DESPITE MAJOR improvements in the treatment of acute lymphoblastic leukemia (ALL) patients during the last two decades, 20% to 30% of children with ALL still relapse.\textsuperscript{1,2} The pathogenesis of leukemia relapse is still poorly understood.\textsuperscript{3} To obtain insight into the mechanisms that determine development of relapse in ALL, several studies have been performed: detection of morphological and immunophenotypic shifts at relapse,\textsuperscript{4,5} detection of shifts in leukemic cell karyotype at relapse,\textsuperscript{6,7} comparative glucose-6-phosphate dehydrogenase studies,\textsuperscript{8} and analysis of Ig and T-cell receptor (TcR) genes at diagnosis and relapse.\textsuperscript{9,10} The development of changes in Ig and TcR gene rearrangement patterns was related to remission duration, suggesting an increasing chance of continuing rearrangement processes with time. These immunogenotypic changes at relapse occurred in a hierarchical order, with changes in IgH and TcR-\(\delta\) genes occurring after only 6 months of remission duration, whereas changes in other Ig and TcR genes were generally detectable after 1 to 2 years of remission duration. The heterogeneity reported here in Ig and/or TcR gene rearrangement patterns at diagnosis and relapse might hamper polymerase chain reaction (PCR)-mediated detection of minimal residual disease (MRD) using junctional regions of rearranged Ig or TcR genes as PCR targets. However, our data also indicate that in 75% to 90% of ALLs, at least one major rearranged IgH, TcR-\(\gamma\), or TcR-\(\delta\) band (allele) remained stable at relapse. We conclude that two or more junctional regions of different genes (IgH, TcR-\(\gamma\), and/or TcR-\(\delta\)) should be monitored during follow-up of ALL patients for MRD detection by use of PCR techniques. Especially in biclonal/oligoclonal precursor B-ALL cases, the monitoring should not be restricted to rearranged IgH genes, but TcR-\(\gamma\) and/or TcR-\(\delta\) genes should be monitored as well, because of the extensive changes in IgH gene rearrangement patterns in this ALL subgroup.

Analysis of Ig and T-Cell Receptor Genes in 40 Childhood Acute Lymphoblastic Leukemias at Diagnosis and Subsequent Relapse: Implications for the Detection of Minimal Residual Disease by Polymerase Chain Reaction Analysis

By Auke Beishuizen, Marie-Anne J. Verhoeven, Elisabeth R. van Wering, Karel Hählen, Herbert Hooijkaas, and Jacques J.M. van Dongen

The rearrangement patterns of Ig and T-cell receptor (TcR) genes were studied by Southern blot analysis in 30 precursor B-cell acute lymphoblastic leukemias (B-ALLs) and 10 T-ALLs at diagnosis and subsequent relapse. Eight precursor B-ALLs appeared to contain biclonal/oligoclonal Ig heavy-chain (IgH) gene rearrangements at diagnosis. Differences in rearrangement patterns between diagnosis and relapse were found in 67% (20 cases) of precursor B-ALLs (including all eight biclonal/oligoclonal cases) and 50% (five cases) of T-ALLs. In precursor B-ALLs, especially changes in IgH and/or TcR-\(\delta\) gene rearrangements were found (17 cases), but also changes in TcR-\(\beta\), TcR-\(\gamma\), Ig\(\alpha\), and/or Ig\(\lambda\) genes (11 cases) occurred. The changes in T-ALLs concerned the TcR-\(\beta\), TcR-\(\gamma\), TcR-\(\delta\), and/or Ig\(\lambda\) genes. Two precursor B-ALLs showed completely different Ig and TcR gene rearrangement patterns at relapse, suggesting the absence of a clonal relation between the leukemic cells at diagnosis and relapse and the development of a secondary leukemia. The clonal evolution in the other 23 ALL patients was based on continuing rearrangement processes and selection of subclones. The development of changes in Ig and TcR gene rearrangement patterns was related to remission duration, suggesting an especially concerns the junctional regions of rearranged Ig and TcR genes, because they are regarded as tumor-specific markers.\textsuperscript{20-22} Based on this assumption, several investigators have used the polymerase chain reaction (PCR) for amplification of the tumor-specific junctional regions of rearranged Ig heavy-chain (IgH), TcR-\(\gamma\), and TcR-\(\delta\) genes to detect minimal residual disease (MRD) during follow-up of ALL patients.\textsuperscript{23-27} However, it should be noted that multiple rearranged IgH genes occur in 30% to 40% of precursor B-ALLs.\textsuperscript{16,28-30} When excluding hyperdiploidy of chromosome 14, the multiple IgH gene rearrangements are most probably due to subclone formation,\textsuperscript{16,28,30} which is suggested to be related to poor prognosis in childhood ALL.\textsuperscript{28,30} This subclone formation can be explained by continuing rearrangement processes such as VH to DJH joining or VH replacements.\textsuperscript{28,30}

