdot}001$). Mean fluorescence values correlated with bcr–abl protein levels, as determined by Western blot analysis ($r = 0{\cdot}62$). Laser scanning cytometry allowing automated analysis of large numbers of cells confirmed the results. Thus, fluorescence *in situ* PCR provides a novel and quantitative approach for monitoring tumour load and bcr–abl transcript levels in CML.

Keywords: CML, bcr–abl mRNA, *in situ* amplification.

The Philadelphia chromosome (Pedersen, 1967), the reciprocal translocation between chromosomes 9 and 22 (Rowley, 1973) that is associated with the bcr–abl translocation (Groffen *et al*, 1984), is a hallmark of chronic myelogenous leukaemia (CML) and leads to the transcription of a fusion mRNA (Kloetzer *et al*, 1985). This mRNA is translated into a bcr–abl protein with deregulated tyrosine kinase activity (McWhirter *et al*, 1993; Pendergast *et al*, 1993; Puil *et al*, 1994) that phosphorylates cellular substrates (ten Hoeve *et al*, 1994; Liu *et al*, 1996; Sattler *et al*, 1996) and has autophosphorylating activity (Liu *et al*, 1993), which is at least in part responsible for the pathogenesis of Philadelphia chromosome-positive (Ph^+) leukaemia (McWhirter *et al*, 1993; Puil *et al*, 1994). It contributes to the malignant growth of the cells (Pendergast *et al*, 1993) and their resistance to apoptotic death (Bedi *et al*, 1994; McGahon *et al*, 1994; Cortez *et al*, 1995). Transfection of the fusion gene in mice has been shown to recreate conditions similar to CML (Daley, 1993).

For many years, the gold standard for detecting and monitoring the Ph^+ clone has been metaphase cytogenetic analysis (Nowell *et al*, 1992). This method is confined to dividing cells, analysing only few metaphases, and sufficient numbers of metaphases cannot always be retrieved. Therefore, we and colleagues (Tkachuk *et al*, 1990; Bentz *et al*, 1994; Sinclair *et al*, 1997) have developed techniques utilizing fluorescence *in situ* hybridization (FISH) to identify interphase cells carrying the Ph^1 translocation. This method detects clonal cells independently from the proliferative status of the cells. This is also true for the most sensitive method, the polymerase chain reaction (PCR), which uses the bcr–abl fusion mRNA as a template for reverse transcription polymerase chain reaction (RT-PCR) (Cross *et al*, 1993). As one can assume that more than a single RNA molecule is transcribed from the fusion gene,

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this makes RT-PCR more sensitive than PCR from DNA templates. Indeed, positive results are obtained in CML in continuous complete remission following treatment with chemotherapy (Gaiger *et al*, 1995), interferon (Pardini *et al*, 1994) or allogeneic and autologous transplantation (Miyamura *et al*, 1994; Lin *et al*, 1996). The clinical significance of minimal residual disease is not entirely clear (Goldman, 1997) but, whereas metaphase genetics only detects proliferating and thus multiplying cells responsible for the clonal expansion of the malignant cells, RT-PCR analyses the whole population that may be comprised of dividing and non-dividing cells. In addition, it raises the question, to what extent quantitative RT-PCR results can be translated into numbers of tumour cells if Ph^+ cells are not all equally transcriptionally active and vary in their expression of bcr–abl transcripts?

To address these problems, we have modified our previously published method (Spann *et al*, 1991; Pachmann *et al*, 1994) for amplification of gene sequences directly in cells on slides, for application to RT-PCR of bcr–abl mRNA. Cells of the Ph^+ clone were identified based on hybridization of a fluorochrome-labelled probe to amplified PCR products of the fusion mRNA. Results from patients at different stages of disease were compared with percentages of Ph^+ metaphases from standard cytogenetic examination, with hypermetaphase (Seong *et al*, 1995) and interphase FISH analyses, and with Western blot analysis of the bcr–abl′ protein (Guo *et al*, 1994) of the same samples; these tests are routinely performed on patients treated in our institution.

PATIENTS AND METHODS

Samples. These were obtained from patients with CML following informed consent in accordance with institutional guidelines. The Ph^+ BV173 cell line was kindly provided by Dr Wei Zhang (at The University of Texas M.D. Anderson Cancer Center). Bone marrow or blood samples of CML patients were treated with ammonium chloride lysis buffer (NH_4Cl 8·02 g, $NaHCO_3$ 0·84 g, EDTA 0·37 g) to lyse red blood cells; cytospin preparations were then prepared, fixed for 10 min in acetone and air-dried at room temperature.

