PERSISTENCE OF CHRONIC MYELOCYTIC LEUKEMIA DESPITE DELETION OF REARRANGED *bcr/c-abl* SEQUENCES IN BLAST CRISIS

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Chronic myelocytic leukemia (CML)[1] is a clonal pluripotent stem cell disease, clinically divided into a chronic phase of a median duration of ~4 yr, followed by an acute phase (blast crisis) lasting for few months (1). Cytogenetic hallmark of most (95%) CML cases is the presence of the Philadelphia (Ph) chromosome, resulting from a reciprocal translocation between chromosomes 9 and 22 (2, 3). Molecular analyses established that, in leukemic cells of all Ph^+ CML patients, the c-*abl* oncogene is transferred from chromosome 9 into the breakpoint cluster region (*bcr*) on chromosome 22 (4, 5); this area is part of a gene of as-yet-unknown function, the *bcr* gene, of which the coding regions are spread over at least 70 kb, including the *bcr* and exons 5′ and 3′ to it (6–8). This genomic rearrangement on the Ph-chromosome results in the transcription of a chimeric 8.5 kb *bcr/c-abl* RNA species, which represents mRNA for an altered c-*abl* protein that differs from its normal counterpart in a higher associated tyrosine kinase activity (8–12).

Herein, we report on a Ph^+ CML patient cytogenetically characterized by the appearance of a second Ph chromosome during blast crisis. Southern blot analyses and in situ hybridization studies suggested a deletion of rearranged 5′ *bcr* and c-*abl* sequences in acute phase of this case. Northern blots of blastic cells detect normal *bcr* and *abl* RNA species instead of the fused 8.5 kb transcript usually observed in Ph^+ CML. These data may indicate that the altered *abl* protein is of subordinate importance for the maintenance of the leukemic state once Ph^+ CML has entered blast crisis.

Materials and Methods

Patient. CML was diagnosed in a 49-yr-old male exhibiting a standard Philadelphia translocation, t(9;22)(q34;q11). Despite initial benefit from busulfan treatment, CML accelerated towards lymphoid blast crisis 45 mo later. Blast cells were of T phenotype and genotype (T cell receptor β chain gene rearrangement), and showed a second Ph chro-

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[1] *Abbreviations used in this paper:* CML, chronic myelocytic leukemia; Ph, Philadelphia chromosome.

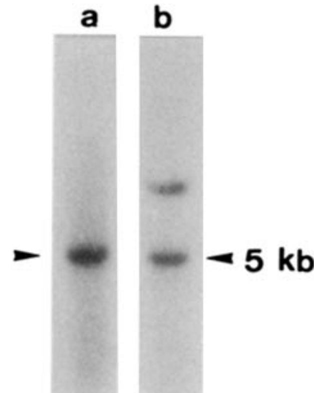

FIGURE 1. Southern blot analysis of blast cell DNAs (15 μg) digested with Bgl II, electrophoresed on a 0.7% agarose gel and hybridized to a 2 kb Bgl II/Hind III 5′ *bcr* probe (lane *a*) and to a 1.2 kb Hind III/Bgl II 3′ *bcr* probe (lane *b*) that detect 5 kb germline fragments.

mosome as the only additional chromosomal aberration; both Phs were of similar size as compared with the single Ph chromosome in chronic state. Combination chemotherapy achieved a clinical remission, and the patient has been in chronic phase for 13 mo; cytogenetic studies performed on three occasions during the latter period revealed two Phs in all metaphases investigated.

DNA Analysis. Bone marrow DNA (15 μg) was digested with restriction enzymes (C. Boehringer and Soehne, Mannheim, Federal Republic of Germany), electrophoresed on a 0.7% agarose gel, blotted, and hybridized to a 2 kb Bgl II/Hind II 5′ *bcr* probe, a 1.2 kb Hind III/Bgl II 3′ *bcr* probe, and a 1 kb 5′ 8E *bcr* cDNA probe, as described (13, 18). λ DNAs were included as mol wt standards. After hybridization, filters were washed under high stringency (10% saline citrate, 65°C) and exposed to XAR-2 film (Eastman Kodak Co., Rochester, NY) for up to 3 d at −70°C with DuPont Lightning Plus intensifying screens (DuPont Instruments, Wilmington, DE).

