ELSEVIER

LEAD ARTICLE

Autologous Transplantation of In Vivo Purged PBSC in CML: Comparison of FISH, Cytogenetics, and PCR Detection of Philadelphia Chromosome in Leukapheresis Products

Georg Hess, Claudia Reifenrath, Anne Friedrich-Freksa, Vera Beyer, Sabine Naumann, Brigitte Schuch, Christoph Huber, Thomas Fischer, and Hans-Jochen Decker

ABSTRACT: *To determine the effectiveness of different methods for the detection of tumor cell contamination of collected peripheral stem cells, we performed a study on 39 chronic myelogenous leukemia (CML) patients who were consecutively treated at our department. Analyses of tumor cell contamination by fluorescence in situ hybridization (FISH), conventional cytogenetics, and polymerase chain reaction (PCR) showed marked differences in the percentage of evaluable results: Quantitative analysis of tumor cell contamination was feasible in 60 of 105 (57%) samples evaluated with the use of conventional cytogenetic analysis and in 105 of 107 (98%) samples analyzed by FISH. PCR was evaluable in all 85 samples tested (100%). Both methods were shown to be adequate overall in determining the number of BCR-ABL positive cells, although cytogenetics tended to produce slightly higher percentages. Based on these results, we conclude that FISH performed on leukapheresis products is a rapid and reliable method for assessing the quality of these products and should be used for routine evaluation of tumor cell contamination of CML stem cell products.*

INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematologic disease characterized by a three-phase clinical course. An initial chronic phase with expanded myelopoiesis is followed by an accelerated phase that finally leads to fatal blast crisis. The disease is cytogenetically characterized by the reciprocal translocation (9;22)(q34;q11), resulting in the Philadelphia (Ph) chromosome. The product of this rearrangement is the *BCR-ABL* fusion gene, which serves as the pathognomonic marker for CML.

Therapeutic options were limited before the introduction of interferon-alpha (IFN) therapy and the use of allogeneic bone marrow transplantation (BMT), and most patients died within a period of 6 years. Therapy with interferon, besides a high rate of side effects, leads to a marked cytogenetic response in a proportion of patients and prolongation of survival but does not cure [1, 2].

Allogeneic bone marrow transplantation is actually the only cure option for patients with CML, although therapy-related morbidity and mortality are considerably high. However, a high percentage of patients do not respond to IFN therapy or they are not eligible for allogeneic BMT, because there is no suitably matched related or unrelated donor or because of advanced age. In the search for therapeutic alternatives for these patients, autologous transplantation has become of interest in the past few years. Recent studies have shown a survival benefit for patients treated by this approach, because the chronic phase of the disease is prolonged [3, 4]. Especially, young age and short duration from the time of diagnosis to transplantation are associated with improved outcome [5]. Despite the fact that relapse may be related to persistent tumor cells owing to an insufficient conditioning regimen, one of

From III Medizinische Klinik, Johannes Gutenberg-Universität (G. H., C. R., S. N., B. S., C. H., T. F., H.-J. D.), Mainz, Germany; and the Institut für Klinische Genetik (A. F.-F., V. B.), Mainz, Germany.

Address reprint requests to: Dr. H.-J. Decker, III Medical Clinic, Haematology, Oncology, and Pneumology, Johannes Gutenberg-University Hospital, Langenbeckstr. 1, 55131 Mainz, Germany.

Received May 26, 1999; accepted June 15, 1999.

655 Avenue of the Americas, New York, NY 10010

0165-4608/00/$–see front matter
PII S0165-4608(99)00133-8

the major limitations of this form of treatment is the reinfusion of clonogeneic cells. Although laboratory methods to purge the leukapheresis product fail, efforts of in vivo purging have been made to reduce this fraction of clonogeneic cells by the use of different mobilization chemotherapies. Especially, more aggressive chemotherapy regimens resulted in a partly Ph negative leukapheresis product when evaluated only by conventional cytogenetics [6–8]. However, there are technical limitations that may interfere with the results of cytogenetics. More-sensitive molecular methods are always able to detect *BCR-ABL* positive cells in leukapheresis products.

Conventional cytogenetics, fluorescence in situ hybridization (FISH), and the polymerase chain reaction (PCR) have proved to be powerful tools for the monitoring of residual disease in CML. FISH and PCR show a sensitivity that is highly superior to that of conventional cytogenetics. In contrast with conventional cytogenetics, FISH analysis is not dependent on metaphases but can be performed on interphase cells. For the diagnosis of residual disease in CML, FISH has been shown to be as sensitive and reliable as conventional cytogenetics. However, for the evaluation of leukapheresis products, to our knowledge, a gold standard of diagnostic procedures has yet to be defined. For this reason, we performed an analysis on 39 consecutive CML patients treated at our department to determine the effectiveness of FISH, conventional cytogenetics, and PCR in the analysis of leukapheresis products.