In situ amplification. For *in situ* amplification, a 35-μl final volume of a conventional one step RT-PCR mixture with RT-PCR buffer (final concentration: 1× RT buffer, Boehringer Mannheim, Indianapolis, IN, USA), $Mn(OAc)_2$ (2·5 mmol/l), dNTPs (each of four at 300 μmol/l concentrations), abl and bcr primers designed according to the sequence published by Heisterkamp *et al* (1985) (bcr: 5′-AGGGTGCACAGCCGC AACGGC-3′, abl: 5′-GGCTTCACTCAGACCC TGAGA-3′, 450 μmol/l each), and 2·5 U of Tth polymerase (Boehringer Mannheim) was added to the cytospin preparation and all sealed in a plastic chamber (Perkin Elmer, Norwalk, CT, USA). The plastic chamber was firmly attached to the slide with a metal clamp (Perkin Elmer). After an initial heating step at 94°C for 2 min, reverse transcription was run for 60 min at 60°C in a Gene Amp *in situ* PCR thermocycler (Perkin Elmer). The subsequent amplification was performed according to the conditions established for conventional PCR except that the times were extended. Thus, after denaturation at 94°C for 2 min, 10 cycles were run with denaturation at 94°C for 1 min 30 s, annealing at 61°C for 1 min 30 s and extension at 72°C for 2 min. Ten more cycles were run with an extension of 5 s for each cycle. This number of amplification steps had been shown to be in the linear range of amplification. After the clamp with the plastic chamber was removed, a 2-μl sample of the supernatant was reamplified and analysed using gel electrophoresis for generation of the expected band. For detection of the amplified product in the cells, the slides were washed in 0·1× saline sodium phosphate EDTA (SSPE), overlaid with 5 μl of fluorochrome-labelled probe and 25 μl of hybridization solution (6× SSPE, 5× Denhardt's solution), resealed, heated to 94°C for 2 min and incubated for 12 h at 47°C. The slides were then washed in two changes of 0·1× SSPE for 10 min at 65°C and covered with anti-fade Vectashield H-1000 (Vector Lab, Burlingame, CA, USA) and a coverslip. The fluorochrome-labelled specific probe was a 262-bp amplification product from the mRNA of the cell line BV173, that was fluorochrome labelled during amplification according to the manufacturer's instructions (Boehringer Mannheim) and purified over a Qiagen quick spin column (Qiagen, Chatsworth, CA, USA). The fluorochrome-labelled control probe was an amplification product of the neo gene using the primers neo-1 5′-CAAGATGGAT TGCACGCAGG-3′ and neo-2 5′-GAGCAAGGTGAGATGACA GG-3′.

Fluorescence analysis. This was performed using a Zeiss Axioplan microscope equipped with a Nu200 CCD camera (Photometrics, Tucson, AZ, USA) connected to a MacIntosh Quadra 840AV computer. Using a Zeiss Plan Neofluar objective 100 × 1·30 oil, images of the fluorescent cells were captured using the IP LAB image analysis program (Signal Analysis, Vienna, VA, USA). The integrated fluorescence of individual cells was analysed and the fluorescence intensity distribution of 100–200 randomly measured cells displayed as a histogram. The percentage of bcr–abl mRNA-positive cells was calculated by subtraction of the fluorescence intensity distribution histogram of cells hybridized with a fluorochrome-labelled control (neo) probe from the histogram of the same cells hybridized with the specific bcr–abl probe using a modified histogram subtraction technique (Overton, 1988). The increase in fluorescence intensity of the positive cells was calculated as fold increase of the mean fluorescence intensity (MFI) of the positive cells over the MFI of the control population from the same patient. Some cell preparations were also measured using a laser scanning cytometer (Compucyte, Cambridge, MA, USA). For this purpose, the cytospins were dipped after hybridization into phosphate-buffered saline (PBS) containing 1 μg/ml propidium iodide (PI) and covered as above. The cells were contoured using red nuclear fluorescence of PI recorded in a photomultiplier equipped with a red long-pass filter in a 40× objective as a threshold parameter with the background determined dynamically for each cell. Integrated green fluorescence recorded with a second photomultiplier equipped with a green filter combination was then determined and compensated for the red PI fluorescence. The fluorescence data were analysed using the WINCYTE program provided by the company (Compucyte) and

displayed as scattergrams, histograms, percentages and mean values of cells calculated from the region gating over single cells.

Statistical analysis. This was performed using the two-sample *t*-test assuming unequal variances and ANOVA regression analysis.

Western blot analysis. This was performed with an anti-ABL (8E9) monoclonal antibody, as previously described (Guo *et al*, 1994). In brief, frozen cells were lysed in boiling sodium dodecyl sulphate (SDS) sample buffer for 5–7 min and the lysate was clarified by centrifugation. Aliquots of the extracts corresponding to 1×10^7 cells were applied to each lane. Samples were electrophoresed through 6·5% polyacrylamide gel, the gels were electroblotted at 4°C overnight and transferred to Immobilon filters (Millipore, Bedford, MA, USA). The ECL Western blotting detection system was used to probe for BCR–ABL protein according to the manufacturer's protocol (Amersham, Arlington Heights, IL, USA). Filters were preblocked by washing with 10% non-fat milk in Tris-buffered saline–Tween 20 (TBS-T) buffer (20 mmol/l NaCl, 0·0038 mmol/l HCl) for 2 h and then incubated with 1:15,000–20 000 dilution of 8E9 in 5% non-fat milk TBS-T buffer overnight at room temperature. The filters were then incubated with a 1:3,000–3500 dilution of horseradish peroxidase-labelled sheep anti-mouse IgG (Amersham, Cat. No. NA 9310) for 2 h. The filters were mixed with ECL reagents and exposed to X-ray film for 1–10 min. The intensities of p210 BCR–ABL, p190 and p145 ABL within appropriate autoradiograms were scanned with a soft laser densitometer (Zeineh Biomed Instruments, Fullerton, CA, USA).

Ph^+ metaphases. Percentages of Ph^+ metaphases were analysed using standard G-banding cytogenetics (CG) obtained on the same day, thus allowing comparison with the other methods.

Hypermetaphase FISH. This was performed as described previously (Seong *et al*, 1995). For FISH detection of the Ph chromosome, the E6B probe from 5 MB of human DNA spanning the breakpoint on chromosome 9q34 involved in the Ph translocation was labelled with biotin for visualization with fluorescein. Criteria for evaluating Ph^+ and Ph^- cells were published previously (Seong *et al*, 1995). Fluorescence *in situ* hybridization for bcr–abl was first described by us (Tkachuk *et al*, 1990) and applied here using a commercially available bcr–abl dual-colour kit (Vysis, Downer's Grove Il, USA), following the manufacturer's instructions. At least 100 cells were counted for each sample and only values surpassing the number of cells with fusion signal above the background of 14% (including two standard deviations) were reported as 'positive'(Schenk *et al*, 1998).