Detailed analysis of the leukemic cells at diagnosis and relapse by use of Ig and TcR gene rearrangement patterns may give insight into the heterogeneity at diagnosis (process of subclone formation) and at relapse (selection of subclones, possibly related to development of therapy resistance), and into the implications of this heterogeneity for MRD detection by PCR-mediated amplification of junctional regions. So far, only a few studies on limited numbers of patients have been published.\textsuperscript{13-18} These studies were restricted primarily to IgH, Ig\(\lambda\), and/or TcR-\(\beta\) genes, and demonstrated changes in rearrangement patterns in 15% to 40% of ALLs.\textsuperscript{13-16} Therefore, we performed detailed comparative Southern blot analyses of IgH, Ig\(\lambda\), Ig\(\kappa\), TcR-\(\beta\), TcR-\(\gamma\), and TcR-\(\delta\) gene rearrangement patterns in 30 precursor B-ALLs and 10 T-ALLs at diagnosis and subsequent relapse.
ANALYSIS OF Ig AND TcR GENES IN ALL AT DIAGNOSIS AND RELAPSE

MATERIALS AND METHODS

Cell samples. Peripheral blood (PB) and/or bone marrow (BM) samples from children with ALL (30 precursor B-ALLs and 10 T-ALLs) were obtained at initial diagnosis and at first relapse in 32 cases and at first and at second relapse in eight additional cases (six precursor B-ALLs and two T-ALLs). In one T-ALL, we received bilateral testis biopsies at relapse as well. Twenty-one children were treated at the Sophia Children’s Hospital, Rotterdam. Their diagnosis and relapse cell samples were collected by the Department of Immunology, Erasmus University, Rotterdam. The other 19 children were treated at different hospitals in The Netherlands and their diagnosis and relapse cell samples were collected by the Dutch Childhood Leukemia Study Group (DCLSG), The Hague.

The diagnosis of acute leukemia was made according to the French-American-British (FAB) classification, based on cytomorphology of BM smears stained with May-Grünwald Giemsa and cytochemistry (periodic acid–Schiff, acid phosphatase, Sudan black B, myeloperoxidase, and α-naphthylesterase). Each diagnosis and relapse was independently confirmed by the laboratory of the DCLSG.

Mononuclear cells (MNC) were isolated from PB and/or BM samples by Ficoll-Paque centrifugation (density = 1.077 g/cm³; Pharmacia, Uppsala, Sweden). The freshly obtained MNC samples were subjected to a detailed immunological marker analysis according to standard protocols.50,59 Remaining MNC were stored in liquid nitrogen. A leukemia was considered to be a precursor B-ALL if the malignant cells were positive for terminal deoxynucleotidyl transferase (TdT) and/or CD10, CD19, and HLA-DR (null ALL), or for TdT, CD10, CD19, and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR, and cytoplasmic Ig heavy-chain μ (pre-B-ALL).40 A leukemia was considered to be a CD3+ T-ALL if the malignant cells were positive for TdT and cytoplasmic CD3 (CyCD3), as well as for CD2, CD5, and/or CD7. A CD3+ T-ALL was diagnosed if the malignant cells were positive for TdT and membrane TcR-CD3 complex expression as well as for CD2, CD5, CD7, and CD4 or CD8.40 A leukemia was considered to be an acute myeloid leukemia (AML) if the malignant cells were positive for CD13, CD33, CDw65, and/or myeloperoxidase, as well as for HLA-DR, CD34, CD14, CD15, and/or CD36.79

Southern blot analysis. DNA was isolated from frozen MNC as described previously.14,15 Control DNA was obtained from granulocytes or other cell samples with germline Ig and TcR genes. Fifteen micrograms of DNA was digested with the appropriate restriction enzymes (Pharmacia). Completeness of digestion was checked with λ-phage DNA and plasmid DNA in parallel digests as described previously.42 The restriction fragments were size-fractionated in 0.7% agarose gels and transferred onto Nytran-13N nylon membranes (Schleicher & Schuell, Dassel, Germany) as described previously.42 To take care that the diagnosis and relapse lanes of each patient during the gel electrophoresis contained comparable amounts of DNA, the DNA contents of the digested samples were checked (and adapted, if necessary) before loading into the agarose gel. This was further verified by staining of the agarose gel with ethidium bromide.42

