Lymphocytic Progenitor Cell Origin and Clonal Evolution of Human B-Lineage Acute Lymphoblastic Leukemia

By Frederic Davi, Christopher Gocke, Stephen Smith, and Jeffrey Sklar

At presentation, bone marrow specimens from over 25% of B-lineage acute lymphoblastic leukemias (ALL) displayed more than two clonal rearrangements of immunoglobulin heavy chain (IgH) genes in Southern blot analyses. Nucleotide sequence analysis has shown predominantly different \( V_{H}D_{J_{H}} \) junctions among these genes, leading to the frequent description of such cases as oligoclonal leukemias. In the present study, we have analyzed the IgH genes from four patients whose leukemic cells contained different patterns of IgH gene rearrangements between presentation and relapse. Nucleotide sequence analysis of the IgH genes showed that three mechanisms could account for these differences: de novo \( V_{H}D_{J_{H}} \) rearrangement, \( V_{H} \) to \( D_{J_{H}} \) rearrangement, and \( V_{H} \) replacement. In all cases, more than two totally different \( V_{H}D_{J_{H}} \) rearrangements appeared during evolution of the disease, formally consistent with the conclusion that these tumors were composed of apparently unrelated clones. However, the retention of some of the antigen receptor gene rearrangements, as well as the persistence of a chromosomal marker in two cases, indicated that these leukemias had a monoclonal origin. These findings support the hypothesis that some ALLs arise from a lymphoid progenitor cell at a stage of lymphocyte development before the onset of IgH gene rearrangement. These leukemic lymphocyte progenitors generate malignant daughter cells capable of in vivo maturation that involves the completion of multiple different IgH rearrangements as well as the modification of preexisting rearrangements by \( V_{H} \) to \( D_{J_{H}} \) rearrangement or by a \( V_{H} \) replacement.

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MATERIALS AND METHODS

Patients, samples, and cell lines. All tissues were obtained with the informed consent of patients. Malignant cells were obtained from patients with ALL at the time of presentation and at relapse. In each case, bone marrow aspirates and peripheral blood samples contained >80% leukemic cells. Diagnosis of ALL was established by standard morphological, cytochemical, and immunophenotypic methods. The precursor B phenotype was based on the presence of malignant cells positive for TdT, CD19, and HLA-DR (null ALL), or for TdT, CD19, CD20, CD45, and HLA-DR.

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CD10, CD19, and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR and cytoplasmic μ chains (pre-B ALL). Establishment of cell lines, as well as their cytogenetic analysis, was performed using techniques previously described.22 Normal bone marrow was obtained from donors for allogeneic bone marrow transplantation. Normal blood was obtained from healthy volunteers.

Southern blot analysis. High molecular weight DNA was isolated and analyzed using the Southern blot hybridization procedure, as previously described.23 Probes used for hybridization consisted of cloned DNA fragments containing human Jμ, Cμ, Cδ1, and Cδ2 regions of the IgH genes and the Jμ regions,24 the Jδ regions,25 and Jγ region26 of the T-cell receptor (TCR) genes. The following restriction enzymes were used for analyses of the various genes: IgH—BamHI, EcoRI, HindIII and Bgl II; Igκ—EcoRI; TCR β—BamHI, HindIII, and EcoRI; TCR γ—BamHI and Bgl II; TCR δ—BamHI, EcoRI, and HindIII.

Polymerase chain reaction (PCR). Amplification of IgH gene rearrangements using the PCR was performed in a two-part procedure. In the first step, 50 ng to 1 μg of DNA was amplified for 20 to 25 cycles with a set of Vμ family-specific primers and a consensus Jμ primer. Ten percent of this amplified material was then reamplified for 15 to 20 cycles using a second set of nested Vμ family-specific and consensus Jμ primers. PCR was performed using nucleotides, buffer, and enzyme provided in the Perkin-Elmer Cetus Gene Amp Kit (Perkin-Elmer Cetus, Norwalk, CT), according to the manufacturer’s specifications. All reactions contained 150 ng of each primer and were performed in a 50-μL volume. Each amplification cycle consisted of a denaturation step at 94°C for 1 minute (3 minutes in the first cycle), a primer annealing step at a temperature ranging from 45°C to 55°C for 1 minute, and a primer extension step at 72°C for 1 minute (and 7 minutes in the last cycle). Negative controls were prepared by performing amplifications on samples lacking DNA or containing DNA from polyclonal specimens (bone marrow or blood from normal individuals). Precautions to avoid contamination with amplified material were followed, as described in Kwock et al.27 and Sarkar et al.28 PCR products were analyzed by electrophoresis in 1% agarose and 6% polyacrylamide gels.