RESULTS

Samples from the Ph^+ cell line BV173 and from 25 patients with CML were investigated for cells positive for the bcr–abl mRNA by *in situ* RT-PCR using the image analysis system. In six patients, CML was recently diagnosed with 90–100% Philadelphia chromosome-positive (Ph^+) metaphases; these

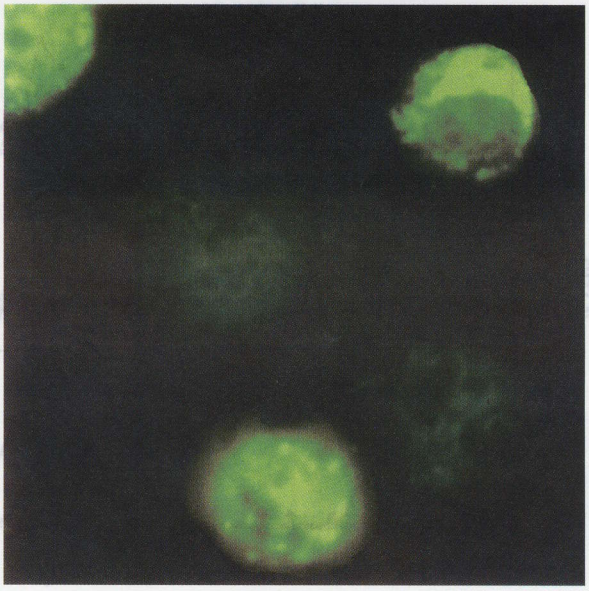

Fig 1. Microfluorograph of cells of a patient in partial remission amplified by *in situ* RT-PCR for bcr–abl transcripts and probed with a fluorochrome-labelled bcr–abl-specific probe (Magnification 100×). Hybridization with the bcr–abl-specific probe can be detected in three cells, whereas in two cells fluorescence is dim and they are negative.

patients had not been treated or had only been minimally treated. One of these patients was again monitored while receiving interferon therapy. Seven additional patients achieved a cytogenetic response with different levels of residual Ph^+ metaphases. Five patients failed to respond to interferon and received other therapies without cytogenetic response, two of whom had relapsed, one after autologous and one after a matched unrelated transplant. Seven patients were in blast crisis, one of them had Ph^- CML. Peripheral blood cells from four healthy persons and bone marrow cells from one healthy donor and three patients with breast cancer were used as controls.

Figure 1 shows a fluorescent micrograph from the cells of a CML patient (patient 7) who had 56% bcr–abl-positive cells (85% Ph^+ metaphases). After *in situ* amplification, the cells were probed with the fluorochrome-labelled specific bcr–abl probe. Three positive cells can be clearly distinguished from the two negative cells. Cells of the Ph^+ BV173 cell line and from 25 CML patients were measured for the fluorescence intensity values. Between 100 and 200 individual cells from preparations hybridized with the bcr–abl probe and the neo control probe were recorded and plotted as fluorescence intensity distribution histograms (Fig 2). Over 90% of the specifically hybridized cells of the cell line BV173 had values surpassing the values of the control histogram. It should be mentioned that autofluorescence of the cell line cells was higher than that of blood and bone marrow cells, perhaps owing to the dye used in the growth medium. Measurements of parallel preparations of the cell line gave almost identical results and this was also true for patients' cells. Repeated preparations ($n = 10$) of cell line cells at different time intervals always

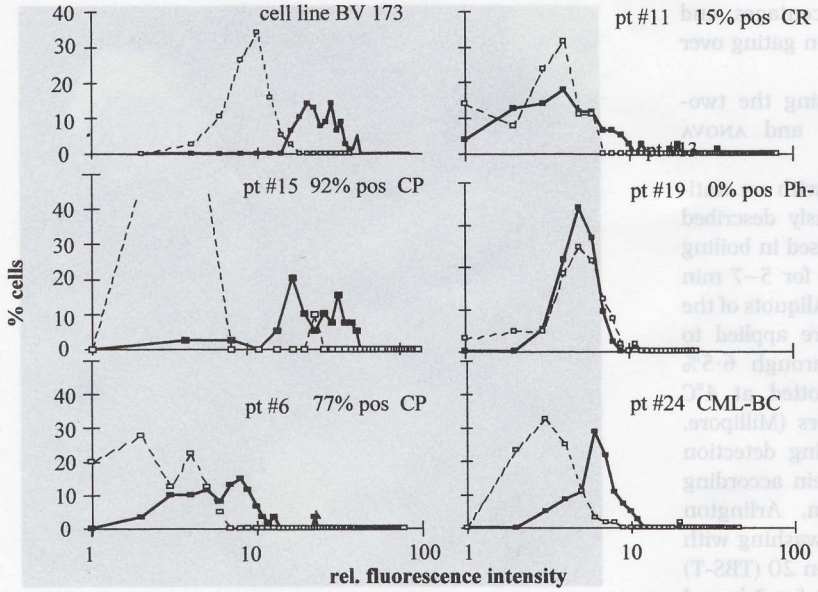

Fig 2. Fluorescence intensity distribution histograms of cells amplified for the bcr–abl translocation probed either with an fluorescein isothiocyanate (FITC)-labelled specific bcr–abl (solid line) or an unspecific neo probe (dashed line). Between 100 and 200 cells were measured individually for their integrated fluorescence intensity using a chilled CCD and the IPLAB image analysis program. Typical fluorescence intensity distribution histograms of cells from the cell line BV173 and five different patients amplified for bcr–abl transcriptsare shown. Two patients are in chronic phase (CP), one patient is in complete remission (CR) under interferon treatment and one patient is in blast crisis (CML-BC).

