Ig heavy-chain (IgH) and partial V62-D63 T-cell receptor (TCR) gene rearrangements were investigated, by polymerase chain reaction (PCR) amplification and sequence analysis, in 52 patients at presentation and first relapse and in 14 at both first and second relapse of B-lineage acute lymphoblastic leukemia. In combination, these techniques amplified one or more clonal markers at presentation in 90% of patients (IgH-PCR, 75%; V62-D63-PCR, 46%; both, 33%). Changes in the pattern of amplification between presentation and first relapse were seen in 31% of patients positive by IgH-PCR at presentation and in 25% of those positive by TCR-PCR. Only 3 patients showed complete change in their rearrangements, which is suggestive of relapse with a new clone. Furthermore, despite the high reported rates of oligoclonality and clonal evolution at the IgH locus, the results presented show that false-negative minimal residual disease (MRD) detection can be avoided by designing D-N-J probes to all presentation rearrangements. Using a PCR approach for both gene markers, false-negative testing because of clonal evolution would have only occurred in 3 (8%) of the IgH-positive patients, in contrast to 5 (21%) of V62-D63-positive patients. Combining these two systems increases the proportion of patients open to study to 90%, allows comparative studies of the sensitivities of the two methods, and reduces the rate of false-negative assessment of MRD caused by clonal evolution to less than 10%. We conclude that large prospective PCR studies of MRD detection should examine gene rearrangements at multiple loci to maximize their applicability and to minimize false-negative relapse prediction.

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BM mononuclear cells were isolated by centrifugation on either a Ficoll/Hypaque (Flow Laboratories, Irvine, CA) or 60% Percoll (Pharmacia, Uppsala, Sweden) gradient, washed in phosphate-buffered saline (PBS), pelleted, and stored at −70°C. DNA preparation was performed either by a guanidium isothiocyanate-caesium trifluoroacetate technique or by lysis of the mononuclear cells with NP-40 followed by sodium dodecyl sulfate/protease K digestion, phenol/chloroform extraction, and ethanol precipitation. Where stored cells were not available, DNA was extracted from stored BM slides by the following technique. Briefly, an area of BM smear varying from 0.5 to 1 cm² (depending on the thickness of the film) was effaced from each slide in 100 μL of PBS, decanted into a small Eppendorf tube, and centrifuged at 15,000g for 5 minutes. After removal of the supernatant PBS by gentle aspiration, 20 μL of PCR buffer (10 mmol/L Tris HCl, pH 8.3; 50 mmol/L KCl; 3 mmol/L MgCl₂; 0.001% gelatin) was added, followed by 2 μL of decanted DNA. These 22 μL were then amplified with 15-μL, 25-μL, or 50-μL Master mixes including primers and template DNA to complete PCR reactions.

MATERIALS AND METHODS

Bone marrow (BM) samples were studied from 55 children (aged 3 months to 16 years) with B-lineage ALL presenting with the following:

A Polymerase Chain Reaction Study of the Stability of Ig Heavy-Chain and T-Cell Receptor δ Gene Rearrangements Between Presentation and Relapse of Childhood B-Lineage Acute Lymphoblastic Leukemia


Royal Hospital for Sick Children (Bristol, UK) or the Hospital for Sick Children (London, UK). A total of 52 children were investigated from presentation to first relapse, and 14 from first to second relapse. All children were diagnosed according to standard morphologic and immunophenotypic criteria. The immunophenotypes of the 55 children were as follows: null ALL (3 cases), common ALL (cALL; 46), pre-B-ALL (5), and B-ALL (1).