Oligonucleotide primers. Design of the 5′ primers was based on sequence homology within the framework 1 region of each Vμ family, according to the published human germline Vμ genes.29-35 The 3′ antisense primers were chosen to match consensus sequences within the six human Jμ genes.36,37 Restriction sites were incorporated into the set of nested primers to facilitate the ligation of the PCR products into vectors for cloning in Escherichia coli. The sequences of the Vμ and the Jμ primers are given in Table I. Clone-specific oligonucleotides were designed to correspond to the N-regions of clonal IgH rearrangements found within the leukemic cells of individual patients. All primers were constructed with an automated DNA synthesizer (Applied Biosystems, Foster City, CA).

DNA sequencing. PCR products were digested with the restriction enzymes EcoRI and BamHI or Bgl II (BRL, Gaithersburg, MD). After purification in low melting point agarose, the fragments were ligated into DNA of the M13 sequencing vector. Multiple M13 clones grown in E coli were isolated and subjected to sequence analysis using the Sequanase Kit (US Biochemical Corp, Cleveland, OH). Only sequences found repeatedly in at least four M13 clones were considered to represent clonal rearrangements. The specific identity of individual Vμ segments was not determined.

N-oligonucleotide hybridization. PCR products amplified from tumor DNA, dilutions of tumor DNA in control DNA (bone marrow from normal individuals), and control DNA by itself, were separated by electrophoresis on 1% agarose gel and transferred to Genatran nylon membranes (Paso, Woburn, MA). Alternatively, the PCR products were directly applied to the membrane using a slotted manifold apparatus (Schleicher and Schuell, Keene, NH). Membranes were hybridized for 4 hours at 46°C in 5XSSPE (1XSSPE is 0.15 mol/L sodium chloride, 10 mM sodium phosphate, 1 mM EDTA) and 0.5% SDS (sodium dodecyl sulfate) with clone-specific oligonucleotide probes which had been 5′ end-labeled with 32P. After hybridization, membranes were washed twice in 2XSSPE, 0.1% SDS for 2 minutes at room temperature, and then once in 5XSSPE, 0.1% SDS for 15 minutes at 2°C below the melting point of the oligonucleotide sequence. Blots were exposed on Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 2 to 16 hours.

RESULTS

Patient 1. Patient 1 (Figs 1 and 2) was diagnosed with pre-B ALL containing the Philadelphia chromosome (Ph). Two cell lines, SUP-B13 and SUP-B15, were established from cells collected at the time of first and second bone marrow relapses, 2 years and 26 months, respectively, after initial diagnosis.28 For this and all other cases except one (patient 3), digestion of DNA with HindIII restriction enzyme, rather than with BamHI or EcoRI, seemed to display a greater number of bands in Southern blot analyses of the IgH genes. Using HindIII, the presentation bone marrow from this case showed a germline band, an equally intense rearranged band, and a second weakly hybridizing band, which probably also represented a clonally rearranged gene (this weakly hybridizing band occurred at the position of a minor cross-hybridizing germline band; the interpretation that this band may have represented a rearrangement in the presentation specimen was based on its relative increased intensity). At both the first and second relapses (bone marrow was analyzed for the first relapse and blood for the second) and in each cell line, the intense rearranged band was lost and a new, strongly hybridizing nongermline band appeared. A band at the position of the weakly hybridizing rearranged band was retained.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Outer Primers</th>
<th>Inner Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vμ1/7</td>
<td>GCTGAGGTGAAGAAGCTGGGG</td>
<td>TCGGATCCTCA/GGTGAAGGGGTC/TTCTGGC</td>
</tr>
<tr>
<td>Vμ2</td>
<td>CAGGTCACTTGAAGAGCTGG</td>
<td>GAAAGTCTCAGTGACCTGACCT/GTC</td>
</tr>
<tr>
<td>Vμ3</td>
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</tr>
<tr>
<td>Vμ4</td>
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<tr>
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<tr>
<td>Vμ6</td>
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<td>GAAAGTCTCAGTGACCTGAGTTAC</td>
</tr>
<tr>
<td>Jμ</td>
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<td>TGGAAATTCAG/T1GGTG/CTTCGT/TGGCGCCAG</td>
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The BamHI, Bgl II, and EcoRI restriction sites are underlined.