gave more than 90% positivity. Further controls included the omission of primers or polymerase, amplification steps with the unrelated neo primers and hybridization of these nonsense amplified preparations with the neo probe. All these controls yielded only background fluorescence. Examples of four different typical fluorescence intensity histograms obtained from CML patients with decreasing numbers of positive cells, one patient with Ph^- CML and one patient in CML blast crisis are shown in Fig 2. The number of positive cells was calculated by subtracting the control histogram from the positive histogram: 92% of the cells of patient 15 hybridized with the specific probe surpassed the fluorescence intensity of the control histogram. Patient 6, analysed at the first time-point, was typical for cases in which two peaks were observed, one coinciding with the histogram of the control cells and the other representing the positive cells with higher fluorescence values. In this case, positive and negative populations of cells were not clearly separated. The positive cells formed a peak and a shoulder, indicating that part of the cells contained low amounts of amplified material and few cells contained very high amounts. In several patients without Ph^+ metaphases, positive cells were still detected (e.g. patient 11). In a patient with Ph-negative CML (patient 19) with 0% positive metaphases, all cells were negative for bcr–abl mRNA. In this patient and in normal controls (not shown), the control neo and the specific bcr–abl hybridization histograms were identical. A sample from a patient with Ph^+ blast crisis (patient 24, as other samples from blast crisis) exhibited surprisingly low expression of bcr–abl (Fig 2). In spite of their low fluorescence, the shift of the whole histogram to higher values indicated that most cells were positive.

To determine the detection limit of the method and to test the accuracy of the analysis method, three separate experiments were performed with three different dilutions (1:1, 1:10, 1:100) of the cell line BV173 in normal peripheral white blood cells (after lysis of red blood cells) and amplified for bcr–abl (Fig 3). Expected and observed values showed a high correlation ($r = 0{\cdot}99$).

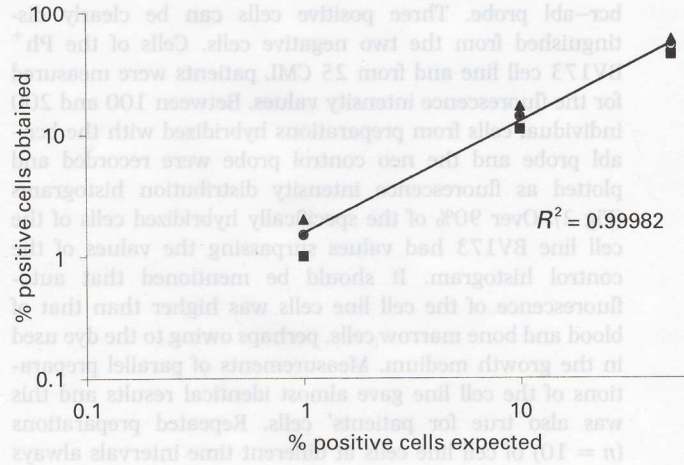

Fig 3. Cells from the cell line BV173 were mixed in different proportions as indicated with normal peripheral white blood cells, amplified for bcr–abl transcripts and measured individually for their integrated fluorescence intensity. Percentages of positive cells from three different experiments (exp. 1, ▲; exp. 2, ●; exp. 3, ■) were determined and compared with the expected values

Table I. Comparison between cytogenetic results for Ph^+ metaphases, FISH, hypermetaphase FISH (HMF),%positive cells by *in situ* PCR for bcr–abl transcripts, increase in mean relative fluorescence intensity of positive cells over control for bcr–abl mRNA (Mean pos. cells rel FI) and results from Western blot analysis for bcr–abl protein for 25 patients with CML.

Patient number	Diagnosis	Date	Treatment	Disease stage	Cytogenetics	FISH	HMF	% cells +ve *in situ*	Mean pos. cells rel FI
1	CML	25.11.96	HU	New	20/20 Ph^+			100%	2·1
2	CML	03.02.97	HU	New	20/20 Ph^+			86%	3·95
3	CML	27.10.96	IFN/AraC/HU	New	17/17 Ph^+			76%	4·74
4	CML	02.03.97	IFN/AraC	New	18/20 Ph^+	89%	50%	66%	3·36
5	CML	20.01.97	HU	New	20/20 Ph^+			68%	1·77
6	CML	27.10.96	No treatment	New	20/20 Ph^+		95%	77%	3·3
	CML	26.02.97	IFN/AraC	Cytogen. resp.	9/20 Ph^+			10%	5·19
7	CML	20.11.96	IFN	Late CP	16/19 Ph^+			56%	5·62
8	CML	09.02.97	IFN	Late CP	3/20 Ph^+			30%	3·19
9	CML	10.11.96	IFN/G-CSF	Haematological CR	Insuff. 3 dipl			10%	5·12
10	CML	12.02.97	IFN/AraC	Cytogen. resp.	0/7 Ph^+			14%	5·7
11	CML	21.10.97	IFN/AraC	Cytogen. resp.	0/20	30%	8%	15%	10
12	CML	05.11.97	IFN/AraC	Cytogen. resp.	1/20 Ph^+	26%	0%	4%	12
13	CML	20.10.96	IFN	Cytogen. resp.	0/20			10%	10
14	CML	11.12.96	HU	Late CP	20/20 Ph^+			100%	31·79
15	CML	11.12.96	HHT	Late CP	20/20 Ph^+			92%	6·98
16	CML	18.09.96	ASCT	Late CP	19/19 Ph^+			100%	5·89
17	CML	25.09.96	HU	Accelerated	17/17 Ph^+			69%	3·73
18	CML	17.09.96	3/96 MUD		1/19 Ph^+			15%	6·67
	CML	10.12.96		Relapse	20/20 Ph^+		43%	66%	4·57
	CML	06.01.97			20/20 Ph^+		70%	88%	8·08
19	CML PH^-	11.12.96	Exp.chemo.	Persistent BC	No abnormalities			0%	
20	CML	13.10.96	Exp. chemo	Lymph BC	20/20 Ph^+ clonal evolution			97%	1·67
21	CML	01.01.97	IFN	Progression to BC	60/40 Ph^+			90%	3·4
22	CML	02.12.97	Exp.chemo.	BC	20/20 Ph^+			70%	1·43
23	CML	06.11.97	Exp.chemo.	BC	18/19 Ph^+	81%	90%	81%	2
24	CML	10.12.96	Exp.chemo.	BC	20/20 Ph^+			60%	2·22
25	CML	13.01.97	Mini MUD	2nd lymph BC	14/20 Ph^+			34%	1·77