RNA Analysis. RNA was isolated from bone marrow cells according to the LiCl/urea method (17); poly(A)$^+$ RNA was obtained after two passages over oligo(dT)-cellulose; 10 μg of poly(A)$^+$ RNA was electrophoresed on a 1.2% agarose gel in the presence of formaldehyde (15). After blotting, nitrocellulose filters (Schleicher and Schuell, Dassel, Federal Republic of Germany) were hybridized to a 0.6 kb Eco RI/Bam HI c-*abl* probe or a 2 kb Bgl II/Hind III 5′ *bcr* probe, as described (13). Filters were exposed to Kodak XAR-5 film using DuPont Lightning Plus intensifying screens for up to 4 d at −70°C.

In Situ Hybridization. Chromosomes obtained from bone marrow were prepared according to standard techniques. After RNase treatment, chromosomes were denatured in 70% formamide in 2× SSC at 70°C for 2 min, rinsed in 2× SSC, and dehydrated. The probe, a 1:1 mixture of human 3′ 1.1 kb Hind III/Eco RI and 5′ 0.6 kb Bam HI c-*abl* plasmids (17) was labelled by nick-translation using [^{3}H]dCTP and [^{3}H]dTTP (New England Nuclear, Boston, MA) to a sp act of 1.2×10^7 cpm/μg. The probe was denatured for 5 min at 70°C at a concentration of 0.2 μg/ml in 50% formamide in 2× SSC with 10% dextran sulfate added to the chromosomes, and hybridized for 10 h at 37°C; slides were rinsed in three changes of 50% formamide/2× SSC at 39°C, followed by 6 h washing in 2× SSC at room temperature, exposed to Kodak NTB2 emulsion for 12 d at 4°C, developed, and stained with quinacrince mustard as described (13).

Results

Southern blot analysis of leukemic cells obtained from this case during blast crisis show a 5 kb germline band after hybridization to 5′ *bcr* sequences (Fig. 1, lane *a*). In contrast to all Ph$^+$ CML patients investigated thus far, a rearranged

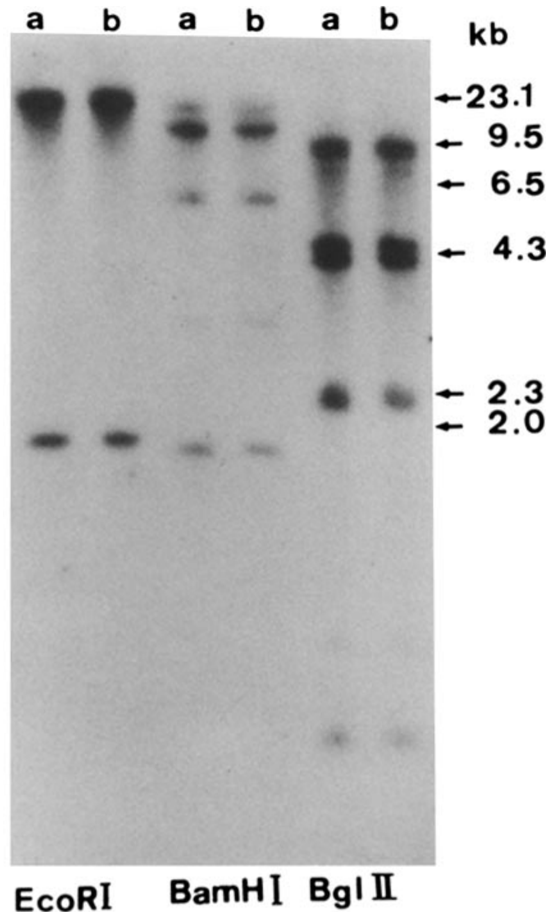

FIGURE 2. Southern blot analysis of human placenta (lanes *a*) and blast cell DNAs (lanes *b*). 15 μg DNAs were digested with Eco RI, Bam HI, or Bgl II as indicated, electrophoresed, and hybridized to a 1 kb 5′ 8E *bcr* cDNA probe.