IgH gene rearrangements were detected with a 32P random oligonucleotide-labeled joining (ΔJ) probe. The ΔJ probe (IGH6) was a 1.0-kb probe, which recognizes sequences just 3' of the ΔJ gene segments.43 The configuration of the Igs genes was analyzed with a 32P-labeled ΔJ probe, a 32P-labeled constant (ΔC) probe, and a 32P-labeled κ deleting element (ΔKde) probe. The ΔJ probe was a 0.55-kb HaeIII fragment, which recognizes sequences just 3' of the ΔJ gene segments,42 the ΔC probe a 2.5-kb EcoRI fragment,42 and the ΔKde probe (IGKDE) a 0.5-kb probe (A. Beishuizen et al, unpublished results). All diagnosis and relapse DNA samples were analyzed by use of the ΔJ, ΔC, and ΔK probes in BgIII digests and BamHI-HindIII double digests and by use of ΔKde probe in BgIII and HindIII digests. If necessary for confirmation, EcoRI and/or HindIII digests were also used. The configuration of the IgK genes was analyzed with the 32P-labeled ΔK3 probe (a 0.6-kb BgIII-EcoRI fragment) in EcoRI-HindIII double digests and/or in EcoRI digests.42

TcR-β gene rearrangements were detected with 32P-labeled Jβ1, Jβ2, and Cβ probes in EcoRI, and HindIII digests, and for confirmation in BgIII digests.42 The configuration of the TcR-γ genes was analyzed by use of the 32P-labeled Jγ1.1, Jγ1.2, Jγ1.3, and Jγ2.1 probes in EcoRI digests, and for confirmation in KpnI and/or BgIII digests.43 The configuration of the TcR-δ genes was analyzed by use of 32P-labeled Jδ1, Jδ2, and Cδ probes in EcoRI, HindIII, and BgII digests.44 In 24 cases, the TcR-δ gene rearrangements were further analyzed with 32P-labeled variable (V)ψ1, Vδ2, Vδ3, VδREC, diversity (D)ψ1, and ψJa probes in EcoRI, HindIII, and BgII digests.44

RESULTS

Immunological marker analysis. Immunological marker analysis of the 30 precursor B-ALLs showed that two were null ALL, 17 were common ALL, and 11 were pre-B-ALL. Changes in immunophenotypic classification occurred in seven precursor B-ALLs. In two of them, an interlineage shift occurred to AML (Table 1).

The 10 T-ALLs could be divided into six CD3+ T-ALLs and four CD3− T-ALLs (three TcR-αβ+ T-ALLs and one TcR-γδ+ T-ALL). No changes in immunophenotypic classification were found in the T-ALLs (Table 2).

Southern blot analysis of 30 precursor B-ALLs at diagnosis. DNA samples from 30 precursor B-ALLs at diagnosis were examined for the presence of rearranged Ig and TcR genes. At diagnosis, rearrangements and/or deletions of the IgH, Igs, and Igκ genes were found in 100% (all 30 cases), 57% (17 cases), and 17% (five cases) of precursor B-ALLs, respectively (Table 1 and Fig 1). In 10 cases, multiple rearranged IgH gene bands, generally differing in density, were found at diagnosis.49 In eight patients, we could exclude hyperdiploidy of chromosome 14 as a cause of these multiple rearranged bands. In the other two patients (no. 2665 and 4553), no cytogenetic data were available, but the density of the three rearranged IgH gene bands were comparable, suggesting trisomy 14. In patient no. 2665, this was further supported by the presence of one germline and two rearranged TcR-δ gene bands of comparable density (Table 1). Therefore, we have concluded that in eight precursor B-ALLs, the presence of multiple rearranged bands was due to the presence of two or more subclones (biclonality/oligoclonality) with differently rearranged IgH genes (Table 1 and Fig 1).40

Rearrangements and/or deletions of the TcR-β, TcR-γ, and TcR-δ genes at diagnosis were found in 33% (10 cases), 53% (16 cases), and 83% (25 cases) of precursor B-ALL, respectively (Table 1 and Fig 2).

Southern blot analysis of 10 T-ALLs at diagnosis. DNA samples from 10 T-ALLs at diagnosis were examined for the presence of rearranged Ig and TcR genes. Rearrangements of the IgH genes at diagnosis were found in 40% (four cases) of T-ALL (Table 2). No rearrangements and/or deletions of the Ig light-chain (IgL) genes were found (Table 2). Rearrangements and/or deletions of the TcR-β, TcR-γ, and TcR-δ genes were found in all 10 T-ALLs at diagnosis (Ta-
ble 2 and Fig 2). In one T-ALL (patient no. 3288), three rearranged TcR-β2 gene bands with different density were found at diagnosis, suggesting subclone formation at the TcR-β gene level (cytogenetic analysis showed diploidy of chromosome 7).

Changes in Ig and TcR gene rearrangement patterns in precursor B-ALL at relapse. Comparative analysis of Ig and TcR gene rearrangement patterns at diagnosis and relapse were performed in all 30 precursor B-ALLs (Table 1). Differences in IgH, Igκ, and Igλ gene rearrangement patterns between diagnosis and relapse were found in 40%, 28%, and 43% of precursor B-ALL (with rearrangements or deletions of these genes), respectively (Table 3). The precursor B-ALLs with these immunogenotypic shifts included all precursor B-ALL with biclonal/oligoclonal IgH gene rearrangements. Differences in TcR-β, TcR-γ, and TcR-δ gene rearrangement patterns were found in 45%, 33%, and 44% of precursor B-ALLs (with rearrangements or deletions of these genes), respectively (Table 3).