PATIENTS AND METHODS

Patients

Between 1995 and 1998, 39 patients with CML were enrolled in two prospective studies at our institution. Studies were performed according to the Declaration of Helsinki and were approved by the local ethics committee. Inclusion criteria are presented elsewhere [9]. In total, three different chemotherapy/mobilization procedures were applied: "5 ± 2": cytarabine 100 mg/m^2/day by continuous infusion over 24 hours, days 1–5; idarubicin 12 mg/m^2/day, days 1–2; "mini-ICE": cytarabine 800 mg/m^2/day, days 1–3; idarubicin 8 mg/m^2/day, days 1–3; etoposide 150 mg/m^2/day, days 1–3, followed by application of human recombinant granulocyte colony stimulation factor (G-CSF, filgrastim, Neupogen) 5–10 μg/kg from day 8 postchemotherapy until the end of leukapheresis collection. Some patients who had a cytogenetic response to IFN-therapy received G-CSF (lenograstim, Granocyte) under continuation of IFN therapy, 150 μg/m^2, without any other prior chemotherapy (steady-state mobilization). In total, the different treatments resulted in successful mobilization in 28 of 39 patients. In 11 patients, no sufficient number of CD34+ cells could be mobilized, or leukapheresis was interrupted because the patient recovered in blast crisis (patient 36) or because of noncompliance (patient 39). Six patients received a second mobilization chemotherapy followed by application of G-CSF (5–10 μg/kg/day) plus human recombinant interleukin 3 (5 μg/kg). In 5 of 6 cases, second therapy resulted in successful mobilization.

Leukaphereses were performed according to standard procedures as described elsewhere [9] and, besides *BCR-ABL* evaluation, quality of the leukapheresis product was analyzed by performing colony assays (CFU-GM).

Fluorescence In Situ Hybridization

Fresh leukapheresis cells were incubated with 0.075 M KCl for 15 minutes at 37°C before fixation with ice-cold methanol:glacial acid (5:1). Cell suspensions were dropped on dry, warm slides. Slides were allowed to air dry. After slides were aged at 60°C for 5–8 hours, FISH was performed by using directly labeled, dual-color probes for BCR and ABL (LSITM bcr/abl translocation probe no. 32-190022, Vysis, Stuttgart, Germany). Fluorescence microscopy was performed according to the manufacturer's instructions. The number of cells in which the count and quality of signals could not be determined unambiguously was used as an internal measurement for hybridization efficiency. When at least 200 cells were suitable for analysis, FISH was considered successful.

Cytogenetics

Leukapheresis samples were cultured 2 and 24 hours at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. Colcemid (0.3 μg/mL medium) was added for the last 20 minutes culture. After centrifugation, cells were treated with 0.075 mol/L KCl for 5 minutes twice and then fixed in freshly prepared methanol/acetic acid in a proportion of 3 to 1, followed by 2 to 1 and 3 to 1. Chromosome preparations were stained with the use of the trypsin-Giemsa and quinacrine dihydrochloride technique.

Reverse Transcription Polymerase Chain Reaction

Leukapheresis materials were collected after leukapheresis, stored overnight at 4°C, and processed immediately the following morning. Cells were prepared by Ficoll-Hypapaque (Sigma, St. Louis, MO, USA), and total RNA was extracted by the method of Chomczynski [10] by using commercially available kits (Boehringer Mannheim, Germany; Cinna/Biotecx Lab, Inc., USA).

Reverse transcription was performed for 40 minutes at 42°C by using 50 U of MuLV-reverse transcriptase (Boehringer Mannheim, Germany) and random hexamer, or a one-step reverse transcriptase polymerase chain reaction (RT-PCR) was performed with an initial transcription at 50°C for 30 minutes with a commercially available kit (Titan One Tube, Boehringer Mannheim, Germany) and downstream primer. A two-round nested PCR reaction was applied and reaction products were electrophoresed on a 2% agarose gel. PCR reactions were performed on a MWG thermocycler (MWG, Ebersberg, Germany). The following reaction conditions and primers were used: First round PCR: 94°C for 5 minutes, 35 cycles of 40 seconds 94°C, 60 seconds 65°C, 60 seconds 72°C. Second round (nested) PCR: 94°C for 5 minutes, 35 cycles of 40 seconds 94°C, 60 seconds 65°C, 60 seconds 72°C with a final extension of 10 minutes at 72°C with the use of 1.25 U of Taq-polymerase (PE, Weiterstadt, Germany) and the buffers supplied. The following primers were used: First PCR: (A)

5′-TGTGATTATAGCCTAAGACCCGGAG-3′ and (B) 5′-GAAGAAGTGTTTCAGAAGCTTCTCCC-3′. Nested PCR: (C) 5′-TCCATTGGCCACAAAATCATACAGT-3′ and (D) 5′-GTGAAACTCCAGACTGTCCACAGCA-3′.

