Detection of Minimal Residual Disease in Acute Lymphoblastic Leukemia by In Vitro Amplification of Rearranged T-Cell Receptor \( \delta \) Chain Sequences

By Thomas E. Hansen-Hagge, Shouhei Yokota, and Claus R. Bartram

Human T-cell receptor (TCR) \( \delta \)-chain diversity mainly originates from high junctional variability, since only a limited number of germline elements is available. This extraordinary diversity at the V-J junction, due to the use of two D\( \delta \) elements and extensive incorporation of N nucleotides, constitutes a specific clonal marker for cell populations exhibiting rearranged TCR\( \delta \) genes. To this end we amplified in vitro by polymerase chain reaction (PCR) the TCR\( \delta \) junctional region of five acute lymphoblastic leukemias (ALL), isolated respective DNA fragments, and used them directly as clonospecific probes. The combination of PCR technology and hybridization to clonalospecific probes permitted the detection of leukemia DNA at dilution of 1:100,000 in all five cases. Moreover, we were able to investigate one of the ALL patients 11 months after achieving continuous complete remission. Conventional Southern blot analysis failed to detect rearranged TCR genes at this stage. However, residual leukemic cells could readily be detected by PCR technique. We conclude that the strategy proposed here is a very sensitive tool to detect minimal residual disease in a significant proportion of human lymphoid neoplasias.

I N THE MAJORITY of human hematopoietic neoplasias, current induction therapies achieve high remission rates. However, disease relapse following successful remission-induction remains a major clinical problem in the treatment of patients with leukemia and related disorders. Since most of these recurrences are thought to stem from remaining neoplastic cells escaping chemotherapy, the detection of minimal residual disease represents a major challenge of today's oncology. Successful strategies for the identification of small amounts of neoplastic cells would be helpful (eg, in monitoring therapeutic effectiveness, in detecting relapses at early stages, and in determining the quality of autologous bone marrow (BM) samples prior to transplantation). Unfortunately the majority of currently available techniques, such as flow cytometry, in vitro cell culture, cytogenetics, or Southern blot analysis, are limited by a detection level of 1% clonally related cells.1

Recently in vitro amplification of genomic or cDNA target sequences by the polymerase chain reaction has opened new avenues toward the identification of neoplastic cells at frequencies of 1:100,000. This ultrasensitive technique has been successfully applied to leukemias and lymphomas characterized by two distinct chromosomal abnormalities, t(9;22), and t(14;18), respectively.2 However, a preferential use of the V\( \delta \), and J\( \delta \) elements has been observed in different cell clones.9,11 Despite this limited germline repertoire, TCR\( \delta \) shows an extraordinary diversity at the V-J junction due to the presence of two D\( \delta \) elements, imprecise VDJ joining and extensive insertion of template-independent N-region nucleotides.15

The strategy for the detection of minimal residual leukemia by PCR proposed in this manuscript is based on this limited number of V\( \delta \) and J\( \delta \) elements on one hand and on the clonalospecific junctional diversity on the other. It appears to be a particular advantage that our approach does not require sequence analyses of the junctional regions and synthesis of leukemia-specific oligonucleotide probes, in contrast to a recent method proceeding from amplified TCR\( \gamma \) sequences.16

MATERIALS AND METHODS

Patients. Determination of the immunogenotype is performed after informed consent from all patients of the prospective German multicenter acute lymphoblastic leukemia (ALL) trials, ALL/ NHL-BFM86 (children) and ALL/AUL-BMFT (adults). Thus far our laboratory has analyzed 32 AUL, 187 cALL, and 43 T-ALL patients and demonstrated a TCR\( \delta \) gene rearrangement in 75%, 81%, and 87% of cases, respectively (Claus R. Bartram, unpublished results). Among the latter patients, 9% (ie, 19 out of 262 patients screened for TCR\( \delta \) rearrangements) were characterized by a rearrangement of V\( \delta \) sequences (see below). Five of these cases are analyzed in this study (ie, three T-ALL patients [Ha, Si, and Ma, aged 6, 7, and 34 years, respectively] and two cALL [Th and Ra, aged 8 and 11 years, respectively]).