In 10 precursor B-ALL, no changes in immunogenotype at relapse were found. The other 20 precursor B-ALLs showed major or minor changes at relapse (Table 1). Two mono-

Table 1. Southern Blot Analysis of 20 Precursor B-ALL Patients With Differences in Immunogenotype Between Diagnosis and Subsequent Relapse(s)

<table>
<thead>
<tr>
<th>DCLSG No. (Sex/age)</th>
<th>Disease Stage (time from diagnosis)*</th>
<th>Immunogenotypic Classification</th>
<th>Sample</th>
<th>Leukemic Cells (%)*</th>
<th>IgH</th>
<th>Igκ</th>
<th>Igλ</th>
<th>TcR-β</th>
<th>TcR-γ</th>
<th>TcR-δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>2665 (M/5 yr)</td>
<td>D Common ALL</td>
<td>BM</td>
<td>2665</td>
<td>94</td>
<td>R, R, R</td>
<td>D, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2717 (M/1 yr)</td>
<td>D Common ALL</td>
<td>BM</td>
<td>2717</td>
<td>90</td>
<td>R, R</td>
<td>D, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3779 (F/3 yr)</td>
<td>R (48) Common ALL</td>
<td>PB</td>
<td>3779</td>
<td>58</td>
<td>R, R</td>
<td>D, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3966 (M/14 yr)</td>
<td>D Common ALL</td>
<td>BM</td>
<td>3966</td>
<td>95</td>
<td>R, R</td>
<td>D, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4501 (F/5 yr)</td>
<td>D Common ALL</td>
<td>BM</td>
<td>4501</td>
<td>67</td>
<td>R, R</td>
<td>G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4511 (M/4 yr)</td>
<td>D Common ALL</td>
<td>BM</td>
<td>4511</td>
<td>94</td>
<td>R, R</td>
<td>D, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4515 (F/12 yr)</td>
<td>R (18) Common ALL</td>
<td>PB</td>
<td>4515</td>
<td>50</td>
<td>R, R</td>
<td>G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4616 (M/1 yr)</td>
<td>D Pre-B-ALL</td>
<td>BM</td>
<td>4616</td>
<td>69</td>
<td>R, R</td>
<td>R, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4756 (M/8 yr)</td>
<td>D Common ALL</td>
<td>BM</td>
<td>4756</td>
<td>89</td>
<td>R, R</td>
<td>R, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4778 (M/1 yr)</td>
<td>D Common ALL</td>
<td>BM</td>
<td>4778</td>
<td>72</td>
<td>R, R</td>
<td>R, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4788 (M/1 yr)</td>
<td>D Pre-B-ALL</td>
<td>BM</td>
<td>4788</td>
<td>65</td>
<td>R, R</td>
<td>D, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4989 (F/5 yr)</td>
<td>R (29) Common ALL</td>
<td>PB</td>
<td>4989</td>
<td>87</td>
<td>R, R</td>
<td>D, G</td>
<td>G</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: D, diagnosis; R, relapse; BM, bone marrow; PB, peripheral blood.
* The time from diagnosis (in months) is time between diagnosis and (first or second) relapse.
† The leukemic cell load was determined by the percentages of TcR-β, CD19, and/or CD20 cells. In case of AML, the leukemic cell load was determined by the percentages of CD13, CD33, CDw65, and/or MPO cells.
‡ Configuration of Ig and TcR genes: G, allele in germline configuration; g, germline band due to the presence of nonleukemic cells (>25%); R*, rearranged band; R**, weak rearranged band; Rn, new rearranged band, not found at diagnosis; Rn*, weak new rearranged band; D, deletion of the involved gene (segment).
§ Second relapse after BM transplantation.
clonal precursor B-ALLs (patients no. 3779 and 3797) had completely different Ig and TcR gene rearrangement patterns at relapse. In patient no. 3779, each rearranged Ig and TcR gene changed at relapse, while in patient no. 3797 a completely different IgH gene rearrangement pattern was found at relapse with IgL and TcR genes in germline configuration (Table 1). In the other 18 precursor B-ALLs with immunogenotypic changes at relapse, a clonal relation could be established, based on the presence of at least one identically rearranged Ig and/or TcR band (allele) (Table 1). In two oligoclonal precursor B-ALL cases (patients no. 2308 and 3829), the clonal relation could only be proven by weak rearranged IgH gene bands (one minor subclone) at diagnosis, which remained stable at relapse. In patient no. 2308, this subclone probably also had a weak rearranged TcR-β gene band (V62-D63), which remained stable at relapse. This could be proven by a mono-allelic BamHI restriction site polymorphism in the V62 gene segment used in this rearrangement.44,45