Table 1. Sequence of Oligonucleotides Primers Used for IgH PCR

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but it was not possible to distinguish it from the minor cross-hybridizing germline band. The germline band, still present in the marrow relapse, was not seen in the cell lines and only faintly hybridized in the analysis of blood from the second relapse.

Other antigen receptor genes displayed the same pattern of rearrangement in both marrow samples, the peripheral blood sample, and the SUP-B13 cell line. This pattern consisted of the germline configuration for immunoglobulin light chain genes (IgL) and beta T-cell receptor genes (TCR β), deletion of delta T-cell receptor chain genes (TCR δ), and apparent bi-allelic rearrangement of the gamma T-cell receptor genes (TCR γ). The SUP-B15 cell line showed one detectable kappa immunoglobulin light chain (Igκ) gene rearrangement and one weakly hybridizing rearranged band with each of the lambda immunoglobulin light chain (Igλ) and TCR β probes, as well as an additional TCR γ rearrangement.

The Ph1 chromosome was detected cytogenetically in all samples. At relapse, some cells had a more complex karyotype: 46,XY,t(9;22)(q34;q11),t(1;4)(p22;q33),der(14q),16p+. Both cell lines had only this karyotype in all cells. Amplification of IgH DNA in the presentation bone marrow repeatedly yielded three bands, two generated with the VH1-family primers, one with the VH3-family primers. The second VH1 rearrangement involved a VH1 segment (VH1a), the DLR3 segment, and the JH6 segment, all joined in frame. The second VH1 rearrangement used a different VH1 segment (VH1b) joined out of frame to the DLR1 segment and the JH6 segment. The VH3 rearrangement consisted of an in-frame joining of a VH3 segment (VH3a) directly to the JH6 segment, without any obvious D segment. In the samples obtained at relapse and in both cell lines, a single band was obtained, and only using VH3-family primers. This rearrangement had a partial homology with the original VH1b rearrangement identified in the presentation specimen, as both rearrangements shared the same D-N-JH region. However, the VH segment in this new rearrangement segment (VH3b) and the N-region between VH and D segments differed completely from that of the original rearrangement. This could be due to either two different VH segments rearranging to the same DJH joint, or the replacement of the VH1b gene by the VH3b gene. The later process, well characterized in mice, has been shown to occur in cultures of virally transformed pre-B cells and in B-cell lymphoma. It involves a recombination process mediated through a heptamer sequence located in the 3′ portion of most VH genes.

Theoretically, the sequences amplified from the blood or bone marrow in this patient could correspond with rearrangements of nonmalignant lymphocytes cells admixed with tumor cells rather than bona fide clonal rearrangements contained in leukemic cell populations. To confirm the origin of these sequences from tumor cells, the frequencies of the sequences obtained were roughly quantitated within the specimens by hybridization studies using oligonucleotides specific for the individual rearrangements. Radiolabeled oligonucleotides (N-oligonucleotides) complementary to the sequences of the N-regions for each of the four rearrangements amplified in this case were hybridized to IgH DNA amplified from the total specimen DNA before or after dilution of that DNA with the DNA of normal bone marrow. Each oligonucleotide hybridized to product amplified from DNA of the appropriate specimen and not to product from normal DNA or from the DNA of specimens from the patient in which the rearrangement could not be detected by PCR (data not shown; similar results are illustrated for patient 3 below). Moreover, the hybridization occurred to product amplified from specimen DNA that had been diluted several orders of magnitude before amplification. The results indicate that the rearrangements represented by the N-oligonucleotides were relatively abundant within the specimens and probably reflected a significant clonal proliferation of cells containing the rearrangements.

Although the VH1b rearrangement was presumably generated by modification of a preexisting rearrangement (DJH or VH1DJH), the tumor contains at least two apparently distinct clonal lines because three different VH1DJH recombinations and a germline band were present in the presentation specimens. These clonal lines may have originated from a leukemic progenitor with both IgH alleles in the germline configuration. Alternatively, these lines may represent subclones that were derived from a tumor cell having one common rearranged allele, but which diverged as a result of different rearrangements on the second germline allele.