Each analysis included a positive (K562) and a nucleic acid-free control. Specificity of the reaction was proved by transfer of randomly chosen probes to a nylon membrane and hybridization with *BCR-ABL*-specific oligonucleotide (5′-AAGCCGCTCGTTGGAACTCCAAGGA-3′).

Statistical Methods

Analysis of the correlations between the percentage of Ph-positive metaphases and the proportion of positive interphase cells was performed by using the Spearman's rank correlation coefficient. The regression line was calculated by the least-squares method. Results obtained by cytogenetics and FISH for the different response groups were compared by using the chi-square test. Comparison of the average number of positive cells by FISH and cytogenetics in the different leukaphereses was performed by Student's *t*-test.

RESULTS

Patients

Data of the patients studied are shown in Table 1. Overall, 39 patients were treated; characteristics of some patients have been shown elsewhere [9]. Average age of patients at diagnosis was 46.7 years (range 22–64 years), with predominance of male sex (M:F = 25:14). Mean duration of

Table 1 Patient characteristics, mobilization, and leukaphereses

UPN	Age at diagnosis	Sex	Diagnosis-mobil (months)	Disease status at mobil	Prior therapy	Mobil chemo	Number of LP performed	Second mobil
1	28	F	120	CP	HU, IFN (9)	2 + 5	1	N
2	57	M	84	AP	HU, IFN (6)	Mini-ICE	4	N
3	48	F	65	CP	HU, IFN (38)	Mini-ICE	3	N
4	51	M	64	CP	HU, IFN (60)	Mini-ICE	2	N
5	32	F	37	CP	HU, IFN (1)	2 + 5	4	Y (5 LP)
6	55	M	34	CP	HU, IFN (22)	2 + 5	2	Y (1 LP)
7	46	F	30	CP	HU, IFN (18)	2 + 5	5	N
8	22	F	29	CP	HU, IFN (29)	2 + 5	4	Y (0 LP)
9	44	F	37	CP	HU, IFN (14)	Mini-ICE	3	N
10	22	M	20	CP	HU	2 + 5	1	Y (7 LP)
11	44	M	35	CP	HU, IFN (5)	Mini-ICE	2	N
12	55	M	15	CP	HU, IFN (1)	2 + 5	1	Y (8 LP)
13	51	M	22	AP	HU, IFN (19)	2 + 5	6	Y (3 LP)
14	50	M	12	CP	HU, IFN (9)	2 + 5	3	N
15	49	M	5	CP	HU, IFN (5)	2 + 5	9	N
16	50	F	14	BC	HU, IFN (12)	Mini-ICE	2	N
17	36	M	10	CP	HU	2 + 5	7	N
18	51	M	8	CP	HU	2 + 5	2	N
19	57	M	10	CP	HU, IFN (3)	2 + 5	3	N
20	28	F	7	CP	HU	2 + 5	1	N
21	50	M	5	CP	HU, IFN (4)	2 + 5	6	N
22	42	M	6	CP	HU, IFN (4)	2 + 5	6	N
23	53	F	2	CP	HU, AraC	Mini-ICE	0	N
24	49	M	54	CP	HU, IFN (59)	Mini-ICE	0	N
25	49	M	52	CP	HU, IFN (40)	Mini-ICE	3	N
26	53	F	9	CP	HU, IFN (8)	Mini-ICE	2	N
27	55	M	3	CP	HU	Mini-ICE	3	N
28	55	M	3	CP	No	Mini-ICE	2	N
29	52	M	33	CP	HU	Mini-ICE	2	N
30	49	M	20	CP	IFN (7)	Steady state	1	N
31	64	M	63	BC	IFN (18)	Mini-ICE	3	N
32	48	F	10	CP	HU, IFN (5)	Mini-ICE	3	N
33	26	M	10	CP	IFN (10)	Steady state	3	N
34	53	F	7	CP	HU, IFN	Mini-ICE	2	N
35	59	F	30	CP	HU, IFN (16)	Mini-ICE	6	N
36	62	M	26	BC	HU	Mini-ICE	1	N
37	27	F	81	CP	HU, IFN	Steady state	2	N
38	40	M	34	CP	IFN (25)	Steady state	2	N
39	59	M	19	CP	HU, IFN (15)	Steady state	1	N

Abbreviations: UPN, unique patient number; CP, chronic phase; AP, accelerated phase; BC, blast crisis; HU, hydroxyurea; IFN, interferon-alpha; Y, yes; N, no; LP, leukapheresis; mobil, mobilization.

disease before mobilization was 28.8 months (range 3–120 months). Most of the patients received a therapy consisting of hydroxyurea or IFN or both before they entered the mobilization procedure. The average number of leukapheresis performed was 2.9 (range 1–9). Patients 23 and 24 had to be excluded from this analysis, because no leukapheresis could be performed owing to insufficient mobilization.