In four precursor B-ALLs (patients no. 2665, 3510, 3869, and 3963), loss of one rearranged IgH gene band (patients no. 2665, 3510, and 3963) and/or one rearranged TcR-β gene band (all four cases) was found at relapse, indicating loss of one subclone or loss of one copy of chromosome 14. In two monoclonal precursor B-ALLs (patients no. 4511 and 4616), the Igλ genes changed from germline at diagnosis to one rearranged allele at relapse, with no changes in the other Ig genes. In five precursor B-ALLs (patients no. 2717, 3869, 3968, 4515, and 4778), changes in TcR-β, TcR-γ, and/or TcR-δ genes were found on one or both alleles, while the configuration of the IgH and IgL genes remained stable. Several examples of changes in Ig and TcR gene rearrangement patterns in precursor B-ALL at relapse are shown in Figs 1 and 2. Changes in Ig and TcR gene rearrangement patterns in T-ALL at relapse. Comparative analyses of Ig and TcR gene rearrangement patterns at diagnosis and relapse were performed in all 10 T-ALLs. Differences in IgH gene rearrangement patterns between diagnosis and relapse were found in two of the four T-ALLs with IgH gene rearrangements at diagnosis (Tables 2 and 3). Differences in TcR-β, TcR-γ, and TcR-δ gene rearrangement patterns were found in 30%, 20% and 10% of T-ALL, respectively (Tables 2 and 3). This concerned five patients, including patient no. 3288 with subclone formation at the TcR-β2 gene level at diagnosis. In all five T-ALLs, a clonal relation between diagnosis and relapse could be proven based on the presence of at least three identically rearranged Ig and/or TcR alleles (Tables 2 and 3). Immunogenotypic shifts are related to immunogenotypic shifts at relapse in precursor B-ALL. In six of seven precursor B-ALLs, the changes in immunogenotypic classification of the leukemic cells could be correlated with changes at the Ig and/or TcR gene level. In four cases, an intralineage immunogenotypic shift occurred: in two cases (patients no. 2717 and 3963) to an immature phenotype, and in two cases (patients no. 2678 and 4501) to a more mature phenotype. In the two other precursor B-ALLs (patients no. 3797 and 3991), an interlineage immunogenotypic shift to AML was observed (Table 1). In one patient, an immunogenotypic shift from pre-B-ALL to common ALL occurred, while no immunogenotypic changes at relapse were found (data not shown). In patients no. 3779 and 3797, no clonal relation in immunogenotype between diagnosis and relapse could be established. In patient no. 3797, this immunogenotypic shift correlated with the interlineage shift from common ALL at diagnosis to AML at relapse, while patient no. 3779 with

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Table 2. Southern Blot Analysis of Five T-ALL Patients With Differences in Immunogenotype Between Diagnosis and Subsequent Relapse(s)

<table>
<thead>
<tr>
<th>DCLS No. (sex/age)</th>
<th>Disease Stage (time from diagnosis)*</th>
<th>Immunogenotypic Classification</th>
<th>Sample</th>
<th>Leukemic Cells (%)</th>
<th>Ig Genes</th>
<th>TcR Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TcR-β</td>
</tr>
<tr>
<td>3288</td>
<td>D</td>
<td>TcR-β+ T-ALL</td>
<td>PB</td>
<td>65%</td>
<td>G</td>
<td>D, R, R</td>
</tr>
<tr>
<td>(f/6 yr)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D, D</td>
</tr>
<tr>
<td>3810</td>
<td>D</td>
<td>CD3+ T-ALL</td>
<td>BM</td>
<td>95%</td>
<td>G</td>
<td>D, G, R</td>
</tr>
<tr>
<td>(M/6 yr)</td>
<td>R (17)</td>
<td>CD3- T-ALL</td>
<td>BM</td>
<td>95%</td>
<td>G</td>
<td>D, G, R</td>
</tr>
<tr>
<td>3977</td>
<td>D</td>
<td>TcR-β+ T-ALL</td>
<td>BM</td>
<td>85%</td>
<td>G</td>
<td>D, G, R</td>
</tr>
<tr>
<td>(M/8 yr)</td>
<td>R (15)</td>
<td>TcR-β+ T-ALL</td>
<td>PB</td>
<td>90%</td>
<td>G</td>
<td>D, R, R</td>
</tr>
<tr>
<td>4664</td>
<td>D</td>
<td>CD3- T-ALL</td>
<td>PB</td>
<td>90%</td>
<td>G</td>
<td>R, G, G</td>
</tr>
<tr>
<td>(M/3 yr)</td>
<td>R (16)</td>
<td>CD3+ T-ALL</td>
<td>PB</td>
<td>90%</td>
<td>G</td>
<td>R, G, G</td>
</tr>
<tr>
<td>4643</td>
<td>D</td>
<td>CD3- T-ALL</td>
<td>PB</td>
<td>86%</td>
<td>R, R*</td>
<td>D, R, g</td>
</tr>
<tr>
<td>(M/10 yr)</td>
<td>R (14)</td>
<td>CD3+ T-ALL</td>
<td>BM</td>
<td>93%</td>
<td>G</td>
<td>D, R, R</td>
</tr>
</tbody>
</table>