new, newly diagnosed patient; cytogen. resp., cytotenetic response; CR, complete remission; CP, chronic phase; BC, blast crisis; HU, hydroxy urea; IFN, interferon alpha; AraC, cytosine arabinoside; G-CSF, granulocyte colony-stimulating factor; HHT, homoharringtonine; ASCT, autologous stem cell transfusion; MUD, matched unrelated donor transplantation; exp. chemo., experimental chemotherapy at MD Anderson Cancer Center.

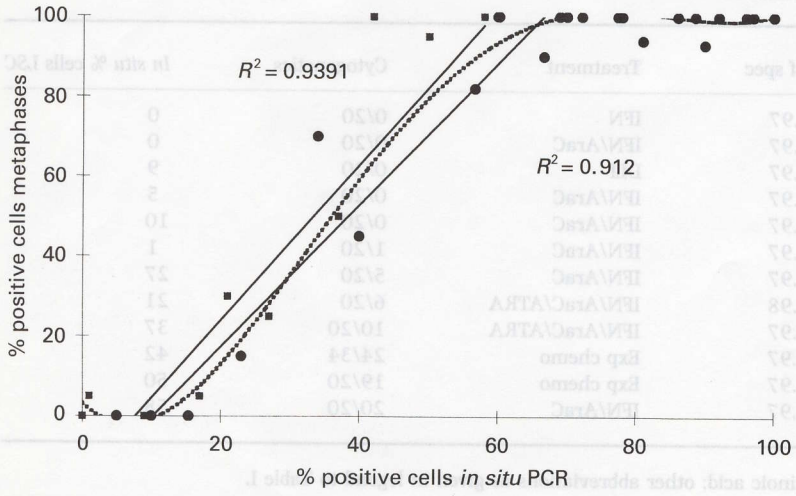

Fig 4. Correlation between the percentage of bcr–abl mRNA-positive cells as determined by image analysis for 24 CML samples(circles) and by LSC for 12 CML samples (squares) and the percentage of Ph^+ metaphases. Correlation coefficient of the linear part: 0·91 for image analysis and 0·94 for LSC measurements.

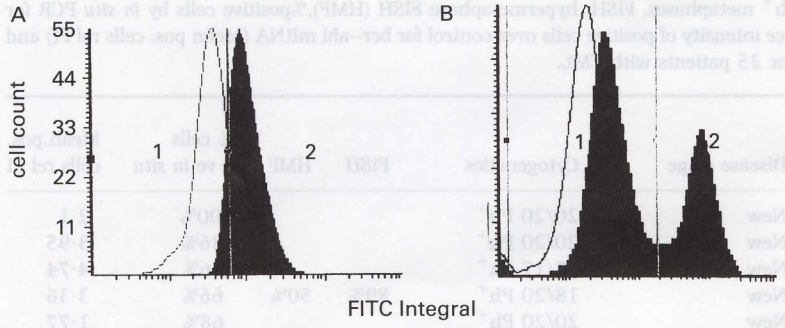

Fig 5. (A) Compensated fluorescence intensity distribution histograms of the cell line BV173 probed with an FITC-labelled specific bcr–abl (solid histogram) or an unspecific neo probe (dashed line) as measured by laser scanning cytometry. (B) Compensated fluorescence intensity distribution histograms of an FITC-labelled specific bcr–abl (solid histogram) or an unspecific neo probe (dashed line) for one typical patient, as measured by laser scanning cytometry.

The *in situ* amplification results obtained for the 25 patients are summarized in Table I and compared with results obtained by FISH, hypermetaphase FISH, cytogenetics and Western blot analysis of the bcr–abl protein. In the six newly diagnosed or only recently treated patients, 68–100% of cells were positive for the bcr–abl message in accordance with cytogenetic results (patients 1–6). The same was true for five patients without cytogenetic response (patients 14–18). In the seven patients who responded to IFN therapy, decreasing percentages of Ph^+ metaphases correlated with decreasing numbers of *in situ*-positive cells, but in some patients with no detectable abnormal metaphases we still detected positive cells using *in situ* PCR. As noted, the patient with Philadelphia-negative CML (patient 19) was also negative in the *in situ* PCR assay.