Patient 2. Patient 2 (Fig 3) was diagnosed with a pre-B ALL containing a t(1;19)(q23;p13). A cell line, SUP-B27, was derived from bone marrow obtained at relapse 1 year later. Southern blot analysis of the DNA from the presentation marrow after digestion with Hind III and hybridization with a JH probe showed a germline and four rearranged bands. The two more intense rearranged bands were conserved at relapse and in the cell line. The relapse marrow also
Fig 2. Patient 1: Summary of immunogenotype and IgH gene rearrangement junctional sequences. (A) Pattern of antigen receptor gene rearrangements by Southern blot analysis. BM, bone marrow; PB, peripheral blood; G, germline; R, rearranged; del, deleted. Arrows indicate identity between rearranged bands; the number above the arrow indicates the number of rearranged bands that were identical. (B) Regions identified as VH, D, and Jn gene segments are overlined. The specific identity of individual VH segments was not determined. N-regions are in small characters. Hatched bars, N-oligonucleotide probes. Vertical parallel bars, identical nucleotides between two rearrangements. (+), in frame; (−), out of frame.

had a germline IgH band and two additional rearrangements, while the cell line showed no germline band. Light chain genes were in the germline configuration in the presentation marrow, while one Igκ and one Igλ rearrangement appeared at relapse and were retained in the cell line. The cell line had four additional Igκ rearrangements, which appeared as bands with much lower intensities. TCR γ genes were in the germline configuration in all samples. A germline and a rearranged band at the similar position were detected with a TCR 6 probe in the presentation and relapse marrows, the latter having two additional rearrangements. The cell line had a different TCR 6 rearrangement, and over time in culture acquired a second TCR γ rearrangement. Finally, hybridization with a TCR δ probe showed a germline and a rearranged band in the presentation marrow, while the relapse marrow and the cell line displayed only the germline configuration of this gene.

Amplification of IgH rearrangements using VH3-family primers yielded the same two products from each sample, likely corresponding to the two conserved bands seen on the Southern blot analysis. One was an in-frame rearrangement of a VH3 segment (VH3c) to the DXP segment, which in turn was joined to the JH6 segment. This rearrangement may account for the cytoplasmic Igκ chain produced by the leukemic cells in this case. The other IgH rearrangement amplified was out-of-frame and involved a different VH3 segment (VH3d), the DXP'1 segment, and the JH5 segment. In addition, VH6-family primers amplified an in-frame VH6-JH4 rearrangement from the presentation marrow. On the other hand, a VH1-DXP'1-JJb rearrangement was amplified solely from the relapse marrow. Therefore, although Southern blot analysis displayed four rearrangements in both presentation and relapse samples, only three of them could be amplified from any specimen in this case. Absence of ampli-
ORIGIN AND EVOLUTION OF B-ALL

**IMMUNOGENOTYPE**

<table>
<thead>
<tr>
<th>Presentation</th>
<th>Relapse</th>
<th>Cell line</th>
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<td>G+4R</td>
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</tr>
<tr>
<td>TCRβ</td>
<td>G+1R</td>
<td>G+3R</td>
</tr>
</tbody>
</table>

**JUNCTIONAL SEQUENCES**

Patient 2. Summary of immunogenotype and IgH gene rearrangement junctional sequences.

Amplification could be explained by the presence of a partial DJH rearrangement, which lacked a rearranged VH segment. Alternatively, insufficient complementarity between rearranged target VH or JH sequences and the consensus oligonucleotides used may have prevented amplification of additional, complete VH-DJH rearrangements. The origin of each of the amplified rearrangements from clonal cell populations was confirmed by hybridization with N-oligomerolnucleotides.

These results suggest that this case contains several clonal lines, with one predominant line that is present at presentation and at relapse and is also represented by the in vitro cell line. In vivo, as well as in vitro, evolution within this line is demonstrated by rearrangements of the IgL and TCR δ genes. At least one additional line at presentation and one at relapse, each bearing different IgH, TCR δ and β rearrangements, were also present. All of these lines appear totally unrelated, at least with regard to their IgH genes. Because three separate leukemogenic transformation events seem unlikely in a single patient, the most plausible explanation for the findings in this case is a common B-lineage committed leukemic progenitor that had its antigen receptor genes in the germline configuration, but could undergo further maturation to the pre-B-cell stage.

Patient 3. Patient 3 (Fig 4) was diagnosed with common ALL. Bone marrow from this patient was analyzed at presentation and at relapse 2 years later. Southern blot analyses performed on an EcoRI digest of DNA from the presentation marrow showed two rearranged IgH genes, as well as a band in the germline position. At relapse the germline band and one rearranged band persisted, while two less intense new rearranged bands also appeared. The patterns of bands in the analyses of IgL genes were identical in both samples and consisted of the deletion of both Igκ alleles, and one Igλ rearrangement. Similarly, the TCR γ gene pattern of a germline and two rearranged bands was unchanged between the two specimens. One of the two TCR δ rearranged bands found at presentation was lost at relapse and one rearranged TCR β band was acquired at relapse.