* The time from diagnosis (months) is time between diagnosis and (first or second) relapse.
† The leukemic cell load was determined by the percentage of TdT+, CyCD3+, or TcR-CD3+ cells.
‡ Configuration of Ig and TcR genes: G, allele in germline configuration; g, germline band due to the presence of nonleukemic cells (=25%); R, rearranged band; R*, weak rearranged band; Rn, new rearranged band, not found at diagnosis; D, deletion of the involved gene (segment).
§ Second relapse after BM transplantation.
common ALL also had a common ALL at relapse (Table 1). Although patient no. 3991 (pre-B-ALL) relapsed with AML with changes on two IgH alleles, the clonal relation was based on seven identically rearranged Ig/TcR alleles at diagnosis and relapse (Table 1 and Fig 1).

The changes at the Ig and TcR gene level in T-ALL were not associated with changes in immunophenotypic classification.

**Changes in Ig and TcR gene rearrangement patterns are related to remission duration.** In the 22 monoclonal precursor B-ALLs, changes at the IgH and TcR-δ gene level were found after remission duration of at least 18 months. The only exception was patient no. 4616 with an Igλ gene rearrangement as single change at relapse (Table 1). Changes at the TcR-β and TcR-γ gene level were found in cases with a remission duration of at least 30 months. Changes in rearrangement patterns on at least four Ig or TcR alleles were also only found after a remission duration of 30 months.

In the eight IgH biclonal/oligoclonal precursor B-ALLs, changes at the Ig and TcR gene level were found in cases with a remission duration of at least 6 months. These changes occurred only at the IgH and TcR-δ gene level. Changes in Igκ, TcR-β, and TcR-γ gene rearrangement patterns were found after remission durations of at least 50 months.

In the series of 10 T-ALLs, changes in IgH and/or TcR gene rearrangement patterns occurred in five patients with a remission duration of at least 1 year.

**DISCUSSION**

Forty ALL patients were analyzed at diagnosis and relapse for the configuration of their Ig and TcR genes by use of Southern blot analysis. Differences in Ig and/or TcR gene rearrangement patterns between diagnosis and relapse were detected in 67% (20 cases) of precursor B-ALL (including all eight biclonal/oligoclonal cases) and 50% (five cases) of T-ALL (Table 3). In precursor B-ALL especially, changes in IgH and/or TcR-δ gene rearrangements were found (17 cases), but also changes in Igκ, Igλ, TcR-β, and TcR-γ genes (11 cases) occurred. The changes in T-ALL concerned the TcR-β, TcR-γ, TcR-δ, and/or IgH genes.

In 15 ALL patients (10 precursor B-ALLs and five T-ALLs), no differences in Ig and TcR gene rearrangement patterns between diagnosis and relapse were found, ie, the original clone caused the relapse (Fig 3). In nine ALL patients with subclone formation at diagnosis (eight precursor B-ALLs and one T-ALL), three different types of immunogenotypic changes occurred. First, in four patients, loss of (faint) rearranged Ig and/or TcR gene bands at relapse was observed, suggesting selection of a major subclone. Second, in two patients, a nearly complete change in immunogeno-
ANALYSIS OF Ig AND TcR GENES IN ALL AT DIAGNOSIS AND RELAPSE

An analysis of Ig and TcR genes in several ALL patients at diagnosis (D) and relapse (R). Control DNA (c) and DNA from ALL patients were digested with the appropriate restriction enzymes, size-fractionated, and blotted onto nylon membrane filters, which were hybridized with 32P-labeled probes. (A) TcR-β gene analyses using EcoRI with the Jβ2 probe. (B) TcR-γ gene analyses using EcoRI with the Jγ1.3 probe. (C) TcR-δ gene analyses using BglII with the Jδ1 probe. The germline bands (c) are indicated. Detailed information concerning the configuration of the different TcR genes is given in Tables 1 and 2.

Fig 2. Southern blot analysis of TcR genes in several ALL patients at diagnosis (D) and relapse (R). Control DNA (c) and DNA from ALL patients were digested with the appropriate restriction enzymes, size-fractionated, and blotted onto nylon membrane filters, which were hybridized with 32P-labeled probes. (A) TcR-β gene analyses using EcoRI with the Jβ2 probe. (B) TcR-γ gene analyses using EcoRI with the Jγ1.3 probe. (C) TcR-δ gene analyses using BglII with the Jδ1 probe. The germline bands (c) are indicated. Detailed information concerning the configuration of the different TcR genes is given in Tables 1 and 2.