Table 2 Results of laboratory analyses

UPN	Method	LP 1	LP 2	LP 3	LP 4	LP 5	LP 6	LP 7	LP 8	LP 9
1	FISH	82								
	CYTO	100(22)								
	PCR	Nd								
2	FISH	36	43	51	61					
	CYTO	29(14)	Nd	Nd	Nd					
	PCR	Pos	Pos	Pos	Pos					
3	FISH	39	50	Nd						
	CYTO	100(21)	80(5)	Ne						
	PCR	Pos	Pos	Nd						
4	FISH	10	11							
	CYTO	10(20)	Ne							
	PCR	Pos	Pos							
5	FISH	Nd	32	Nd	Ne					
	CYTO	100(14)	100(6)	100(30)	Ne					
	PCR	Nd	Nd	Nd	Nd					
6	FISH	66	83							
	CYTO	93(15)	100(25)							
	PCR	Nd	Nd							
7	FISH	35	32	27	Nd	19	41			
	CYTO	Nd	67(24)	Nd	Nd	Nd	Nd			
	PCR	Nd	Nd	Nd	Nd	Nd	Nd			
8	FISH	Nd	30	26	27					
	CYTO	33(3)	Nd	0(3)	Ne					
	PCR	Nd	Nd	Nd	Nd					
9	FISH	Nd	Nd	Nd						
	CYTO	Nd	Nd	Nd						
	PCR	Pos	Pos	Pos						
10	FISH	33	35	40	58	Nd	45	Nd		
	CYTO	33(15)	44(9)	100(11)	100(10)	80(15)	13(15)	Nd		
	PCR	Nd	Nd	Nd	Nd	Nd	Nd	Nd		
11	FISH	29	31							
	CYTO	20(30)	16(25)							
	PCR	Pos	Pos							
12	FISH	9	12	20	20	21	35	Nd		
	CYTO	Nd	35(20)	Ne	Ne	30(20)	Ne	Ne		
	PCR	Nd	Nd	Nd	Nd	Nd	Nd	Nd		
13	FISH	Nd	Nd	Nd	45	Nd	34			
	CYTO	Ne	Ne	Ne	73(26)	100(40)	100(1)			
	PCR	Pos	Pos	Pos	Nd	Nd	Nd			
13	FISH	16	48	44						
	CYTO	63(19)	Nd	Nd						
	PCR	Pos	Pos	Pos						
14	FISH	7	3	17						
	CYTO	3(31)	0(26)	0(7)						
	PCR	Nd	Nd	Nd						
15	FISH	25	22	Nd	24	Nd	22	Ne	51	28
	CYTO	13(23)	46(13)	40(20)	66(15)	100(5)	85(20)	95(20)	Ne	Ne
	PCR	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd
16	FISH	28	32							
	CYTO	0(2)	0(2)							
	PCR	Pos	Nd							
17	FISH	2	3	Nd	Nd	1	4	Nd		
	CYTO	Nd	3(30)	5(22)	6(17)	4(23)	0(16)	3(35)		
	PCR	Pos	Pos	Pos	Pos	Pos	Pos	Pos		

(*continued*)