Amplification of IgH DNA from the presentation bone marrow yielded PCR products with Vκ2 and Vμ4 primers. One product consisted of a Vκ2 segment (Vκ2a) joined in frame to a D-D fusion (DK4 and DM2 segments), which, in turn, joined to the Jκ6c segment. The second product consisted of a Vμ4 segment (Vμ4b), parts of the DM 1 and DLR 5 segments and a Jμ segment that could not be precisely identified because of extensive 5’ deletion. Two contiguous
stop codons in the DLR5 segments interrupted the reading frame of this rearrangement. The V{sub}H4 rearrangement was also found at relapse, while the V{sub}H2 rearrangement was not detected. Instead, a V{sub}H5 and a different V{sub}H4 rearrangement were amplified. The V{sub}H5 rearrangement contained the V{sub}H5b segment joined in frame to the J{sub}H6 segment. The second V{sub}H4 rearrangement was clearly related to the rearrangement previously detected, as they shared the same N-D-N-J{sub}H region, but the V{sub}H4 segment used in this case (V{sub}H4b) was different (substitutions of nucleotides at 10 separate positions distinguished the portions of the rearrangements amplified). This IgH modification most likely reflects a V{sub}H replacement, in which there is retention of sequences located 3' to the conserved internal V{sub}H gene heptamer (TACTGTG) recognized as the signal for V{sub}H to V{sub}H recombination. An alternative explanation for these observations is a gene conversion event within the V{sub}H sequence; however, the 18-bp deletion found within the rearrangement at the 3' end of the V{sub}H4b segment, is more likely to have been generated during V{sub}H replacement, when exonucleolytic digestion of rearranging gene segments, as in de novo V-D joining, commonly occurs. Figure 5 illustrates the results of N-oligonucleotide hybridization in this case.

Therefore, it appears that the bone marrow of patient 3 contained a leukemic clone bearing a V{sub}H4 rearrangement that persisted through the course of the disease, but underwent a V{sub}H replacement event within a subclone. At different times, the leukemic cells in this patient also possessed additional IgH rearrangements, including the V{sub}H2 rearrangement found at presentation and the V{sub}H5 rearrangement found at relapse. These must have been produced by a V{sub}HDJ{sub}H recombination in a precursor neoplastic clone with germline IgH genes. Whether both alleles were in the germline configuration in this precursor, or only one allele was germline and the other had already undergone the V{sub}H4 rearrangement cannot be resolved, although the deletion of IgK genes at both presentation and relapse favors the latter interpretation.

Patient 4. Patient 4 (Figs 6 and 7) was an infant diag-
Additionally, the second relapse marrow showed another Igλ rearrangement. A common TCR γ rearrangement was found in all samples, while the second relapse marrow had an additional rearrangement. After prolonged exposure, four more faint bands appeared in analyses of the presentation sample and the first relapse sample, only two of which migrated at the same position. Four TCR δ rearrangements were detected in the presentation sample, but only a single shared rearranged band was observed in both relapse samples. Exclusively germline configurations of TCR β genes were detected in all samples from this case.

Four IgH rearrangements were amplified from the DNA of the presentation marrow. Three of these had similar D-N-JH regions composed of the DN4 segment joined to the JH3b segment. However, the ViH-N regions were different and involved either a ViH2 (ViH2b), a ViH5 (ViH5c), or the ViH6 segment. The fourth rearrangement had a totally unrelated sequence, created by a ViH3 segment (ViH3e) joined to the JH4 segment. All four rearrangements were out of frame. In both relapse samples, a rearrangement was amplified that displayed partial homology with the first rearrangement detected in the presentation bone marrow; it had the same DN4-JH3b recombination identified previously, but this sequence was joined in frame to a ViH4 segment (ViH4c). Two additional rearrangements were obtained from the first re-

