Detection of Minimal Residual Leukemia by Polymerase Chain Reactions *

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Introduction

Disease recurrence following successful remission induction by polychemotherapy or bone marrow transplantation represents a major clinical problem in the treatment of leukemia patients. Sensitive methods for the identification of neoplastic cells escaping therapeutic interventions and potentially causing relapse are important for the monitoring of therapeutic effectiveness. The in vitro amplification of genomic or complementary deoxyribonucleic acid (cDNA) target sequences by polymerase chain reaction [1] has opened new avenues toward the detection of minimal residual leukemic cells at frequencies of 1:10000 to 1:1000000. In the following we will briefly summarize our recent experience with the application of polymerase chain reaction (PCR) strategies to patients with chronic myelocytic leukemia (CML) and acute lymphoblastic leukemia (ALL) in complete remission according to clinical and laboratory parameters.

Materials and Methods

Cell Samples

Since 1986, bone marrow (BM) or peripheral blood (PB) cell specimen obtained from 332 children with ALL have been referred to our laboratory for immunogenotype analysis as part of the prospective German multicenter BFM (Berlin-Frankfurt-Münster) ALL/NHL-1986 trial [2]. Details of molecular genetic and immunological data and their correlation to clinical features will be described elsewhere after completion of the trial. Clonospecific probes were prepared from 17 patients (eight T-ALL, nine cALL) exhibiting a suitable T cell receptor (TCR) δ gene rearrangement, and used for evaluation of the remission status by PCR. We also investigaged three pediatric Ph-positive ALL. The study of Ph-positive CML patients treated by allogeneic bone marrow transplantation (BMT) at the Department of Internal Medicine III, University of Ulm, was restricted to 40 cases being in complete remission at the time of initial PCR analysis according to clinical, hematological, cytogenetic, and Southern blot criteria [3].

Polymerase Chain Reaction

For the detection of Ph-positive leukemic cells we used PCR protocols described previously [4–6]. After the synthesis of cDNA by reverse transcriptase, messenger ribonucleic acid (mRNA) from the hybrid BCR/ABL or normal ABL alleles was amplified by a set of nested primers. As internal ABL control we amplified a fragment of 173 bp with oligomers complementary to ABL exon II and III sequences (Fig. 1). Rearranged BCR/ABL molecules were amplified by primers derived from either BCR exon I and ABL exon II to detect 271 bp fragments corre-

* Supported by the Deutsche Forschungsgemeinschaft, Deutsche Krebshilfe, and Förderkreis für tumor- und leukämiekranke Kinder Ulm. S. Yokota is the recipient of an Alexander-von-Humboldt fellowship.

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Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

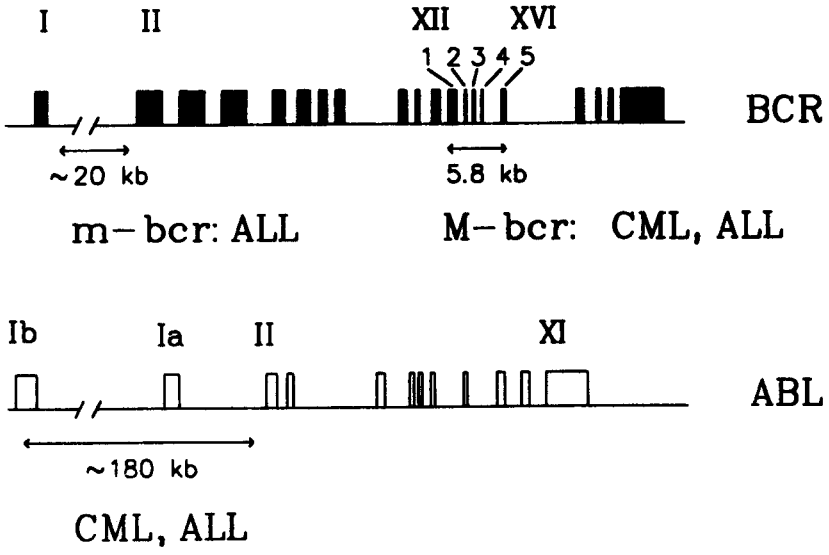

Fig. 1. The Ph translocation is molecularly characterized by a rearrangement of the BCR and ABL genes. The breakpoints on chromosome 22 are clustered within two limited areas (*M-bcr* and *m-bcr*), the breaks on chromosome 9 occur in the first ABL intron