5′ *bcr* fragment is not visible. However, a recombination within the *bcr* could be established by hybridization of Bgl II digests to 3′ *bcr* sequences that are usually transferred to chromosome 9q+ in Ph$^+$ CML (Fig. 1, lane *b*). A Bgl II polymorphism as an explanation for the latter result could be ruled out, since digests using different restriction enzymes (Bam HI, Eco RI, and Hind III) showed a similar pattern (data not shown). To investigate the possibility that, in this patient, the break on chromosome 22 has occurred outside the *bcr* region but still within the *bcr* gene, we hybridized different digests of blast cell DNAs to a 1 kb cDNA probe covering the most 5′ *bcr* sequences known thus far (18). Again no rearranged fragments are detected as compared with control DNAs (Fig. 2). These results suggested a deletion of rearranged 5′ *bcr* sequences in blast cells. However, the cDNA probe might not cover all chromosomal *bcr* sequences, and we therefore cannot entirely rule out the possibility that a recombination took place in such a region. Since cell samples from the chronic state of this patient were not available for Southern blot analysis, we decided to perform in situ

TABLE I
Results of In Situ Hybridization to c-abl Probes

Chromosome	Chronic phase		Blast crisis	
	Grains observed	Expected	Grains observed	Expected
1	7	9.0	24	18.1
2	12	8.8	14	17.7
3	4	7.3	12	14.6
4	9	7.0	7	14.0
5	6	6.7	11	13.4
6	2	6.3	6	12.6
7	5	5.8	10	11.6
8	2	5.3	8	6.5
9	13	2.5	34	5.0
9q+	5	3.0	7	5.7
10	2	4.9	8	9.7
11	3	5.0	6	10.0
12	0	4.9	11	9.8
13	6	3.9	4	7.8
14	1	3.7	5	7.5
15	2	3.5	2	7.1
16	3	3.2	9	6.5
17	5	3.1	8	6.2
18	0	2.9	5	5.9
19	1	2.3	5	4.5
20	0	2.5	0	5.0
21	0	1.7	1	3.4
22	3	0.9	0	1.8
Ph	9	0.5	4*	2.2*
X	4	2.8	7	5.6
Y	0	1.0	3	1.9

Grains were counted on complete, well-spread metaphases. Number of grains expected was determined according to DNA content (16).
* On two Ph chromosomes.

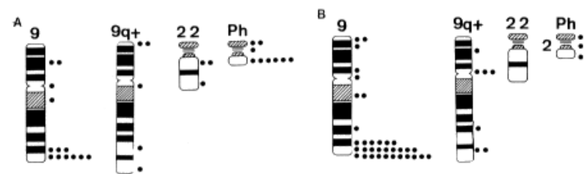

FIGURE 3. Distribution of silver grains on relevant chromosomes obtained from chronic phase (*A*) and blast crisis (*B*) of the CML patient hybridized in situ to c-*abl* probes (see Table I).

hybridization studies of c-*abl* sequences to metaphases obtained from both states, to investigate a possible concurrent deletion of this oncogene.

Distribution of 104 silver grains obtained from analysis of 21 metaphases of chronic phase was uniform and at random on all chromosomes except specific signals ($p < 0.01$) on chromosomes 9 and 22q− (Table I and Fig. 3*A*). However, in situ hybridization on c-*abl* sequences to 49 metaphases obtained from blast crisis showed a significant grain accumulation ($p < 0.01$) only on chromosome 9, while distribution of silver grains on both Phs and all other chromosomes was

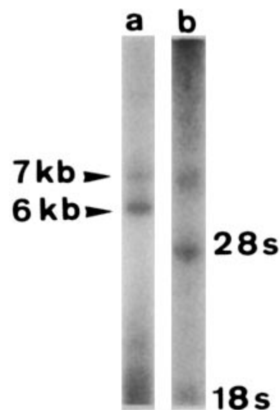

FIGURE 4. Northern blot analysis of poly(A)$^+$ RNA (10 μg) obtained from blast cells of the CML patient hybridized to a c-*abl* (lane *a*) and 5′ *bcr* probe (lane *b*). The position of ribosomal 28 S and 18 S RNA is indicated.

uniform (Table I and Fig. 3*B*). These results unequivocally demonstrated a deletion of c-*abl* sequences from the blast phase Ph-chromosomes.

Finally, we performed Northern blot analyses of blastic cells. Hybridization to c-*abl* and *bcr* sequences exhibited normal 6 and 7 kb c-*abl* as well as 4.5 and ~7 kb *bcr* transcripts, respectively (Fig. 4). Neither probe detected the hybrid 8.5 kb *bcr*/*abl* RNA species usually found in Ph$^+$ CML.