Southern blot analysis. High mol wt DNA was prepared from cryopreserved mononuclear BM cells by standard technique. Ten micrograms of DNA was digested with EcoRI, HindIII, and BamHI (Boehringer Mannheim, Mannheim, FRG), electrophoresed on a
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0.7% agarose gel, blotted and hybridized as described. Following hybridization, the filters were washed under stringent conditions and exposed to XAR-5 film (Kodak, Rochester, NY) using intensifying screens for 24 hours at -70°C. To demonstrate TCRβ rearrangements we used probe J6s16, kindly provided by Drs T. Boehm and T.H. Rabbitts. In HindIII digests, presence of a rearranged 9.6-kb fragment suggests a V,D,Jβ rearrangement. We reanalyzed respective cases by a Vβ-specific probe created by PCR-directed amplification (see below) of Vβ sequences of an ALL patient using oligomers 1 and 4. Indeed, this 0.65 kb Vβ probe hybridized to the very 9.6-kb HindIII fragment previously identified by probe J6s16, while cases exhibiting rearranged TCRβ fragments of different sizes with J6s16 revealed a germline configuration following hybridization to the Vβ probe (data not shown).

Polymerase chain reaction. PCR was essentially performed as described by Saiki et al. The reaction mixture contained 300 ng chromosomal DNA, 30 pmol of each 5' and 3' oligonucleotide primer, 0.2 mmol/L of each deoxynucleotidetriphosphate, 10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl2, and 0.01% gelatine (wt/vol) in a total volume of 100 μL. Synthetic oligonucleotides were prepared using the solid-phase phosphate triester method according to TCRβ sequences published by others. Respective amplimers are listed in Table 1, and their positions within the TCRβ locus are indicated in Fig 1. The mixture was incubated at 95°C for 12 minutes to denature double-stranded DNA and then cooled at 56°C for five minutes. Primer extension was started by addition of 0.5 unit Taq polymerase (Perkin-Elmer/Cetus, Norwalk, CT) and allowed to proceed at 74°C for 90 seconds. Following this initial-round denaturing, annealing and extension steps were performed at 95°C for 75 seconds, at 56°C for 90 seconds, and at 70°C for 120 seconds, respectively, for 30 cycles.

Hybridization with clonospecific probe. Amplified DNA (2 ng) was either spotted together with 2 μg of Sau3AI-digested human placenta DNA onto nylon filters (Nytran, Schleicher & Schuell, USA) for 24 hours at 20°C followed by washing under stringent conditions and exposure to XAR-5 film (Kodak, Rochester, NY) using intensifying screens for 24 hours at -70°C. To demonstrate TCRβ rearrangements we used probe J6s16, kindly provided by Drs T. Boehm and T.H. Rabbitts. In HindIII digests, presence of a rearranged 9.6-kb fragment suggests a V,D,Jβ rearrangement. We reanalyzed respective cases by a Vβ-specific probe created by PCR-directed amplification (see below) of Vβ sequences of an ALL patient using oligomers 1 and 4. Indeed, this 0.65 kb Vβ probe hybridized to the very 9.6-kb HindIII fragment previously identified by probe J6s16, while cases exhibiting rearranged TCRβ fragments of different sizes with J6s16 revealed a germline configuration following hybridization to the Vβ probe (data not shown).

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Table 1. Oligomers Used for Polymerase Chain Reactions

<table>
<thead>
<tr>
<th>Oligomers</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Vβ, L)</td>
<td>5´ GTGTGATTGGGTGGGCTTCAGCTAC 3´</td>
</tr>
<tr>
<td>2 (Vβ, 6)</td>
<td>5´ ACTCAAGCCAGCTCATCAGTA 3´</td>
</tr>
<tr>
<td>3 (Vβ, 3)</td>
<td>5´ TCACGCAAAGTATGGTTCCTGTTG 3´</td>
</tr>
<tr>
<td>4 (Jβ, 5)</td>
<td>5´ TTCCTTCTCTCTCTTTCCAAGGATGAG 3´</td>
</tr>
<tr>
<td>5 (Jβ, 3a)</td>
<td>5´ GAGTTACUACTGGGUCCAC 3´</td>
</tr>
<tr>
<td>6 (Jβ, 3b)</td>
<td>5´ CCCAGGACTTGGTGCCAC 3´</td>
</tr>
</tbody>
</table>

See Fig 1 for position of oligomers; in oligomer 4 a G (asterisk) was substituted for wild-type A, thus creating an artificial FokI cleavage site.
DNA Probe

<table>
<thead>
<tr>
<th>DNA</th>
<th>Ha</th>
<th>Ma</th>
<th>Si</th>
<th>Th</th>
<th>Ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ha</td>
<td>●</td>
<td></td>
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<td></td>
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<tr>
<td>Ma</td>
<td>●</td>
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<td>Si</td>
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<tr>
<td>C</td>
<td>●</td>
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<td>●</td>
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<tr>
<td>Ra</td>
<td>●</td>
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</table>

Fig 2. Hybridization of clonospecific probes obtained from five ALL patients (Ha, Ma, Si, Th, Ra) to amplified DNA samples obtained from leukemia cells of the same cases as well as peripheral blood cells of a healthy control (C). Two nanogram of DNAs were spotted onto nylon membranes and hybridized consecutively to the five probes. Note the lack of any cross hybridization.