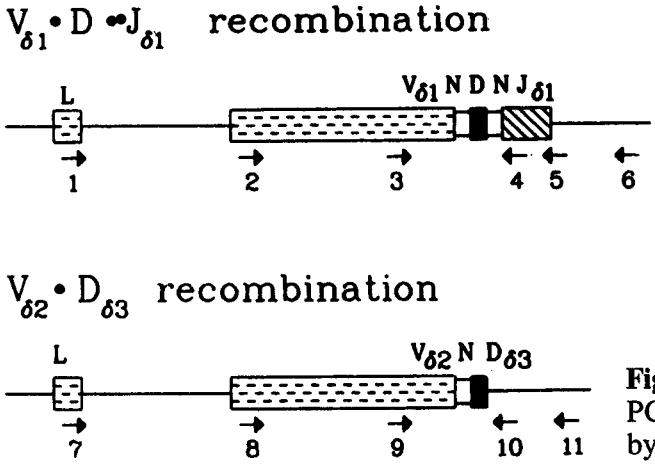

Fig. 2. Position of oligomers (*arrows*) used for PCR analysis of ALL patients characterized by $V_{\delta 1}DJ_{\delta 1}$ or $V_{\delta 2}D_{\delta 3}$ rearrangements

sponding to breaks within m-bcr, or M-bcr exon 1 + 2 and ABL exon II resulting in 395 or 320 bp fragments depending on the presence or absence of M-bcr exon 3 in the hybrid transcript (Fig. 1).

Isolation and hybridization of clono-specific TCRδ probes for the detection of residual ALL followed a PCR strategy recently described by us in detail [7]. The position of nested primers used for probe preparation (oligomers 1/6 + 3/4 or 7/11 + 9/10) and detection of residual leukemic cells (oligomers 1/6 + 2/5 or 7/11 + 8/11) is indicated in Fig. 2.

Results and Discussion

Ph-Positive Leukemias

The Ph translocation is based on a recombination of the BCR and ABL genes [8]. The breaks vary over a distance of more than 180 kb on chromosome 9, but cluster within two limited regions of the BCR gene on chromosome 9, called the major (M) and minor (m) breakpoint cluster region (bcr) (Fig. 1). In virtually all Ph-positive CML patients the breaks occur in introns between exon 2/3 or 3/4 of the M-bcr, while in Ph-positive ALL either of the two areas is involved. Despite dif-

Table 1. PCR analysis of 40 Ph-positive CML patients in CCR after BMT

Patients (*n*)	Clinical features	Initial PCR status	Follow-up
6	Long-term survivors (> 5 years)	all negative	CCR
6	T-cell depleted BMT, initial PCR 19–48 months after BMT	all positive	2 clinical relapses 1 genetic relapse
28	Initial PCR 1–37 months after BMT	11 positive	4 PCR negative 2 genetic relapses 3 clinical relapses
		17 negative	1 genetic relapse 1 clinical relapse

ferences at the genomic level, Ph-positive leukemias become accessible to PCR analysis because BCR exons are always spliced to ABL exon II [4, 5].