Table 2 Continued

UPN	Method	LP 1	LP 2	LP 3	LP 4	LP 5	LP 6	LP 7	LP 8	LP 9
18	FISH	14	13							
	CYTO	21(14)	0(6)							
	PCR	Pos	Pos							
19	FISH	28	27	22						
	CYTO	0(22)	0(10)	13(24)						
	PCR	Pos	Pos	Pos						
20	FISH	90								
	CYTO	Ne								
	PCR	Pos								
21	FISH	23	31	49	61	74	Nd			
	CYTO	33(9)	Nd	92(13)	75(4)	67(27)	Nd			
	PCR	Pos	Pos	Pos	Pos	Pos	Pos			
22	FISH	33	38	42	50	55	55			
	CYTO	Nd	Ne	Nd	Nd	91(20)	93(30)			
	PCR	Pos	Pos	Pos	Pos	Pos	Pos			
25	FISH	11	19	17						
	CYTO	0(48)	Ne	0(27)						
	PCR	Pos	Pos	Pos						
26	FISH	15	8							
	CYTO	0(25)	7(30)							
	PCR	Pos	Pos							
26	FISH	53	37	39						
	CYTO	96(20)	98(45)	Nd						
	PCR	Pos	Pos	Pos						
27	FISH	Nd	16	13						
	CYTO	Ne	Ne	31(16)						
	PCR	Pos	Pos	Nd						
28	FISH	4	7							
	CYTO	0(36)	0(36)							
	PCR	Pos	Pos							
29	FISH	Nd	23							
	CYTO	Nd	32(22)							
	PCR	Pos	Pos							
30	FISH	2	3							
	CYTO	Ne	80(15)							
	PCR	Pos	Pos							
31	FISH	6	6	7						
	CYTO	11(17)	5(39)	8(25)						
	PCR	Pos	Pos	Pos						
32	FISH	47	42	55						
	CYTO	100(30)	100(30)	97(30)						
	PCR	Pos	Pos	Nd						
33	FISH	24	16	14						
	CYTO	80(30)	67(30)	50(30)						
	PCR	Pos	Pos	Pos						
34	FISH	43	46							
	CYTO	96(25)	100(25)							
	PCR	Pos	Pos							
35	FISH	31	43	24	45	39	46			
	CYTO	55(20)	85(20)	85(20)	94(29)	87(21)	Nd			
	PCR	Pos	Pos	Pos	Pos	Pos	Pos			
36	FISH	56								
	CYTO	0(16)								
	PCR	Pos								
37	FISH	6								
	CYTO	0(2)								
	PCR	Pos								
38	FISH	9	Nd							
	CYTO	Ne	Ne							
	PCR	Pos	Pos							
39	FISH	Nd	Nd							
	CYTO	Nd	Nd							
	PCR	Pos	Pos							

FISH and cytogenetic studies depict percentage of Ph-positive cells in each harvest; numbers in parenthesis denote the number of metaphases analyzed.

Abbreviations: Pos, positive; Nd, not done; Ne, not evaluable.

Laboratory Evaluation

Results of cytogenetic analysis, FISH analysis, and RT-PCR performed on leukapheresis samples are shown in detail in Table 2.

Cytogenetic Analysis

A total of 105 cytogenetic analyses have been performed on leukapheresis material. In 85 of 105 (81%) samples, at least one metaphase could be analyzed. Because low metaphase counts do not give representative results, a cutoff for evaluation was set; it required at least 10 metaphases for each analysis to be scored quantitatively evaluable. In respect to this cutoff, 60 (57%) of all samples were suitable for quantitative analysis (Fig. 1).

FISH Analysis

A total of 107 FISH analyses have been performed. Of them, 105 (98%) were evaluable because at least 200 cells could be analyzed. In respect to the number of cells counted, all samples were suitable for quantitative analysis (Fig. 1). A cutoff was set for Ph+ <5% of all cells analyzed. In respect to this cutoff, 97 (90%) of all analyzed samples were positive for *BCR-ABL* detection.

RT-PCR

A total of 85 samples were analyzed by qualitative RT-PCR (Fig. 1). All of these samples proved to be positive for the detection of *BCR-ABL*.

Average Number of Ph+ Cells in the Different Leukaphereses

Because of the low number of patients requiring more than six leukaphereses, only the first six leukaphereses were chosen. For more homogeneity, patients with accelerated or blastic phase were excluded. In addition, patients with steady-state mobilization were excluded. When samples with fewer than 10 evaluable metaphases were excluded, 86 FISH and 61 cytogenetic results were available for the anlaysis. Figure 2 shows the average number of Ph+ cells during leukaphereses 1–6 when measured by FISH and conventional cytogenetics. As shown, there is a marked difference between both methods (from 12.3 to 30.7%), with cytogenetics showing the higher average number of Ph-positive cells. This difference did not show statistical significance, because of the low number of leukaphereses in our study. On average, both methods revealed results very similar on the different days, with no statistically significant differences between the different leukaphereses; however, there were some patients with less tumor contamination during the first leukaphereses (patients 2, 15, 21, and 22).

Matched Pair Analysis of FISH and Cytogenetics Results

Figure 3 shows the matched pair analysis for FISH and cytogenetics, provided that at least 10 metaphases could be evaluated by cytogenetics. In total, 62 pairs were compared (Fig. 3). Regression analysis supported the view that, in comparison with FISH, cytogenetic analysis tended to reveal higher-percentage figures.

Correlation of Cytogenetic and FISH Results with Response Criteria

Table 3 shows the correlation of FISH results with the response graded by cytogenetics. Responses obtained by cytogentics and FISH analysis were defined as follows: Complete response (CR): 0% positive metaphases by cytogenetics, less than 6% positive cells by FISH. Major response (MR): <35% Ph-positive cells by cytogenetics or FISH. Minor response (mR): 35–95% Ph-positive cells by either method. No response (NR): >95% Ph-positive cells by cytogenetics or FISH. There was a good correlation of both methods, classifying the degree of response to therapy (Table 3). In respect to the characteristics of FISH to present less-extreme results, there was a tendency toward

Figure 1 Qualitatively and quantitatively evaluable results. Cytogenetics: more than 10 metaphases evaluable. The observed difference in the number of samples suitable for quantitative analysis is significant ($P = 0.0142$ by chi-square test).