The comparison between *in situ* amplification and cytogenetic results for the patients is shown in Fig 4; the correlation coefficient for all Ph^+ samples measured in image analysis was 0·877, $P < 0·0001$, and the coefficient of precision was $r^2 = 0·76$. It is, however, obvious from Fig 4 that the relationship between the two parameters consists of different parts: in patients in which all metaphases, i.e. dividing cells, belong to the malignant clone, up to 40% of the bone marrow cells can be of non-clonal origin, whereas in patients with less than 100% Ph^+ metaphases, there is a strict correlation between the two methods ($r^2 = 0·91$); again, in patients with no detectable clonal metaphases, *in situ* PCR-positive cells can range between 0% and 15%. Consistent with these results, the correlation between interphase FISH and *in situ* PCR was 0·95 ($P = 0·001$) and was 0·85 ($P = 0·001$) between hypermetaphase FISH and *in situ* PCR. These fluorescence measurements obtained from several hundred individually analysed cells using a cooled CCD camera and image analysis were supplemented with results obtained with the recently introduced automated method of laser scanning cytometry (LSC). LSC allows analysis of 1×10^3–10^4 cells in 10–20 min. As shown in Fig 5A, when 3×10^3 cells were analysed, histograms obtained with LSC for the cell line BV173 were similar to those obtained using image analysis shown in Fig 2. In Fig 5B, results of one typical patient are shown with a clear separation between positive and negative cells, which was better than that obtained by CCD camera image analysis. The previous good correlation between *in situ* PCR results analysed by image analysis and cytogenetics was corroborated by the LSC results measuring between 1000 and 2000 cells (Fig 4). In contrast to the first 25 samples, the 12 subsequent samples were taken mainly from patients in partial and complete cytogenetic remission. The trendlines for both methods are almost superimposable and the precision between the percentage of Ph^+ metaphases and *in situ* PCR in the linear

Table II. Comparison between cytogenetic results for Ph^+ metaphases and percentage of positive cells by *in situ* PCR for bcr–abl transcripts using laser scanning cytometry for 12 patients with CML.

Patient number	Diagnosis	Date of spec	Treatment	Cytogenetics	*In situ* % cells LSC
1	CML	14.04.97	IFN	0/20	0
2	CML	17.09.97	IFN/AraC	0/20	0
3	CML	11.11.97	DLI	0/20	9
4	CML	13.11.97	IFN/AraC	0/20	5
5	CML	21.10.97	IFN/AraC	0/20	10
6	CML	05.11.97	IFN/AraC	1/20	1
7	CML	17.12.97	IFN/AraC	5/20	27
8	CML	22.02.98	IFN/AraC/ATRA	6/20	21
9	CML	22.10.97	IFN/AraC/ATRA	10/20	37
10	CML	28.09.97	Exp chemo	24/34	42
11	CML	17.03.97	Exp chemo	19/20	50
12	CML	07.09.97	IFN/AraC	20/20	58

DLI, donor lymphocyte infusion; ATRA, all *trans* retinoic acid; other abbreviations as given in legend to Table I.

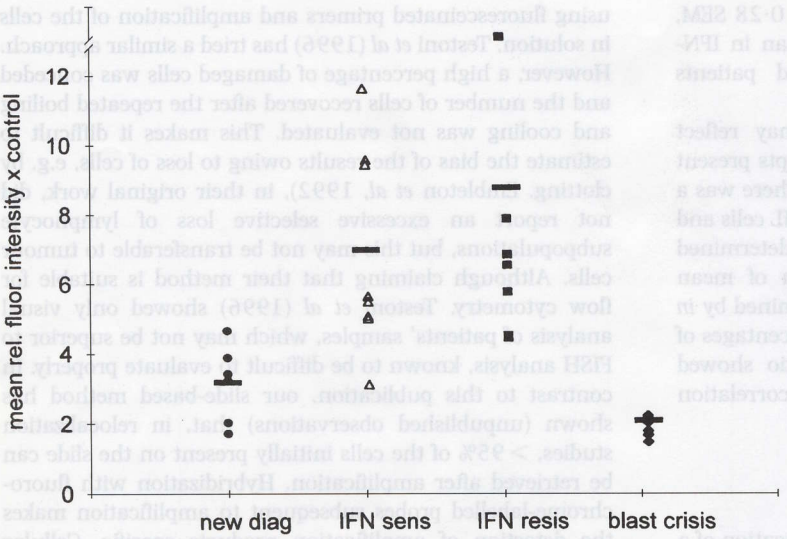

Fig 6. Increase in mean fluorescence intensity (MFI) of positive cells over MFI of control cells for CML patients at different stages of disease. new diag, six newly diagnosed patients; IFN sens, eight patients with disease sensitive to treatment with interferon alpha; IFN resis, seven measurements from five patients with disease resistant to treatment with interferon alpha; blast crisis, six patients in CML blast crisis. Differences between newly diagnosed and IFN sensitive: $P = 0{\cdot}004$; between IFN sensitive and IFN resistant: not significant; between IFN sensitive and blast crisis: $P = 0{\cdot}0011$; between newly diagnosed and blast crisis: $P = 0{\cdot}034$.

part of the curve in for the LSC results was $r^2 = 0{\cdot}97$. In essence, the relationship between metaphase cytogenetics and *in situ* PCR results would best be described using a sigmoid curve (dotted line in Fig 4). The lower part of the curve is formed by samples from patients, in which Ph^+ metaphases are no longer detected and *in situ* amplifications seems to be more sensitive. The uppermost part of the curve represents samples with 100% Ph^+ metaphases but with a different extent of replacement of the normal bone marrow with tumour cells. Table II compares results obtained by LSC analysis with cytogenetic results for 12 additional patients.