Type at relapse occurred, except for stable rearranged IgH and/or TcR-δ gene bands, which were faint at diagnosis, suggesting selection of a minor subclone (Table 1 and Fig 3). In the remaining three biclonal/oligoclonal cases, as well as in 14 of the 16 monoclonal ALL patients, the differences at the Ig and/or TcR gene level between diagnosis and relapse were probably due to continuing rearrangement processes in the original clone (Fig 3). However, in the latter ALL, we cannot exclude the expansion of a minor subclone, which was not detectable at diagnosis due to the Southern blot detection limit of approximately 5% (Fig 3). It should be noted that especially in ALL with subclone formation at diagnosis, combinations of the mentioned processes of clonal evolution might occur. Only two of 25 ALL patients with immunogenotypic changes showed completely different Ig and TcR gene rearrangement patterns at relapse, suggesting the absence of a clonal relation between the leukemic cell populations at diagnosis and at relapse and the development of a secondary leukemia (Fig 3).

The changes in immunogenotype could be correlated with

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<th>Table 3. Changes in Ig and TcR Gene Rearrangement Patterns Between Diagnosis and Relapse in Childhood ALL</th>
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<td><strong>Ig Genes</strong></td>
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The frequencies only concern ALL with Ig and/or TcR gene rearrangements. If no Ig or TcR gene rearrangement was found at diagnosis and/or relapse, the ALL was excluded from the calculations.
changes in immunophenotypic classification of the leukemic cells in six precursor B-ALL cases. In two precursor B-ALLs (patients no. 3797 and 3991), an interlineage shift to AML occurred, suggesting the development of a secondary leukemia. In one of them (patient no. 3797), indeed, a complete change in immunogenotype at relapse was found, supporting the diagnosis of secondary leukemia. However, in the other case (patient no. 3991), seven identically rearranged IgH/TcR alleles were still present at relapse, indicating that the AML was related to the precursor B-ALL, and thereby excluding the development of a secondary leukemia. In a third precursor B-ALL (patient no. 3779), the immunophenotype at diagnosis and relapse was identical, but a completely different immunogenotype at relapse was found. Based on the long remission duration (4 years) and the extensive immunogenotypic shifts (on at least ten alleles), a secondary precursor B-ALL was suggested. These three cases emphasize the difficulties in defining a secondary leukemia and the importance of cytogenetic analysis of malignant cell samples to determine the clonal relation or clonal evolution between diagnosis and relapse. Unfortunately, cytogenetic data in ALL patients are not always available, as was the case in patients no. 3779 and 3991.

Interestingly, the development of changes in Ig and TcR gene rearrangement patterns was related to remission duration, suggesting an increasing chance of continuing Ig and TcR gene rearrangement processes with time. This phenomenon has also been reported by Wasserman et al, who analyzed IgH gene junctional regions in 12 leukemias at diagnosis and relapse. A second phenomenon was the finding that these changes in Ig and/or TcR gene rearrangement patterns occurred in a hierarchical order, i.e., changes in IgH and TcR-δ genes were already detectable after 6 months of remission duration, whereas changes in other Ig and TcR genes were generally found after 1 to 2 years of remission duration. This hierarchical order might be related to the hierarchical order in Ig and TcR gene rearrangements during B- and T-cell differentiation.

As indicated earlier, the PCR technique can be used for the amplification of tumor-specific junctional regions of rearranged IgH, TcR-γ, and/or TcR-δ genes to detect MRD in ALL. Recently, the significance and prognostic implications of MRD detection by PCR in childhood ALL at different time points during therapy has been determined. Potter et al concluded that PCR detection of high levels of residual disease at the end of induction therapy identifies patients at increased risk for relapse during therapy. Furthermore, they concluded that absence of detectable MRD at the end of chemotherapy is not sufficient to assure that the patient is cured, indicating that after treatment frequent serial monitoring is required for the early prediction of relapse.

It should be noted that the extensive changes reported here in Ig and/or TcR gene rearrangement patterns between diagnosis and relapse (clonal evolution) might hamper the detection of MRD by PCR-mediated amplification of leukemia-specific Ig and/or TcR gene junctional regions, especially in case of biclonal/oligoclonal precursor B-ALL. This might lead to false-negative MRD results. It has been argued that changes in Ig and/or TcR gene rearrangement patterns as detected by Southern blotting do not necessarily lead to false-negative PCR results, because a change in Southern blot band pattern does not automatically imply that the complete sequence of the junctional region of a rearranged Ig or TcR gene has changed. For example, Vβ replacements in completely rearranged IgH genes generally do not affect the original V-D-JH junctional regions, and also the D-JH junctional region will generally remain stable in subclones, which rearranged different Vβ gene segments to an identical D-JH precursor.