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IgH - PCR

Primer Sequences:

- FR3A: 5'-ACACGGG(C/T)(C/G)TGTATTACTGT-3'
- FR1: 5'-(C/G)AGGT(A/G)CAGCTG(C/G/T)(A/T)G(C/G)AGTC-3'
- JPS: 5'GTGACCAGGG(C/T)CC(C/T)TGGCCCAG-3'
- Vδ2: 5'CTTGCACCATCAGAGAGA-3'
- Dδ3: 5'AGGGAAATGGCACTTTTGCC-3'

Vδ2-Dδ3 - PCR

Fig 1. Positions of primers used in IgH- and Vδ2-Dδ3-PCR are shown. The variable (V) regions of the IgH gene are composed of well-conserved framework regions (FR) and more varied complementarity determining regions (CDR). The CDR III is massively diverse, being created by juxtaposition of one each of a large number of variable, diversity (D), and joining (J) regions, variable excision of terminal bases from the regions involved, and random insertion of "N" nucleotides during the rearrangement process. Consensus primers were used to the most conserved areas of the FR1 or FR3 and J regions. Partial Vδ2-Dδ3 rearrangements can be amplified using specific Vδ2 and Dδ3 primers. The Dδ3 primer is derived from intronic sequence between Dδ3 and J6l, which may be excised during further recombination into J6. Sequence diversity at this locus derives from random excision and "N" nucleotide insertion and, possibly, occasional involvement of D61 and/or D62, during the joining process.
STABILITY OF GENE REARRANGEMENTS IN ALL

A

<table>
<thead>
<tr>
<th>V SEQUENCE</th>
<th>N SEQUENCE</th>
<th>D SEQUENCE (DQ1/10 or DXP1)</th>
<th>N SEQUENCE</th>
<th>J SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRESENTATION REARRANGEMENT</td>
<td>GCA</td>
<td>CGTACGTAGGGCCACCT</td>
<td>GSSCAGT</td>
<td>CAGCCAAGAACCA</td>
</tr>
<tr>
<td>RELAPSE REARRANGEMENT</td>
<td>GCAAGA</td>
<td>GCTGAGGGAGGACCT</td>
<td>GSSCAGT</td>
<td>CAGCCAAGAACCA</td>
</tr>
</tbody>
</table>

Fig 2. Clonal evolution at the IgH locus by V-V replacement is shown. Patient no. 7 was investigated by IgH-PCR using consensus FR3 and JH primers. BM samples were studied at presentation, at isolated CNS relapse (13 months from diagnosis), at BM harvest before allogeneic BM transplantation (at 19 months), and at second (BM) relapse (28 months). Sequence analysis (A) showed the rearrangement seen at second relapse to be a V-V replaced version of the presentation rearrangement. D-N-J sequence is fully preserved, although the V-N sequence is corrupted (common N sequence is underlined). Therefore, the tracking of residual disease is not affected if a D-N-J probe is used, as shown in (B) where a Southern blotted polyacrylamide gel is probed with a 32P-labeled oligonucleotide designed to presentation D-N-J sequence (position shown in bold). The additional band in the presentation lane probably represents a lower-level subclone, which resolves rapidly following chemotherapy. BM disease persists at the time of "isolated" relapse. This subclone is eradicated (at least to below the maximum sensitivity of this probe, 10^{-6}) by BM transplantation, and relapse occurs with a new predominant subclone. No hybridization is seen to normal BM mononuclear cell DNA (N).

Polyacrylamide Southern Blot Analysis

One patient (no. 7), whose IgH rearrangement underwent V-V replacement between presentation and second relapse, was further investigated by Southern blotting of polyacrylamide-resolved PCR products (Fig 2). PCR products from amplification of BM mononuclear DNA at presentation and BM relapse were diluted 1 in 10,000. Thirty-microliter aliquots of these diluted products, together with equivalent volumes of undiluted product from patient BM mononuclear cell DNA at both central nervous system (CNS) relapse and harvest and from normal BM mononuclear cell DNA, were electrophoresed through 8% polyacrylamide. This gel was soaked for 5 minutes in 0.4% sodium hydroxide solution and then blotted by a standard Southern technique onto Hybond N+ nylon membranes (Amersham International) using the same solution. Oligonucleotide probes were synthesized (a 391 DNA Synthesizer [Applied Biosystems, Foster City, CA]) to presentation D-N-J sequence and end-labeled using 32P-γ-ATP. Hybridization and washing were performed at 1°C below the calculated melting temperature of the probe, and autoradiography was performed for up to 4 hours.