Taken together, the occurrence of a *bcr*/*c-abl* rearrangement in this case during generation of CML is deduced from the demonstration of a c-*abl* oncogene translocation by in situ hybridization in chronic state samples and the detection of rearranged 3′ *bcr* sequences in Southern blots from acute state. A deletion of recombined sequences of both genes in acute state, probably during duplication of the Ph chromosome, is suggested by the failure to detect (*a*) translocated c-*abl* sequences on both Phs, (*b*) rearranged 5′ *bcr* sequences, and (*c*) the hybrid 8.5 kb *bcr*/*abl* transcript during blast crisis.

Discussion

The involvement of c-*abl* and *bcr* sequences in the development of Ph$^+$ CML has been deduced from (*a*) the consistent observation of a rearrangement of both genes on the Ph chromosome in all cytogenetic subtypes of this leukemia, (*b*) the concurrent detection of a novel 8.5 kb chimeric *bcr*/*c-abl* RNA transcript, and (*c*) the expression of an altered p210 c-*abl* protein that differs from the normal p145 counterpart as to its associated tyrosine kinase activity (4–12, 19, 20). However, neither the precise physiological function of the products encoded by both genes nor the meaning of their altered versions in Ph$^+$ CML has been elucidated as yet. Moreover, recent reports on Ph$^-$ CML characterized by a *bcr* rearrangement without juxtaposition of c-*abl* (21), as well as Ph$^+$ ALL cases showing a c-*abl* translocation to other sequences on the Ph instead of *bcr* (22) may indicate that both genes could interact with other genes to generate similar leukemic entities. Several Ph$^-$ CML patients have been observed (23, 24) that developed the Ph during later stages of their leukemia, suggesting that acquisition

of Ph is not the primary event in the generation of this disease. Other patients with typical Ph^+ CML entered acute phase with blast cells lacking the Ph chromosome, consistent with the existence of Ph^- leukemic cells within the same patient (25). Finally, glucose-6-phosphate dehydrogenase isoenzyme analysis showed the presence of Ph^- leukemic cells in Ph^+ CML patients (26). These data suggest a multistep pathogenesis of CML in which the development of the cytogenetically visible Ph chromosome, as well as the molecularly detectable *bcr*/c-*abl* rearrangement is a second event in this process.

Along this line, transition of chronic towards acute phase of Ph^+ CML would represent a third step, characterized by marked differences in the biology of leukemic cells, and associated with karyotypic evolution exhibiting additional nonrandom chromosomal aberrations (1, 27). The detection of identical rearranged *bcr* fragments as well as comparable levels of the 8.5 kb *bcr*/*abl* RNA species in blast cells and chronic phase cells of the same patients also suggest that genes other than *bcr* and c-*abl* induce this terminal shift of biological properties within leukemic cells (9, 28, and Bartram, unpublished results).

However, the data presented here do not necessarily rule out a possible influence of rearranged *abl*/*bcr* sequences for the course of CML blast crisis. In this respect it seems to be noteworthy that the patient has survived the acute phase for already >1 yr in remarkably good condition. Thus the deletion of the rearranged *abl*/*bcr* sequences, i.e., the withdrawal of the second step on the way to CML blast crisis, may result in the manifestation of a leukemic state that differs from the much more aggressive blast crisis usually observed in CML.

On the other hand, the results reported here may indicate that once a leukemic cell has entered blast crisis, the rearranged *abl*/*bcr* sequences and their respective protein product may have a modulating effect on the clinical course of blast crisis, but are no longer essential for the maintenance of a leukemic state itself. While the present case appears to be a unique in vivo model to study the necessity of altered *bcr*/*abl* sequences in CML blast crisis, it would be of considerable interest to investigate in vitro the effect of either antisense RNA or monoclonal antibodies directed against altered *bcr*/*abl* sequences in leukemic cells obtained from chronic phase of Ph^+ CML patients. It seems tempting to speculate that these in vitro studies could prove a crucial function of these genes in the chronic phase of CML, while our present investigation can only address their respective role in blast crisis.

Summary

We report on a Ph^+ chronic myelocytic leukemia (CML) case, cytogenetically characterized by the occurrence of a second Philadelphia (Ph) chromosome in lymphoid blast crisis of T cell lineage. In situ hybridization analyses showed a deletion of translocated c-*abl* sequences, present on the Ph during chronic state, from both Ph in acute state. Moreover, Southern blot analyses of blastic cells exhibited a rearrangement within *bcr*, but a deletion of 5′ *bcr* sequences, and Northern blots failed to detect the hybrid 8.5 kb *bcr*/c-*abl* transcript usually observed in Ph^+ CML.

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