RESULTS

Isolation of clonospecific DNA probes. The strategy for the preparation of DNA probes specifically characterizing different lymphoblastic leukemias is outlined in Fig 1. The TCRδ locus is nested within the TCRα gene cluster at chromosome 14q11. Neoplastic lymphocytes show a preferential use of Vδ and Jδ regions in VDJ recombinations of the TCRδ chain but reveal a clonospecific junctional diversity due to D elements and junctional flexibility coupled with extensive incorporation of N-region sequences. To isolate a probe containing the (ND)NDN sequences unique to an ALL clone, two rounds of PCR (30 cycles each) are performed. A first step using oligonucleotides 1 and 6 as primers amplifies a rather large fragment of 650 bp containing rearranged Vδ, leader (L), Vδi, and Jδi sequences, which can be easily distinguished from amplification artifacts (eg, primer-dimer) as often observed in PCR. A second PCR round using amplimers 3 and 4 further amplifies about 100 to 120 bp of the first amplification product flanking and including the clonospecific-(ND)NDN sequences of variable size. Amplimer 4 contains a single mismatch to the wild-type Jδi sequence, thus creating an artificial FokI cleavage site 9 and 13 bases, respectively, upstream of the FokI recognition site. Upon FokI digestion, the clonospecific fragment of approximately 60 to 80 bp containing 5' universal Vδi and 3' specific (ND)NDN sequences can be isolated after separation by electrophoresis. Finally, this probe is labeled using a hexanucleotide primer identical with Vδi sequences immediately upstream of the (ND)NDN region.

This approach has been successfully applied to all five ALLs exhibiting VδiJδi rearrangements investigated in this study (Fig 2). Most importantly, a highly specific hybridization of the five clonospecific probes was restricted to genomic DNA of only those leukemic cells the different probes had been isolated from. It should be emphasized that...
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Fig 4. Southern blot analysis of HindIII-digested BM DNA (10 μg) obtained from patient Ma prior to (a) and after therapy (b). Following hybridization to TCRβ probe Jα16, a rearranged 9.6-kb fragment (arrow) is visible at presentation, while a germline configuration of the TCRβ locus is observed in remission.

these leukemias had rearranged identical V and J elements but differed specifically as to their (ND)NDN regions. Hybridization of all five clonospecific probes to amplified DNA obtained from peripheral blood or BM samples of 13 healthy controls revealed no positive hybridization signals.

Sensitivity of the PCR assay. To determine the sensitivity of our method in detecting clonal cell populations, genomic DNAs of the leukemias were diluted into DNA obtained from peripheral blood cells of five healthy controls. PCR experiments using primers 1 and 6 amplified all TCRβ fragments containing rearranged V, and J, sequences. However, in contrast to the situation in monoclonal cell populations (leukemias), respective fragments obtained from peripheral blood-cell DNAs of normal controls are polyclonal as to the junctional (ND)NDN region. DNAs without a V,DJ, rearrangement cannot be amplified at all due to the large size of intervening sequences between the V, and J, primers. Following 30 cycles of amplification using primers 1 and 6, we included a second amplification round of 30 cycles primed by amplimers 2 and 5 (Table I, Fig 1) to increase the specificity of the amplification reaction.

A representative result of these analyses is shown for patient Ma (Fig 3). One microgram of DNA was diluted at $10^{-1}$ to $10^{-8}$ and amplified. Aliquots of 100-ng amplified DNA were run on a 1.5% agarose gel and visualized by ethidium bromide staining (Fig 3A). All samples exhibit amplified Vδ, Jδ fragments. The monoclonal origin of this fragment in patient Ma (a) cannot be distinguished from the polyclonal nature of the control sample (j) at this stage. However, 2-ng samples of the same fractions were spotted onto nylon filters and hybridized to the clonospecific Ma probe (Fig 3B). Leukemia DNA is detected when representing as little as 0.001% ($10^{-3}$) of total DNA.