The fluorescence intensity values varied widely between different cells of individual patients and between different patients; therefore, we determined whether these differences were related to stage of disease. In Fig 6, the increase in mean intensities of bcr–abl-positive cells over control values is plotted for patients with newly diagnosed disease, interferon-sensitive and interferon-resistant disease, and blast crisis. In newly diagnosed and minimally treated patients, the fluorescence ratios varied between 1·8- and 4·7-fold over control (mean = 3·20 ± 0·45 SEM, median = 3·33). In patients responding to interferon treatment, the mean values of the positive cells showed mean values ranging between 3·2- and 12-fold (mean = 7·13 ± 1·11 SEM, median = 5·70), and this differed significantly from the newly diagnosed patients ($P = 0{\cdot}004$). Interestingly, one patient (patient 5) was investigated for a second time 4 months after onset of interferon treatment. The percentage of positive cells dropped from 77% to 10% but the mean fluorescence intensity of the positive cells increased from 3·3- to 5·2-fold over control. This patient was included in both groups (newly diagnosed patients and IFN-sensitive patients). The highest values were seen for patients with long-standing CML without cytogenetic response, with values ranging from 4·57- to 31·8-fold (mean = 9·01 ± 3·29 SEM, median = 6·29). These values were, however, not significantly different from the values of positive cells in IFN-sensitive patients ($P = 0{\cdot}30$). Included is one patient who was investigated twice during relapse. During this time, the mean bcr–abl ratio of his positive cells almost doubled. Very low values were determined in samples from patients with clonal evolution and blast crisis, with values

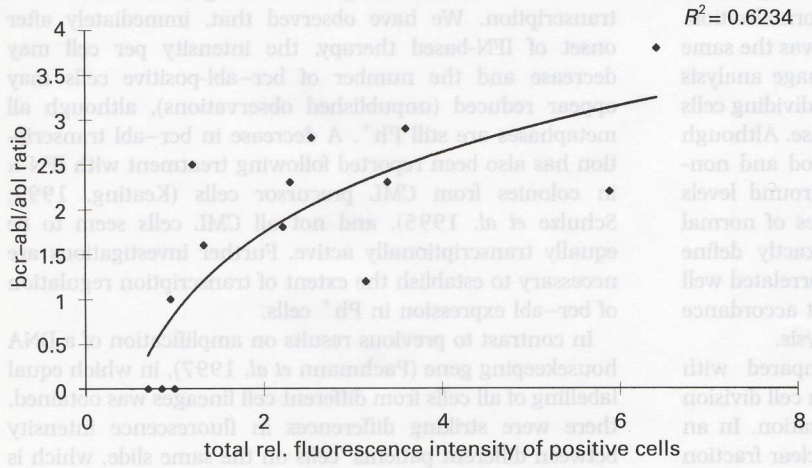

Fig 7. Correlation between increase in mean fluorescence intensity of positive cells over MFI of control cells as determined by *in situ* PCR and the bcr–abl/abl ratio at the same instant as Western blot analysis for 15 CML patients, for which both analyses were available. Correlation coefficient 0·62 and $P = 0{\cdot}07$.

ranging from 1·4- to 2·2-fold (mean = 2·08 ± 0·28 SEM, median = 1·89), a significantly lower range than in IFN-sensitive ($P = 0·0011$) and newly diagnosed patients ($P = 0·034$).

The differences in fluorescence intensity may reflect differences in the amount of amplifiable transcripts present in the cells. Therefore, we investigated whether there was a correlation between the mRNA content of the CML cells and the expression of p210 protein of the same cells, determined as bulk bcr–abl/abl protein ratio. Comparison of mean fluorescence values of the cell populations determined by *in situ* amplification normalized for the varying percentages of positive cells with the bcr–abl/abl protein ratio showed a second order correlation (Fig 7), with a correlation coefficient of 0·62 and $P = 0·003$.

DISCUSSION

In the present report we demonstrate that amplification of a segment of the bcr–abl fusion mRNA *in situ* in individual cells allows the discrimination of cells expressing and not expressing bcr–abl mRNA at the single cell level.

Positive and negative cells could not always be distinguished as unequivocally as shown in the micrograph (Fig 1), therefore the frequency of positive cells was calculated from the fluorescence intensity histograms, using a modified subtraction analysis, applying the same criteria as used in flow cytometry for correction of histogram overlap (Overton, 1988). An overall good correlation with cytogenetic results was obtained. Some important discrepancies were observed, however; for example, the frequency of bcr–abl-positive cells could vary between 60% and 100% when 100% of the metaphases were Ph^+. This discrepancy may be related to the restriction of cytogenetic analysis to dividing mononuclear cells, whereas the *in situ* PCR method analyses all cells of the bone marrow, mature and precursor myeloid cells, erythroid and megakaryocytic precursors, and lymphoid cells, independent of their mitotic activity. *In situ* PCR, thus, rather measures the tumour load of the bone marrow or blood than the proportion of clonal cells within the pool of proliferating mononuclear cells. Another important difference was the detection of bcr–abl-positive cells by *in situ* PCR in patients with complete cytogenetic normalization. As obvious from Fig 4, the offset at the *x*-axis was the same whether measurements were performed by image analysis or LSC. These positive cells may represent non-dividing cells of the malignant clone and, thus, residual disease. Although we did not detect positive cells in normal blood and non-CML marrow samples, methodological background levels have to be firmly established in a larger series of normal bone marrow samples in order to more exactly define minimal residual disease. *In situ* PCR results correlated well with hypermetaphase FISH and were in perfect accordance with results obtained by interphase FISH analysis.

The advantage of the *in situ* PCR compared with hypermetaphase FISH is the independence from cell division and, with interphase FISH, the easy quantification. In an 'in-cell PCR' technique analysing the mononuclear fraction using fluoresceinated primers and amplification of the cells in solution, Testoni *et al* (1996) has tried a similar approach. However, a high percentage of damaged cells was conceded and the number of cells recovered after the repeated boiling and cooling was not evaluated. This makes it difficult to estimate the bias of the results owing to loss of cells, e.g. by clotting. Embleton *et al*, 1992), in their original work, did not report an excessive selective loss of lymphocyte subpopulations, but this may not be transferable to tumour cells. Although claiming that their method is suitable for flow cytometry, Testoni *et al* (1996) showed only visual analysis of patients' samples, which may not be superior to FISH analysis, known to be difficult to evaluate properly. In contrast to this publication, our slide-based method has shown (unpublished observations) that, in relocalization studies, > 95% of the cells initially present on the slide can be retrieved after amplification. Hybridization with fluorochrome-labelled probes subsequent to amplification makes the detection of amplification products specific. Cellular fluorescence was quantified by CCD camera or LSC and the evaluation procedure allowed subtraction of the control histogram from the positive histogram, thus making evaluation objective. The new possibility of LSC analysis will replace the cumbersome and time-consuming procedure of image analysis and allows us now to rapidly screen large numbers of cells (several thousand) in a short time, making the method also superior to interphase FISH, which is still plagued by difficulties in discriminating positives from negatives. Thus, it is now possible to analyse high numbers of cells in a manner almost similar to flow cytometry, but to avoid the drawbacks associated with PCR of cells in solution. The complete procedure including evaluation can be performed in 1 d, so that results are available in a timely fashion for clinical decisions.