Since allogeneic BMT appears to be the only accepted curative therapy for CML resulting in the eradication of the malignant cell clone, we recently determined the remission status of 40 transplanted CML patients by PCR analysis. The result of this study is summarized in Table 1. All long-term survivors (5–9 years) showed no residual disease in at least two independent PCR and might be truly cured from CML. In contrast, all patients receiving a T-cell depleted marrow for prevention of graft-versus-host disease exhibited residual leukemic cells. Two of the patients experienced a clinical relapse within the following year; in a third patient, Ph-positive cells became visible again in Southern blot and cytogenetic analyses. These data might be reconciled with previous reports on a significant increase in clinical relapses after T-cell depleted BMT [9]. In 28 patients initial PCR analysis was performed 1 month to 3 years after BMT (Table 1, Fig. 3). Fifteen patients scored repeatedly PCR-negative during follow-up, while 2/17 cases became PCR-positive and relapsed either clinically or cytogenetically. Among the 11 cases with an initially PCR-positive result, five patients experienced clinical or cytogenetic relapses. Interestingly, four patients became PCR-negative during the following year.

Our data underline the importance of longitudinal PCR analysis in individual patients. Thus in some cases residual leukemic cells may be diluted out during the post-BMT period. However, patients may also become PCR-positive and relapse clinically, despite an initially negative result. We would like to emphasize that the precise clinical relevance of PCR analysis remains to be elucidated in prospective trials including more patients. Along this line it is remarkable that one of the repeatedly PCR-positive cases has been in otherwise complete remission for more than 4 years. Conflicting data as to the frequency of residual leukemia have thus far emerged from reports on PCR analysis in transplanted CML patients [6, 10–16]. These discrepancies could originate from various sources including differences between therapeutic regimen, cell samples, or technical problems. False positive reaction due to contamination with as few as one specific molecule is a major challenge that can only be overcome by rigorously following standardized precautions [17].

The PCR strategy discussed above is also applicable to a significant number of ALL patients. In adults the Ph translocation constitutes the most frequent cytogenetic abnormality, while it is found in only 5% of pediatric cases. Irrespective

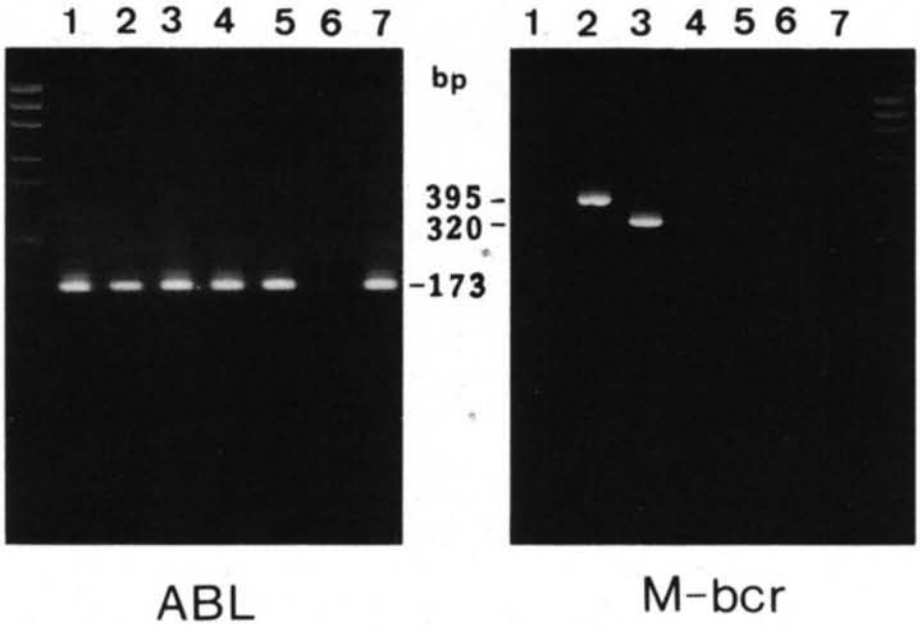

Fig. 3. PCR analysis of six Ph-positive CML patients in complete remission after BMT. Two equal volumes of BM cDNA were amplified by use of oligomers detecting either ABL or rearranged BCR-ABL fragments. All cases exhibit a positive ABL control fragment of 173 bp. Residual leukemic cells characterized by fragments of 395 bp or 320 bp are visible in two patients (*lanes 2 and 3*). *Lane 6* contains a negative control (water). Samples were run in agarose gels and visualized by ethidium bromide staining. *Hae*III digested ∅X174 DNA served as molecular weight marker