Detection of minimal residual leukemia cells in remission. To investigate the value of this strategy for the detection of residual leukemia cells in clinical settings, we fortunately were able to obtain a BM sample from patient Ma, who is in continuous complete clinical/hematologic remission for 11 months. As shown in Fig 4, Southern blot analyses of this BM sample failed to detect the TCRδ gene rearrangement and deletion characterizing leukemia cells of this patient initially. Also a germline configuration of TCRδ and γ genes was observed in this remission sample in contrast to the monoclonal recombination pattern at presentation (not shown). However, minimal residual leukemia cells could be identified undoubtedly at this stage by in vitro amplification of VDJ fragments via PCR and hybridization to the clonospecific probe obtained from this patient at acute phase.

As shown in Fig 5, 100-ng samples of amplified BM DNAs obtained from four ALL patients at presentation, a healthy control, and patient Ma in remission were run on an agarose gel and stained with ethidium bromide (Fig 5A). In
contrast to a single sharp band in three of the ALL cases, in patient Si (Fig 5A, lane b) two fragments are visible, reflecting biallelic Vβ6-Vδ6 rearrangements with different sized junctional regions. This gel was Southern blotted and hybridized subsequently to the clonospecific probes obtained from the ALL patients. A representative result is shown for the probe characterizing patient Si, revealing only a specific hybridization signal to the amplified DNA fragment of this very patient (Fig 5B). Rehybridization of the same filter to a single sharp band in three of the ALL sized junctional regions. This gel was Southern blotted and reflecting biallelic Vβ6-Vδ6 rearrangements with different patient Si (Fig 5A, lane b) two fragments are visible, indicating residual leukemic cells. Dilution experiments of amplified DNA samples from both stages of this disease (not shown) suggest the presence of $10^{-3}$ to $10^{-4}$ leukemic cells in the remission sample of this patient.

**DISCUSSION**

In this study we took advantage of the fact that the TCRδ locus is defined by a limited number of germline elements generating extensive junctional diversity upon somatic recombination. Therefore isolation of clonospecific probes following in vitro amplification of respective junctional regions requires only a limited number of oligonucleotides to prime PCR in various leukemia samples. Although we have focused in the present investigation on cases exhibiting Vδ, DJδ6, rearrangements, the same strategy can be applied to the majority of cases revealing DD and DDJδ6, rearrangements or recombination of less frequently used V or J elements. Since approximately 80% of lymphoproliferative disorders seem to exhibit rearranged TCRγ sequences (see Materials and Methods, Claus R. Bartram, unpublished results), it may be envisaged that this technique is applicable to the majority of lymphoid neoplasias. In this respect it might be of interest that this procedure takes only six days (less than conventional Southern blot analysis) including preparation of a clonospecific probe and its application to monitor the remission quality of the respective patient, provided that a cell or DNA sample from acute state has been stored. Recently a different approach for the assessment of residual disease in ALL based on the in vitro amplification of rearranged TCRγ sequences has been suggested. This method includes the cloning and sequencing of amplified DNA fragments and the subsequent construction of a clone-specific synthetic oligonucleotide probe. In this respect it appears to be a particular advantage that our strategy eludes this labor-intensive and expensive step.

The advent of PCR technology has considerably facilitated the detection of minimal numbers of neoplastic cells in vitro and in vivo. As has been shown previously in cases characterized by t(9;22) or t(14;18), we were able to demonstrate residual leukemia cells in a T-ALL patient felt to be in complete remission with respect to conventional criteria, including Southern blot analysis. Although these cells may indeed represent a fully malignant clone with the capacity to replace normal hematopoiesis and to cause clinical relapse, it should be emphasized that the precise biological and clinical meaning of leukemic cells at such small quantities has not been determined as yet. The phenomenon of transient genetic relapses in continued clinical remission of chronic myelogenous leukemia patients following BM transplantation (BMT) raises an important caveat. However, it should also be stressed that PCR-directed tailor-made methods for the investigation of residual cells in various neoplasias will make it possible for the first time to assess the natural history of such clones and ultimately to define their clinical significance. Along this line prospective studies of the remission status of ALL patients using the strategy discussed in this report have been initiated in our institution.

**ACKNOWLEDGMENT**

This report is dedicated to Professor T.M. Fliedner on the occasion of his 60th birthday. We thank Drs H. Riehm, D. Hoelzer, E. Thiel, and W.D. Ludwig and many physicians of the German Pediatric and Adult Multicenter ALL Trials who referred patient material to us. We also thank J. Lyons and A. Fröhlich for preparation of synthetic oligonucleotides. We gratefully acknowledge the continuous support of Drs E. Kleihauer and H. Seliger as well as the help of A. Erkert in editing the manuscript.

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Detection of minimal residual disease in acute lymphoblastic leukemia by in vitro amplification of rearranged T-cell receptor delta chain sequences

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