RESULTS

Rearrangements Amplified at Presentation

IgH-PCR using the consensus FR3 primer successfully amplified rearrangements in 37 patients at presentation. In 2 others, bright single products were obtained with a consensus FR1 primer; sequence analysis of these showed deletion of bases from the 3' end of the anticipated FR3 priming site, a phenomenon that has been described previously. Therefore, 39 of 52 (75%) patients were open to investigation by IgH-PCR between presentation and first relapse. Only 4 of 39 (10%) patients showed more than 2 rearrangements by IgH-PCR. Three rearrangements were amplified in 2 patients; 1 of whom was trisomic for chromosome 14 on cytogenetic analysis. Five rearrangements were amplified in the other 2 patients, but, in each case, there were only 2 unique sequences; all others were the product of...
Fig 3A and B.
secondary gene rearrangement events. V62-D63–PCR amplified rearrangements in 24 of 52 (46%) patients at presentation; no patient had more than 2 rearrangements amplified. Overall, 47 (90%) of the 52 leukemias studied at presentation showed distinct clonal electrophoretic bands by either IgH- or TCR δ-PCR; 17 (33%) were amplified by both techniques.

**Stability of Rearrangements Between Presentation and First Relapse**

**Stable rearrangements.** Identical rearrangements at presentation and relapse, in both number and sequence, were seen in 27 of 39 patients (69%) by IgH-PCR and in 18 of 24 (75%) by V62-D63–PCR. Examples of 4 patients who relapsed with identical rearrangements by IgH-PCR are shown in Table I. Three examples are shown in Figs 3B and C. The etiology of band loss at relapse is unclear; in no case did cytogenetic analysis show chromosome 14 deletion.

Combining the results for both loci, there were only 3 patients (8%), nos. 6, 18, and 49, for whom no relationship existed between presentation and relapse findings. Patient no. 6 was an infant (previously reported in Potter et al29) who presented and relapsed with bands of completely unrelated sequence by both IgH– and V62-D63–PCR, despite a presentation/relapse interval of only 10 months. Patient no. 18 became PCR-negative by both techniques at relapse. This patient had a normal karyotype at presentation and, although gaining a 1q- deletion, had no evidence of chromosome 14 loss at relapse. Patient no. 49 showed no relationship between the sequences of the amplified IgH rearrangements at the two events, and V62-D63–PCR was negative on both occasions. Cytogenetic analysis confirmed that a different clone was present at relapse.

Overall, 2 of 3 patients who showed complete change of IgH rearrangements from presentation to relapse were V62-D63–PCR-positive; in neither were these TCRδ rearrangements stable. All 5 patients with complete change at the V62-D63 locus were IgH-PCR-positive at presentation. In 3 of these, either partial (D-N-J) or complete IgH rearrangements were retained at relapse.

**Stability of Rearrangements From First to Second Relapse**

All 14 patients studied between first and second relapse were positive on IgH-PCR. Of these patients, 12 (86%) relapsed with identical rearrangements in both number and sequence to those seen at first relapse; 1 lost one of two bands, and another (patient no. 7) had a V-V replaced version of the presentation rearrangement, and the respective sequences are shown in (C). Common N sequence at the V-N-D junction is underlined. Patient no. 51 relapsed with the most predominant of five presentation rearrangements (three being V-V replaced versions of the other two).

![Figure 3](https://example.com/fig3.png)