As we have shown previously (Spann *et al*, 1991; Pachmann *et al*, 1994), amplified material is shed into the supernatant. Even if we assume that amplified material leaks from all cells to the same extent, the possibility exists that some true Ph^+ cells may appear negative. This would be the case in particular for cells expressing low levels of transcripts, as we have shown up to 100-fold differences in mRNA content between the cells. Some Ph^+ cells might also remain undetected owing to downregulation of bcr–abl transcription. We have observed that, immediately after onset of IFN-based therapy, the intensity per cell may decrease and the number of bcr–abl-positive cells may appear reduced (unpublished observations), although all metaphases are still Ph^+. A decrease in bcr–abl transcription has also been reported following treatment with IFN-α in colonies from CML precursor cells (Keating, 1993; Schulze *et al*, 1995), and not all CML cells seem to be equally transcriptionally active. Further investigations are necessary to establish the extent of transcription regulation of bcr–abl expression in Ph^+ cells.

In contrast to previous results on amplification of a DNA housekeeping gene (Pachmann *et al*, 1997), in which equal labelling of all cells from different cell lineages was obtained, there were striking differences in fluorescence intensity between different patients' cells on the same slide, which is

indicative for true differences in mRNA content. We have also shown previously and in our artificial mixtures that transfer of amplified material into negative cells does not seem to play a role. The different levels of positivity observed at different stages of disease, the highest occurring in late-stage CML, were consistent with the high bcr–abl/abl protein ratios reported for patients who are resistant to therapy (Guo *et al*, 1994). This Westen blot analysis necessarily shows lower values for bcr–abl protein for patients with partial responses than for non-responders, as it is an average value obtained from a mixture of normal and abnormal cells. In contrast, the *in situ* technique, analysing individual cells, is able to identify the persisting CML cells in patients with partial responses, which exhibit fluorescence intensities similar to cells from resistant patients. Whether this is owing to preferential survival of highly expressing cells or to upregulation of expression during treatment needs to be further investigated. Others have also reported an increase in bcr–abl transcripts per cell during relapse of CML after bone marrow transplantation (Gaiger *et al*, 1995; Lin *et al*, 1996).

In contrast to patients with late-stage disease and patients in remission, who exhibited a wide variation in bcr–abl mRNA expression, cells from patients in blast crisis showed uniform fluorescence intensity distributions similar to histograms obtained from the BV173 cell line, with almost Gaussian distributions indicating an equal level of expression in all cells. The relatively low intensities consistently obtained from blast crisis cells were supported by the results from the bcr–abl protein Western blots. The low transcriptional activity of cells in blast crisis may indicate that these cells have become independent from bcr–abl-driven proliferation owing to additional transforming events. This finding requires confirmation, but hypermethylation-associated silencing of abl gene expression has been shown recently (Issa *et al*, 1997) and may explain the decreased transcript levels seen by us.

If bcr–abl transcripts can be downregulated (Keating, 1993; Keating *et al*, 1994, Dilloo *et al*, 1995) and expression levels are affected by treatment, monitoring the presence of CML cells, the purging efficacy of stem cell preparations (Fabrega *et al*, 1993; Smith *et al*, 1994) and the persistence of residual cells in patients after stem cell transplantation (Cross *et al*, 1993; Miyamura *et al*, 1994) using amplification of bcr–abl transcripts with RT-PCR as the only test may produce false-negative results (Pardini *et al*, 1994; Thijsen *et al*, 1997). Alternatively, increased positivity in PCR solution prior to overt relapse (Gaiger, 1995; Lin, 1996) may not only be a result of rising numbers of clonal cells, but increased bcr–abl transcription levels of individual cells may also contribute to this phenomenon. Furthermore, increased expression of the bcr–abl was reported during progression of disease in the chronic phase (Amikam *et al*, 1995). Using the proposed *in situ* PCR method will make it possible to investigate such events more closely at the single-cell level. We have provided evidence that cells which are resistant to treatment have higher transcript levels than cells that have not yet been exposed to treatment. Thus, our data suggest that the level of bcr–abl expression in individual cells may be a determinant of their response to therapy.

The bcr–abl fusion protein can induce specific antibodies (van Denderen *et al*, 1993) and reactive T cells (Cheever *et al*, 1993; Fuchs *et al*, 1995; Komatsu & Moriyama, 1996; Choudhury *et al*, 1997), and thus the level of bcr–abl transcripts (Miyamura *et al*, 1994; Hochhaus *et al*, 1995) and immunogenic proteins may influence the balance between initial growth and survival advantage (Kabarowski *et al*, 1994) and cell death in CML. Monitoring the transcriptional activity of bcr–abl in individual cells using *in situ* RT-PCR in CML may thus also contribute to a better understanding of the natural history of CML.

ACKNOWLEDGMENTS

This work was supported in part by grants from NIH CA16672, CA4963 and CA55164, and the Stringer Professorship for Cancer Research and Treatment (M.A.).

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