nosed with a null ALL. Six IgH rearranged bands, in addition to a germline band, were identified in Southern blot autoradiograms of a HindIII digest of DNA from his presentation bone marrow. In a first relapse, 1 year later, a germline band and two strongly hybridizing rearranged bands were present, one of which had already been detected in the previous sample. Furthermore, three faint rearranged bands could also be seen. In the second relapse, 8 months later, the bone marrow displayed only two IgH rearranged bands, identical to the two intensity hybridizing bands seen on the first relapse marrow. The Igκ genes remained in the germline configuration throughout the disease, while one Igλ rearranged band appeared at the same position among all three samples.
Relapse marrow. One involved a VH3 segment (VH3f) joined directly to the JH4 segment; the other, a VH1 segment (VH1d) joined in frame to the DLR2 segment and the JH5b segment. In the second relapse marrow, only one rearrangement could be amplified despite the detection of two IgH rearrangements by Southern blot analysis of this specimen. As mentioned in the discussion of patient 2, this could be due to a partial DJH recombination or to a mismatch between primers and target sequences. Hybridizations to PCR products derived from the DNA of the various specimens in this case were performed using an N-oligonucleotide probe specific for the junctional sequence each rearrangement. As expected, the N-oligonucleotides hybridized to product from amplified DNA and DNA dilutions of the appropriate specimens. An N-oligonucleotide for the VH4c rearrangement yields a strong hybridization signal in both relapse samples, but a negative signal in the presentation marrow (data not shown). Therefore, the common IgH rearranged band seen on the Southern blot analysis in all three samples seems to correspond to a nonamplifiable sequence.

In this patient with a complex pattern of antigen receptor gene rearrangements, four IgH rearrangements were clearly related and came from a common allele bearing a DN4-JH3b recombination. Although it could be possible that multiple VH replacements occurred, it seems more likely that these rearrangements arose in the progeny of a cell containing a partial DJH joining and that several distinct VH segments were subsequently rearranged in different subclones de-

![Diagram](attachment:image.png)
scended from that cell. The presence of other totally unrelated sequences suggests that the original leukemic clone had other antigen receptor genes in the germline configuration. This inference is consistent with the intense germline rearranged sequences and TCR α genes before clonally expanding to produce the second relapse.

DISCUSSION

In this study we have analyzed antigen receptor genes to characterize the origins and evolution of ALL. This work relied heavily on PCR amplification to isolate and sequence IgH gene rearrangements. Previous studies have used single consensus oligonucleotides to 3' VH and JH sequences and have shown IgH rearrangements could be amplified in 60% to 90% of ALL. In our hands, this method had failed to amplify multiple rearrangements in oligoclonal tumors; in general, only one or sometimes two bands were obtained. This could be due to the preferential amplification of sequences having better matches with the consensus VH primer or to more efficient amplification of rearrangements that are more abundant within the sample. In view of these problems, we used VH-family specific primers. This approach enabled us to amplify many more IgH rearrangements. However, the number of sequences obtained by PCR did not strictly correspond with the number of bands detected on Southern blot analyses. Some sequences might not be amplified because they were partial DJH recombinations or were partially mismatched with the primers. Conversely, some sequences amplified were not seen on Southern blot analyses. This reflects the greater sensitivity of PCR techniques over Southern blot analysis for detection of less abundant but still clonal rearrangements, as discussed by Deane et al.

The interpretation that an amplified IgH rearrangement was tumor-related and did not come from background lymphocytes was supported by the following considerations: (1) all sequences were found in multiple M13 clones obtained from independent amplifications; (2) amplification of polyclonal cells (bone marrow or blood from normal individuals) was always performed in parallel and generated a smear of PCR products assayed by polyacrylamide gel electrophoresis, while clonal populations showed one or sometimes two discrete bands (data not shown); and (3) slot blot hybridization of these PCR products with clone-specific N-region oligonucleotide probes showed that the specific sequences could be detected only in the relevant samples and not in control normal bone marrows. Furthermore, rough quantitation indicated that the amplified sequence was present in multiple copies within the tumor sample and did not arise from the amplification of a single background lymphocyte. It should be noted, however, that quantitation by this assay is only approximate and that the extent of dilution at which clonal IgH rearrangements were still detected might not exactly reflect the true abundance of cells with these rearrangements, since many factors, such as the degree of homology with the VH primers, may affect the efficiency of amplification.

The four patients presented in this study had a pattern of IgH gene rearrangements that differed between diagnosis and relapse (Table 2). In three of these patients (patients 2 to 4), a common IgH rearrangement was conserved, indicating a straightforward clonal relationship. This was further supported by the presence of a common rearrangement for some other antigen receptor gene, and in one case by the persistence of a chromosomal translocation t(1;19) (patient 2). In one patient (patient 1), there was no definite conserved IgH rearrangement, but the presence of an identical DJH recombination, as well as the conserved configurations of TCR α and β genes and the persistence of the Ph' chromosome leads to a similar conclusion.

Three different mechanisms were responsible for shifting patterns of Ig gene rearrangement observed over time among the cases in our study (Table 2).