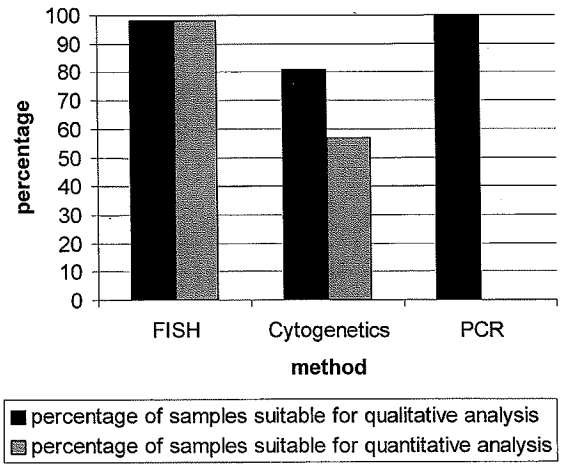
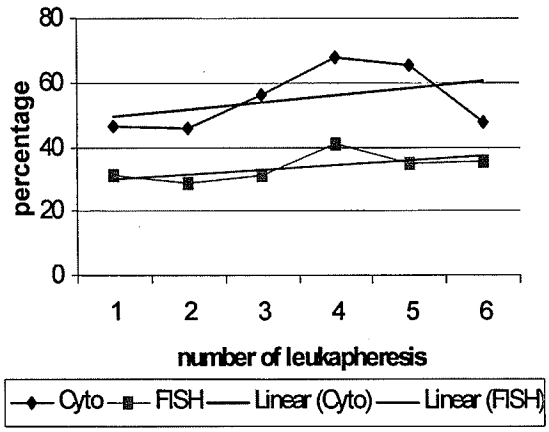


Figure 2 Comparison of different leukaphereses by FISH and cytogenetics. Comparison of the average numbers of positive cells by cytogenetics or FISH analysis for the different leukaphereses (1–6). Only patients with prior chemotherapy in CP were evaluated; at least 10 metaphases had to be evaluable for cytogenetic analysis.



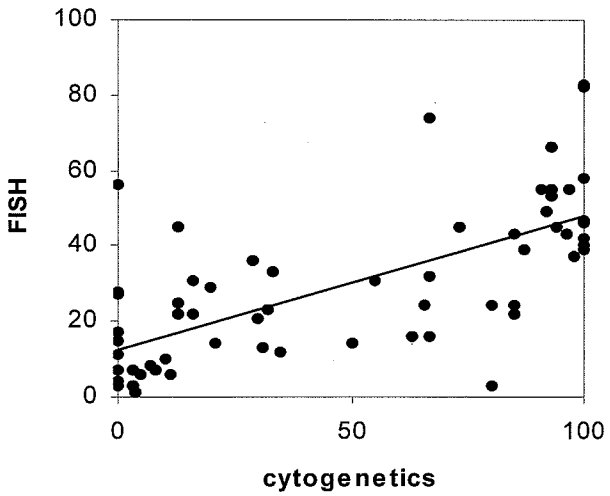


Figure 3 Matched pair analysis of cytogenetics and FISH. All samples that were analyzed by both methods (n = 62) were considered for analysis. At least 10 metaphases had to be evaluable for cytogenetic analysis. R^2 = 0.4684.

more MR and mR by using FISH compared with cytogenetics, which did show more CR and NR. However, the difference did not reach statistical significance, except for the group of nonresponders, which differed significantly between the different methods.

DISCUSSION

Autologous stem cell transplantation seems to be a reasonable choice for patients with chronic-phase CML not eligible for allogeneic BMT. For the long-term outcome, there is concern about the probable influence of clonogeneic cells retransfused. Currently, there are no data available showing a clear correlation between the degree of tumor contamination and outcome. To address this problem, a reliable and sensitive method of evaluating tumor cell contamination has to be chosen. The data that we present here will be helpful in defining an optimal strategy for evaluating tumor cell contamination.

In the majority of studies on autologous transplantation with mobilized stem cells, conventional cytogenetics has been the standard procedure for evaluation of Ph+ cells in patients with CML. However, there are technical limitations for this method, and molecular methods show that even cytogenetically negative samples have contamination with *BCR-ABL*-carrying cells [11]. We performed an analysis evaluating leukapheresis products by conventional cytogenetics, FISH, and qualitative RT-PCR.