Fig 3. PCR analysis of IgH rearrangement at presentation and relapse is shown. (A) Of 39 patients, 27 (69%) presented and relapsed with identical IgH rearrangements. Four examples are shown at presentation (P), first relapse (R1), and in 2 cases also at second relapse (R2). SM, size marker. All bands were cut from the gels and sequenced to confirm their identical nature. The faint step ladder of bands in the background of patient no. 43 (R2) is thought to be caused by amplification of productive rearrangements in normal lymphocytes, whose CDR III sequences vary in length by three base (ie, codon) intervals. (B) Of the 39 patients, 12 (31%) changed their pattern of amplification between presentation and first relapse; 9 presented and relapsed with related rearrangements. Three examples of this are shown. Patient no. 9 relapsed with a single band of identical size to the larger of two presentation bands. Sequence analysis (C) shows perfect presentation of D-N-J sequence between these two rearrangements, excepting the 5' terminal bases of the diversity region DM1. Both rearrangements may have developed independently of the presentation rearrangement. The latter patient presented and relapsed with related subclones of a precursor cell with a DJ rearrangement only. Patient no. 48 relapsed with a V-V replaced version of the presentation rearrangement, and the respective sequences are shown in (C). Common N sequence at the V-N-D junction underlined. Patient no. 51 relapsed with the most predominant of five presentation rearrangements (three being V-V replaced versions of the other two).
relapse, but PCR study of BM mononuclear cell DNA at the time of second relapse amplified a larger V-V replaced band. To further investigate the time course of clonal evolution in this patient, we performed patient-specific oligonucleotide probing of Southern blotted PCR products from presentation and from both relapses (see Fig 2).

Of the 14 patients, 8 were V62-D63-PCR-positive at first relapse; of these 8 patients, 7 had an identical band pattern at relapse and 1 became negative.

Comparison of IgH Southern Blot and PCR Findings

Twelve of the patients studied by IgH-PCR as above had previously been fully studied by Southern blotting using JH probes. Agreement between the findings of the two types of study was generally good. At presentation PCR amplified identical numbers of bands to the number of nongermline bands seen on Southern blot in 9 patients. Where there was discrepancy, Southern blot showed the larger number of rearrangements; one more than PCR showed in 2 patients, and two more in 1 patient.

Differences in the number of rearranged bands seen on Southern blot between presentation and relapse were observed in 4 patients, of whom 3 gained an extra nongermline band at relapse. In 1 of these patients, patient no. 7 as described above, this may be explained by the emergence of a V-V replaced version of the presentation sequence at relapse as shown by PCR. In the other 2, PCR analysis was identical between presentation and relapse, and the identity of the new bands is unclear. Another patient (no. 9) dropped from three to one nongermline band on Southern blot at relapse, and, significantly, this was 1 of the patients who had two bands at presentation but only one at relapse by PCR. The only major discrepancy was 1 patient (no. 18) who was positive by both PCR techniques at presentation and became PCR-negative at relapse but remained identical on Southern blot.

DISCUSSION

PCR analysis of gene rearrangements has shown that many cases of oligoclonality at the IgH locus and clonal evolution at both IgH and TCRδ loci can be explained by secondary gene rearrangements occurring in subclones. Although such changes most profoundly affect the IgH locus in Southern blot studies, it is not clear at which locus PCR studies of MRD will be most compromised. We addressed this question by studying a large group of children by both techniques at presentation and relapse.

At the IgH locus, three mechanisms of secondary rearrangement are described: V-V replacement, independent V-DJ rearrangement, and an open-and-shut mechanism. In all 3 cases, the V-N-D sequence will differ between subclones, but D-N-J sequence will be completely preserved. Therefore, D-N-J sequence is the preferred site when designing oligonucleotide probes for use in monitoring MRD. This approach will avoid false-negative relapse prediction when relapse occurs with a related subclone. Furthermore, it will minimize the number of probes required when multiple related rearrangements are present in subclones.

Oligoclonal rearrangements have been reported in 15% to 45% of B-lineage ALL by Southern blot analysis, yet we only observed these in 10% of patients in this study and 16% in a previous one. There is no doubt that direct visualization of products in the manner described will miss low-level subclones detectable by cloning techniques. Furthermore, incomplete DJ rearrangements will be detected by Southern blotting but not amplified using a V region primer. However, this does not appear to adversely affect the potential for relapse prediction. Most patients relapse with identical or related rearrangements to their one or two predominant presentation rearrangements as amplified by PCR.

In this study 27 of 39 (69%) patients in whom IgH rearrangements were amplified at presentation relapsed with identical rearrangements. Clonal evolution was seen in 12 patients (31%), a rate similar to that reported in most Southern blotting studies. Of these 12 patients, 9 relapsed with subclones clearly related to their presentation disease. They showed varying combinations of loss of presentation rearrangements and new rearrangements derived from V-V replacement or independent V-DJ rearrangement events (see Table 1). However, in all 9 cases, the use of D-N-J oligonucleotide probes designed to all unique presentation rearrangements would have allowed early detection of the evolving subclones. Expanded to the whole study population, this would have required the use of less than 1.5 probes per patient who was IgH-positive at presentation (56 probes for 39 patients).