(1) Loss of rearrangements. Loss of IgH gene rearrangements was seen in all four patients. Loss of TCR gene rearrangements was also detected for three of them: TCR α gene rearrangements for one patient (patient 4), TCR β gene rearrangements for two patients (patients 2, 3, and 4), and TCR γ gene rearrangements for one patient (patient 2). Loss of Ig and TCR gene rearrangements was probably related, at least in some instances, to the loss of the clonal lines bearing these sequences. The disappearance of subclones between presentation and relapse may have been related to their elimination by chemotherapy. On the other hand, certain cells containing a subset of rearrangements observed earlier in the case may have become resistant to chemotherapy and/or acquired mutations leading to a selective growth advantage and overwhelming of competing subclones. A comparable phenomenon took place during the establishment in culture of the in vitro cell line developed for patient 2.

(2) Modification of preexisting rearrangement. Modification of a preexisting rearrangement by a VH replacement clearly occurred in one case (patient 3) and possibly in another (patient 1). Rearrangements of distinct VH genes to a common DJH joining could also account for IgH gene modification in one case (patient 4) and possibly in one other (patient 1). Therefore, in all but one patient (patient 2), clonal
evolution involved, at least for some IgH rearrangements, modification of a preexisting rearrangement by VH replacement or VH to DJH joining, as reported by other investigators.44,46

Two other mechanisms responsible for modification of preexisting rearrangements have also been described: somatic mutations and "open and shut" events. Somatic mutations occur frequently during evolution of some mature B-cell tumors such as follicular lymphoma,14 similar to the process by which postrearrangement diversification of Ig gene sequences takes place during the normal immune response. Recent studies have also shown that somatic mutations may also occur at low frequency in precursor B leukemic cells44 and can modify IgH rearrangements between diagnostic and relapse.47 In any event, somatic mutation is probably not responsible for the IgH gene alterations detected by Southern blot in our patients, as the sequences of PCR products amplified from the tumors show far greater differences than can be explained by somatic mutation. Junctional sequences can be changed by an "open and shut" mechanism.18 In this process, new N-region nucleotides are generated after the rearrangement has reopened and closed following previous deletion and addition of nucleotides. This mechanism has been observed in artificial recombination substrates introduced into murine early B cells15 and more recently in human ALL.44 However, these types of modification were not seen in our study.

(3) De novo rearrangements. De novo rearrangements were observed for three patients (patients 2 to 4). The fourth patient (patient 1) had three different V_HDJH recombinations at presentation. Therefore in all cases, more than two totally unrelated V_HDJH sequences appeared during the evolution of these ALL, formally consistent with oligoclonality of the tumors.

Several explanations could account for this apparent oligoclonality in ALL. (1) Extra chromosome 14 may be present in a monoclonal tumor, as has been shown to occur in some ALL.5 Cytogenetic data were available for two of our patients and did not show any evidence of additional copies of chromosome 14. Nevertheless, we cannot rule out this possibility for the two other patients. (2) Oligoclonality might result from independent transformation of separate lymphoid cells. Indeed, there is a group of tumors, particularly among those arising in immunosuppressed patients, in which independent lines of tumor cells are present, as confirmed by study of polymorphism in Epstein-Barr viral genome commonly found in these tumors.52 Therefore, true multiclonality cannot be excluded a priori. (3) Transformation of a single progenitor lymphoid cell with unarranged Ig genes, followed by the emergence of subclones with independent diverse IgH rearrangements, has been proposed to occur in some lymphocytic tumors.5,19,48,49 Considered in aggregate, our data best fits this overall mechanism for apparent oligoclonality of the ALL cases in our study. Thus, our analysis strongly supports the hypothesis of a transformed lymphocyte progenitor cell that undergoes distinct IgH rearrangements within different subclones. These subclones can further differentiate by completing V_H to DJH rearrangements, V_H replacement, and also IgL rearrangements, the latter being observed for three patients (patients 1, 2, and 4). Maturation of the leukemic cells in the form of Ig gene rearrangements appearing over the course of the disease would seem to reflect the physiologic differentiation of their benign counterpart during normal B-cell development.50 However, rearrangements of TCR γ, δ, and β genes acquired over time may or may not be a property shared by normal developing B cells.

Our results confirm those of some previous studies of oligoclonal B-lineage ALL.19 In contrast, Wasserman et al11 have recently suggested that evolution of the immunogenotype in B-lineage ALL is most often related to V_H replacement or V_H to DJH recombination, ie, modification of a preexisting rearrangement rather than de novo rearrangement. This discrepancy might be due to the usage of different primers for DNA amplification, as we found that V_H family-specific primers could amplify more diverse IgH rearrangements within oligoclonal tumors than the single V_H consensus primer.