of age, however, Ph-positive ALL signals a very poor prognosis. PCR is an important alternative strategy to cytogenetic analysis for the rapid diagnosis and subclassification of Ph-positive leukemias and offers a useful approach for the detection of residual leukemic cells. Thus far we have monitored three children with Ph-positive ALL. Although the patients were otherwise at standard risk and achieved a complete clinical-hematological remission, the leukemic cell clone was never eradicated completely as indicated by PCR. All three patients relapsed 6–9 months after initial diagnosis. It appears to be necessary and worthwhile to extend respective investigations to more patients in multicenter trials.

ALL Patients Characterized by Distinct TCRδ Recombinations

Different PCR strategies based on the specific immunogenotype of ALL patients have been proposed for the detection of residual leukemic cell populations [7, 18, 19]. The approach developed in our laboratory takes into account the striking observation that the vast majority of all ALL patients show a TCRδ recombination and, moreover, a preferential use of specific TCRδ elements depending on the immunological subtype [7, 20–27]. In our series of 332 pediatric ALL cases analyzed prospectively for immunoglobulin and T-cell receptor arrangements, we observed a TCRδ recombination in 96% of T-ALL (58/60) and 81% of cALL patients (163/204). Southern blot analysis demonstrated a hybridization pattern predicting a $V_{\delta 1}DJ_{\delta 1}$ recombination in 25% of T-ALL and a $V_{\delta 2}D_{\delta 3}$ rearrangement in 57% of the cALL patients. This interpretation was confirmed for the 23 cases examined by PCR-directed sequence analysis [27].

Based on Southern blot data we prepared clonospecific probes from leukemia cell DNA of eight T-ALL characterized by a $V_{\delta 1}DJ_{\delta 1}$ rearrangement and nine cALL exhibiting a $V_{\delta 2}D_{\delta 3}$ recombination (Fig. 2). None of the probes showed cross-hybridization to DNA obtained from leukemic cells of the other ALL patients or from healthy probands, con-