It has been suggested that the instability of IgH rearrangements increases as a function of time. In favor of this idea, Wasserman et al identified new rearrangements at relapse in only 1 of 7 patients relapsing within 3 years from diagnosis but in 4 of 5 relapsing beyond that point. Although this could explain why we found less clonal evolution between first and second relapse (average interval, 17 months) than between presentation and first relapse (average interval, 29.9 months), it is perhaps more likely that this reflects inadequate suppression of disease after first relapse.

This study actually provides little support for such an hypothesis. The mean interval from presentation to first relapse for all patients showing clonal evolution at the IgH locus was 29.7 months compared with 32.1 months for those with completely stable rearrangements. Furthermore, new rearrangements at first relapse were seen relatively early at 10 months (an unrelated sequence) and 24, 24, 25, and 28 months (all V-V replaced sequences), respectively, from diagnosis. Therefore, we would advocate that the relative long-term stability of these rearrangements (coupled with the potential of PCR to study minimal amounts of slide material) render them an attractive tool for differentiating second leukemias from late relapses.

Secondary gene rearrangement at the TCRδ locus is thought to take the form of further recombination into Jα, as the first step towards deletion of the δ locus (which lies nested within the TCRα locus) during TCRα rearrangement. This will invariably delete the site of the 3' primer used to amplify V62-D63 rearrangements and, thus, result in false-negative MRD detection. It may account for loss of PCR bands at relapse in 4 of 23 patients (17%) V62-
Dδ3-PCR-positive at presentation in this series. All presentation bands disappeared in 3 of these 4 cases. The exception (patient no. 24) had two rearrangements at presentation but dropped to one at relapse, implying that it may well prove advisable to track all presentation rearrangements at this locus also. This would require only an extra 0.25 probes per patient (30 probes for 24 patients in this study).

Of 47 patients positive by either IgH- or TCRδ-PCR, we found only 3 (6%) who changed their pattern of amplification in a manner suggestive of relapse with entirely unrelated clones. In 2 patients (nos. 6 and 18), complete change occurred at both loci. The third patient (no. 49) only amplified successfully by IgH-PCR. This rate of complete clonal change is in keeping with the findings of large cytogenetic studies of ALL. These have variously reported relapse with entirely unrelated (together with those of other loci, eg, TCRγ33) rather than as mutually exclusive. Using a combination of IgH- and Vδ2-Dδ3-PCR, MRD assessment is possible in 90% of B-lineage ALL using an average of 1.8 oligonucleotide probes per patient positive by either or both systems (86 probes for 47 patients). Approximately 30% of patients will amplify by both systems, allowing direct comparison of the sensitivity of IgH- and TCRδ-PCR. Most importantly, the rate of false-negative relapse prediction as a consequence of clonal evolution will be less than 10%.

In conclusion this study has shown that, contrary to the impression gained from Southern blotting studies, IgH rearrangements provide more reliable markers for relapse prediction in B-lineage ALL than those involving Vδ2-Dδ3. However, these techniques should be seen as complementary (together with those of other loci, eg, TCRγ) rather than as mutually exclusive. Using a combination of IgH- and Vδ2-Dδ3-PCR, MRD assessment is possible in 90% of B-lineage ALL using an average of 1.8 oligonucleotide probes per patient positive by either or both systems (86 probes for 47 patients). Approximately 30% of patients will amplify by both systems, allowing direct comparison of the sensitivity of IgH- and TCRδ-PCR. Most importantly, the rate of false-negative relapse prediction as a consequence of clonal evolution will be less than 10%.

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A polymerase chain reaction study of the stability of Ig heavy-chain and T-cell receptor delta gene rearrangements between presentation and relapse of childhood B-lineage acute lymphoblastic leukemia

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