Recent studies on IgH junctional regions in 13 biclonal/oligoclonal precursor B-ALLs at diagnosis showed that, indeed, most multiple IgH gene rearrangements can be explained by Vβ replacements (seven cases), or rearrangement of different Vβ gene segments to a preexisting D-JH rearrangement (four cases). In two cases, unrelated junctional regions were found, suggesting de novo IgH gene rearrangements. Also, comparison of junctional regions at diagnosis and subsequent relapse showed that in IgH monoclonal precursor B-ALL, the instability of V-D-JH joinings was partly generated via Vβ replacements with a conserved junctional region, partly via rearrangements of different Vβ gene segments to an identical D-JH precursor, and partly due to minor changes in the junctional regions. However, in the two published biclonal/oligoclonal precursor B-ALL cases, extensive changes in V-D-JH junctional regions were found at relapse, which could be explained by outgrowth of a minor subclone, de novo IgH gene rearrangements, and loss of a subclone at relapse. Changes in TcR junctional regions between diagnosis and subsequent relapse were reported in two cases. One case could be explained by the occurrence of continuing rearrangements at the TcR-γ gene level, and, in the other case, the major subclone disappeared as deduced from TcR-δ gene analysis.
PCR due to changes in VH primer annealing, dependent on the family-specific primers for PCR-mediated MRD detection during sequence of the newly rearranged VH gene segment. This pitfall might partly be overcome by using a panel of seven VH regions remained unchanged, it should be noted that differences in VH gene segments might influence the efficiency of the precursor B-ALL with monoclonal IgH genes at diagnosis (n = 22) 18% (4/22) 29% (4/14) 40% (8/20) Precursor B-ALL with biclonal/oligoclonal IgH genes at diagnosis (n = 8) 100% (8/8) 50% (2/4) 57% (4/7) T-ALL (n = 10) 50% (2/4) 20% (2/10) 10% (1/10) The frequencies only concern ALL with IgH, TcR-γ and/or TcR-δ gene rearrangements. If no IgH, TcR-γ or TcR-δ gene rearrangement was found at diagnosis and/or relapse, the ALL was excluded from the calculations. * Patient no. 4511 was excluded from the calculations because both IgH alleles were deleted at diagnosis and at relapse. † In two additional biclonal/oligoclonal cases (patients no. 2308 and 3829), a rearranged band at relapse was identical to a weak rearranged band at diagnosis, suggesting selection of a minor subclone.

Although in several cases, the V-D-Jt or D-Jt junctional regions remained unchanged, it should be noted that differences in Vt gene segments might influence the efficiency of the PCR due to changes in Vt primer annealing, dependent on the sequence of the newly rearranged Vt gene segment. This pitfall might partly be overcome by using a panel of seven Vt FR1 family-specific primers for PCR-mediated MRD detection during follow-up, as suggested by Deane et al. 24

In Table 4, we have summarized our data concerning the changes and stability of IgH, TcR-γ, and TcR-δ gene rearrangement patterns at relapse, because the junctional regions of these genes are generally used as PCR targets for the detection of MRD. Table 4 shows that changes in IgH gene rearrangement patterns at relapse occur at high frequency in precursor B-ALL, especially in biclonal/oligoclonal cases. Changes in TcR-γ and TcR-δ gene rearrangements at relapse are found in both precursor B-ALL and T-ALL, but generally concern only one allele. Despite the high frequency of immunogenotypic changes, at least one major IgH, TcR-γ, and/or TcR-δ rearranged band (allele) remained stable in 75% to 90% of precursor B-ALLs and 90% of T-ALLs. The optimal targets for MRD detection in monoclonal precursor B-ALL are IgH genes, but it might be valuable to monitor the TcR-γ gene rearrangements as well, especially in cases with germline or deleted IgH genes on one or both alleles. In T-ALL, the TcR-γ and TcR-δ genes represent optimal MRD-PCR targets. However, in biclonal/oligoclonal precursor B-ALL (30% to 40% of the total group of precursor B-ALLs), it will be difficult to estimate which minor or major IgH gene band (allele) will remain stable, especially in cases with more than two subclones. This implies that in these leukemias the MRD-PCR monitoring should not be restricted to IgH genes, but that TcR-γ and TcR-δ gene rearrangements should be monitored as well. Therefore, our data indicate that MRD detection in ALL patients by PCR techniques needs monitoring of two or more junctional regions of IgH, TcR-γ, and/or TcR-δ genes to prevent false-negative results.

Our study shows that changes in Ig and TcR gene rearrangement patterns between diagnosis and relapse occur at a high frequency (67% in precursor B-ALLs and 50% in T-ALLs) and are caused by different processes of clonal evolution. These changes in immunogenotype appear to be related to remission duration and seem to occur in a hierarchical order. The heterogeneity reported here in Ig and/or TcR gene rearrangement patterns at diagnosis and relapse has implications for the detection of MRD using PCR-mediated amplification of tumor-specific Ig and TcR gene junctional regions, because it can be foreseen that subclone formation at diagnosis and clonal evolution at relapse might lead to false-negative results. Therefore, it is important to define optimal strategies in exploiting IgH, TcR-γ, and/or TcR-δ gene rearrangements for the detection of MRD by use of PCR techniques.

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