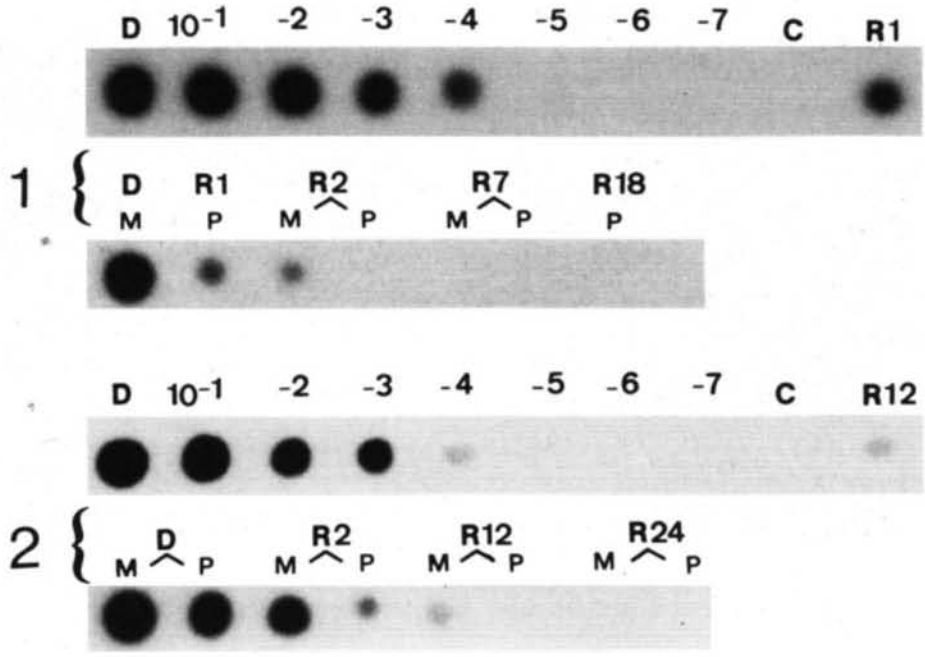

Fig. 4. Detection of minimal residual leukemia in two cALL patients characterized by $V_{\delta 2}D_{\delta 3}$ rearrangements. DNA obtained from leukemic cells at diagnosis (*D*) was diluted into peripheral blood cell DNA of a healthy control (*C*) at 10^{-1} to 10^{-7}. Upon amplification DNA fractions (2 ng) were spotted onto nylon membrane and hybridized to the clonospecific TCRδ probes derived from leukemic cells of each patient, thus establishing a detection limit at 1:10000. PCR analysis of bone marrow (*M*) and peripheral blood (*P*) DNA obtained during continuous complete remission (*R*) 1–24 months after initial diagnosis revealed significant differences in the decrease of residual leukemic cell populations between both patients

firming our previous experiences [7]. To determine the sensitivity of the clonospecific probes in detecting residual disease, we performed dilution experiments (Fig. 4). Following 70 PCR cycles primed by nested amplimers, 10^{-4} leukemic cells were detected in all cases. Due to greater junctional diversity, the sensitivity of clonospecific $V_{\delta 1}DJ_{\delta 1}$ probes was even higher and identified leukemia DNA when representing as little as 0.0001% of total DNA.

We next analyzed BM DNA samples of the 17 ALL patients during complete clinical-hematological remission; none of the samples revealed rearranged TCRδ fragments upon Southern blot analysis suggesting a frequency of residual leukemic cells, if any, below 1%. However, PCR demonstrated residual leukemia in a significant proportion of patients (Table 2). Remarkably, all eight patients investigated 3 weeks to 4 months after starting chemotherapy showed residual leukemic cells (range 10^{-2} to 10^{-5}), while the six patients studied more than 18 months after diagnosis scored negative. Longitudinal studies performed in seven cases indicated that residual neoplastic cells may persist, independent of known

Table 2. PCR analysis of 17 children with ALL in CCR using clonospecific TCR δ probes

Months after diagnosis	Children[a] (*n*)	PCR status of BM cells
1	5	all positive
2–6	9	6 positive 3 negative
7–12	6	3 positive 3 negative
13–23	4	1 positive 3 negative
>24	4	all negative

[a] Consecutive remission samples were analyzed from seven cases.

risk factors, for variable periods in different individuals (Fig. 4). Thus in some patients the leukemic cell clone is obviously eradicated after 3 months, while in other cases residual disease was demonstrated up to 14 months after diagnosis.

We would like to mention some possible limitations of the PCR approach. Thus PCR will also amplify DNA sequences of functionally irrelevant or dead cells and even residual DNA particles originating from leukemic cells. On the other hand, any subpopulation of leukemic cells characterized by further recombination of sequences comprised in a clonospecific probe would escape detection by PCR, despite a potential capacity to proliferate and cause clinical relapse.

Although still preliminary, our data suggest that PCR analysis offers a useful tool to elucidate the biological and clinical significance of residual disease in ALL patients. Ultimately this approach may be part of a rationale to adapt highly aggressive therapeutic regimen to the individual demand of a leukemia patient thereby preventing excess of therapy. For the time being any conclusion drawn from PCR analysis has carefully to consider possible technical pitfalls as well as clinical features and complementary laboratory data.

Acknowledgements. We thank all participants of the BFM/NHL-1986 trial (coordinator: Professor H. Riehm) for continuous support and gratefully acknowledge the close cooperation with Dr. W. D. Ludwig. CML patients were analzyed in fruitful cooperation with Drs. R. Arnold, B. Heinze, and H. Heimpel.

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