Other investigators have reported that only a single pattern of ongoing rearrangement (ie, V_H replacement or V_H to DJH recombination or complete de novo rearrangement) occurred in an individual patient.45 These results suggested that the type of ongoing rearrangement was restricted depending on the development stage and IgH status of the precursor cells at the time of malignant transformation. Our data differ in showing that distinct ongoing IgH rearrangements processes can coexist within the same tumor.

Our study suggests that despite an appearance of biclonality or oligoclonality, all the ALL analyzed here were monoclonal tumors and represented the leukemic transformation of lymphoid progenitors that yield progeny cells in which rearrangements of Ig genes continued well after primary transformation events had already taken place. A similar situation has been found in mice, where pre-B-cell tumors induced by Abelson murine leukemia virus could undergo clonal evolution and exhibit multiple IgH and L rearrangements.17,22 Furthermore, continuing rearrangement of antigen receptor genes is not a phenomenon confined to leukemic cells in vivo; rearrangement of genes may also proceed in vitro (as in the cell line SUP-B27). All of these examples illustrate that transformation does not preclude further maturation and that the concept of tumor cells "frozen" at a specific stage of differentiation has limited relevance. Thus, despite the fact that acute leukemias consist of proliferating cells that fail to fully mature beyond certain stages of differentiation, this stage does not represent the stage at which transformation occurred. Transformation of a lymphocyte progenitor cell followed by clonal evolution has been well

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<th>Table 3. IgH Gene Rearrangement Sequences as Targets for Minimal Residual Disease Analysis</th>
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documented in some cases of ALL, 53 of acute myelogenous leukemia, 54 and in chronic myelogenous leukemia. 55 Some biphenotypic leukemia might also originate from transformation of a pluripotent progenitor cell. 56 Therefore, partial maturation of transformed cells appears to be a common feature of leukemia, although the reasons why cells cannot continue to develop beyond certain immature stages is unclear.

Two of our patients (patients 1 and 2) had a pre-B ALL, one had a common ALL (patient 3), and one had a null ALL (patient 4). Therefore, clonal evolution is not restricted to a specific subtype of B-lineage ALL. Myeloid-associated antigens such as CD13, CD15, and CD33 were also detected on the leukemic cells of two of our patients (patients 1 and 4). These findings add further evidence for the lymphocytic progenitor cell origin of these leukemias and suggest involvement of a pluripotential stem cell in some instances.

Previous studies have shown preferential usage of certain VH, D, and JH gene segments in human ALL. Though the number of IgH rearrangements sequenced in our study was small, similar findings were made. VH gene segments from families Vp4 (15.8%), Vp5 (10.5%), and Vp6 (10.5%) were overrepresented compared with the expected frequencies based on the size of each family (respectively, 10%, 1%, and <1%), as previously reported. 57 The two most frequently used D segments belonged to the DLR and DXP families (30% each), as already noted by Kiyoi et al. 47 Finally, the JH4 and JH6 genes were the most frequently found (38.5% each) as previously shown by several investigators. 47, 57 Therefore, ALLs with unstable IgH gene rearrangements do not display any specific type of repertoire and cannot be distinguished from other ALLs based on their use of certain gene segments.

Detection of minimal residual disease in B-ALL by PCR using clone-specific probes for the N-region of the IgH genes has become increasingly widely used in recent years. 40-42, 58, 59 This approach to monitoring of disease after therapy assumes stability of the rearrangement over the course of the disease. In our study, all cases had lost one or more IgH rearrangements, and more importantly the associated N-regions, between presentation and relapse. However, one IgH rearrangement was at least partially conserved among samples at various stages during the disease (Table 3). Because it is impossible to predict which rearrangement may disappear and which may persist, the most accurate assay would require use of a specific probe for each rearrangement. While this strategy may be impractical, the DJH junction seems overall more often conserved than the VnJD junction, and it would seem reasonable to choose this region as a target for minimal residual disease analysis. TCR γ, which is frequently rearranged in B-ALL, has been suggested as a more stable marker. 50 Indeed, three of the cases in our study had TCRγ rearrangements that were apparently not modified over time. Nevertheless, one case (patient 4) had lost some of the TCRγ rearrangements. Similarly, Tycko et al 51 have reported a case with totally different TCRγ rearrangements at diagnosis and relapse, while IgH rearrangements remained identical. Taken together, these data indicate the instability of antigen receptor gene rearrangement in some cases of ALL represents a potentially important complication in the application of these gene rearrangements as markers for the detection of minimal residual disease.

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