As expected, RT-PCR was able to detect *BCR-ABL* transcripts in all samples evaluated, but did not provide information on the content of clonogeneic cells. For further studies, quantitative PCR may help in addressing this question, because it gives more precise information about the content of *BCR-ABL*-carrying cells. However, it is a quite laborious method. In a comparison of cytogenetics and FISH for overall qualitative evaluability, there was an advantage for FISH over cytogenetics (98% vs. 81% of samples accessible). This difference increased choosing only those samples suitable for quantitative analysis. The following criteria were used: Cytogenetics: at least 10 metaphases evaluable. FISH: at least 200 cells evaluable. Ninety-eight percent of FISH analyses were evaluable, but only 57% of cytogenetic analysis (Fig. 1). This difference proved to be statistically significant (P = 0.014). In the literature, most authors report performance of at least 20 metaphases if possible, but a cutoff for samples with a low number of metaphases analyzed is seldom set. We think that quantitative assessment of Ph+ cells in leukaphereses products requires at least 10 metaphases to be evaluable. Interestingly, in 12 samples that showed more than 10% of interphase cells positive for the Ph chromosome, cytogenetics failed to detect any Ph chromosome. Furthermore, cytogenetic analyses showed a high number of samples with 100% Ph-positive metaphases (n = 14), which probably does not represent the real content of *BCR-ABL* positive cells. We conclude that, overall, FISH has a considerably better reliability for detecting Ph-carrying cells than does cytogenetics.

Comparing levels of Ph positivity over the different leukaphereses revealed a marked difference in the contents of Ph+ cells between both methods, showing a difference of 12.3–30.7%, with cytogenetics always presenting higher levels of Ph-positive cells. For higher homogeneity, only patients in chronic phase, with prior mobilization chemotherapy and leukaphereses 1–6 were compared. Owing to the number of samples tested, this difference did not reach statistical significance. Additionally, we performed a matched pair analysis of all samples that could be evaluated with both methods, applying the definition for quantitative analysis heretofore given. Regression analysis supports the tendency of cytogenetics to show higher figures of positive results (Figs. 2 and 3).

There was a slight tendency toward a higher percentage of *BCR-ABL* positive cells in late leukaphereses. Because the overall number of leukaphereses was small, this difference did not reach statistical significance, and further ob-

Table 3 Correlation of response criteria comparing cytogenetics with FISH

	Complete cytogenetic response (n = 10)	Major cytogenetic response (n = 18)	Minor cytogenetic response (n = 21)	No cytogenetic response (n = 12)
FISH CR (n = 6)	3	2	1	0
FISH MR (n = 30)	6	14	11	1
FISH mR (n = 26)	1	2	9	12
FISH NR (n = 0)	0	0	0	0

servations are needed. Regarding the differences of Ph-positive cells in early and late leukapheresis products, there are only a few observations in the literature. Although some authors have shown a correlation between early leukapheresis and low number of Ph-positive cells [6], other data do not support this result [12–14].

In summary, we suggest FISH to be the method of choice for evaluating leukapheresis samples of patients with CML who are being considered for autologous transplantation. First, cytogenetics is a time-consuming method, requiring the preparation of metaphases, which fails in a substantial proportion of samples. Second, with the use of classical cytogenetics, only cells with potential to divide are analyzed. Therefore the pool of resting interphase cells is missed for evaluation. Third, cytogenetics tends to give more-extreme results (e.g., 0 or 100%). Especially, a result of 100% Ph-positive cells might underestimate the content of normal cells coexisting with the abnormal clone. In contrast, FISH is a feasible, easy, and fast method that gives a higher number of qualitatively and quantitatively evaluable results. It is independent of cell growth, and a large number of cells can be analyzed. Additionally, FISH does not tend to give extreme results and therefore is able to display more realistically the extent of tumor cell contamination. As shown, FISH is suitable for determining the response of treatment adequately to cytogenetics, and therefore it can be used for clinical response monitoring.

Eighteen of the patients noted in this paper have been transplanted with the mobilized stem cells. There has been no therapy-related mortality. Eight patients received an allogeneic transplant. A total of 5 patients died, mainly because of progression to blastic phase or because of mortality associated with allogeneic transplantation.

For the future, it will be necessary to correlate the percentage of Ph-positive cells in mobilized stem cells estimated by FISH and quantitative PCR with the clinical outcome of patients. This correlation will be extremely helpful in defining a predictive value for tumor cell contamination and an optimal treatment regimen. Furthermore, it will be of great interest to compare the results of these different laboratory methods, analyzing exclusively CD34-selected cells.

This work was supported by grants form the Sander-Foundation (93.038.2.) and from the Deutsche José Carreras Leukämie-Stiftung e.V. We gratefully acknowledge Sylvia Ernst for excellent technical assistance.

REFERENCES

1. Kantarjian HM, O'Brien S, Anderlini P, Talpaz M (1996): Treatment of chronic myelogenous leukemia: current status and investigational options. Blood 87:3069–3081.
2. Ohnishi K, Ohno R, Tomonaga M, Kamada M, Onozawa K, Kuramoto A, Dohy H, Mizoguchi H, Miyawaki S, Tsubaki K (1995): A randomized trial comparing interferon-alpha with busulfan for newly diagnosed chronic myelogenous leukemia in chronic phase. Blood 86:906–916.
3. Carella AM, Podesta M, Lerma E, Dejana A, Prencipe E, Vassallo F, Crescenti C, Soracco M, Benvenuto F, Chimirri F, Cralier P, Florio G, Valbonesi M, Frassoni F (1996): Mobilization/transplantation of Ph1-negative blood progenitor cells in chronic myelogenous leukaemia. Ann Oncol 7(suppl 2): 19–22.
4. Boque C, Petit J, Sarra J, Cancelas JA, Munoz J, Espanol JI, de la Banda E, Aventin A, Berlanga J, Ferra C, Amill B, Torrico C, Azqueta C, Llucia M, Garcia J, Granena A (1996): Mobilization of peripheral stem cells with intensive chemotherapy (ICE regimen) and G-CSF in chronic myeloid leukemia. Bone Marrow Transplant 18:879–884.
5. McGlave PB, DeFabritiis P, Deisseroth A, Goldman J, Barnett M, Reiffers J, Simonsson B, Carella A, Aeppli D (1994): Autologous transplants for chronic myelogenous leukemia: results from eight transplant groups. Lancet 343:1486–1488.
6. Carella AM, Podesta N, Frassoni F, Raffo MR, Pollicardo N, Pungolino E, Vimercati R, Sessarego M, Parodi C, Rabitti C (1993): Collection of normal blood repopulating cells during early haematopoietic recovery after intensive conventional chemotherapy in chronic myelogenous leukemia. Bone Marrow Transplant 12:267–271.
7. Chalmers EA, Franklin IM, Kelsey SM, Newland AC, Clark RE, Sproul AM, Cratty G, McCann SR, Fielding A, Goldstone AH, Hepplestone A, Watson W, Sharp RA, Tansey P (1997): Treatment of chronic myeloid leukemia in first chronic phase with idarubicin and cytarabine: mobilization of Philadelphia-negative peripheral blood stem cells. Br J Haematol 96:627–634.
8. Kantarjian HM, Talpaz M, Hester J, Feldman E, Korbling M, Liang J, Rios MB, Smith TL, Calvert L, Deisseroth AB (1995): Collection of peripheral-blood diploid cells from chronic myelogenous leukaemia patients early in the recovery phase from myelosuppression induced by intensive-dose chemotherapy. J Clin Oncol 13:553–559.
9. Fischer T, Neubauer A, Mohm J, Huhn D, Busemann C, Link H, Arseniev L, Büssing B, Novotny J, Ganser A, Duyster J, Bunjes D, Kreiter S, Autlitzky W, Hehlmann R, Huber C (1998): Chemotherapy-induced mobilization of karyotypically normal PBSC for autografting in CML. Bone Marrow Transplant 21:1029–1036.
10. Chomczynski P, Sacchi N (1987): Single step method of RNA isolation by acid guandidium-thyocyanate-phenol-chloroform extraction. Anal Biochem 167:156–159.
11. Johnson RJ, Owen RG, Child JA, Morgan GJ, Branard DL, Dickinson H, Ricketts S, Rawstron A, Evans P, Woodhead V, Major K, Robinson F, Smith GM (1996): Mobilization of Philadelphia-negative peripheral blood mononuclear cells in chronic myeloid leukaemia using hydroxyurea and G-CSF (filgrastim). Br J Haematol 93:863–868.
12. Hughes TP, Grigg A, Szaer J, Ho J, Ma D, Dale BM, Green RM, Norman JE, Sage RE, Hermann R, Cannell P, Schwarer AP, Taylor K, Atkinson K, Arthur C (1997): Mobilization of predominantly Philadelphia chromosome-negative blood progenitors using cyclophosphamide and rHUG-CSF in early chronic-phase chronic myeloid leukaemia: correlation with Sokal prognostic index and haematological control. Br J Haematol 96:635–640.
13. Mehta J, Mijovic A, Powles R, Pagliuca A, Singhal S, Czepulkowski B, Swansbury GJ, Treleaven J, Mufti GJ (1996): Myelosuppressive chemotherapy to mobilize normal stem cells in chronic myeloid leukemia. Bone Marrow Transplant 17:25–29.
14. Archimbaud E, Michallet M, Philip I, Charrin C, Clapisson G, Belhabri A, Guilhot F, Stryckmans P, Adeleine P, Fiere D (1997): Granulocyte colony-stimulating factor given in addition to interferon-alpha to mobilize peripheral blood stem cells for autologous transplantation in chronic myeloid leukaemia. Br J Haemotol 99:678–684.