Clonal Analysis of Childhood Acute Lymphoblastic Leukemia with "Cytogenetically Independent" Cell Populations

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Abstract

Acute lymphoblastic leukemia (ALL) is generally regarded as a clonal disease in which a single abnormal progenitor cell gives rise to neoplastic progeny. Five of 463 cases of childhood ALL with adequately banded leukemic cells were found to have two cytogenetically independent cell populations. In addition, two of the four cases tested had more than two rearranged immunoglobulin genes and (or) T cell receptor genes. To investigate the clonality of these unusual leukemias, we examined the neoplastic cells for X-linked markers extrinsic to the disease. Leukemic cells from each of the three patients heterozygous for an X-linked, restriction fragment length polymorphism showed a single active parental allele, suggesting that both apparently independent cell populations developed from a common progenitor. These cases provide evidence that leukemogenesis involves a multistep process of mutation and suggest that karyotypic abnormalities may be a late event of malignant transformation.

Introduction

Leukemias and lymphomas are generally thought to be clonal in origin, developing from a single, abnormal progenitor cell capable of expansion by indefinite self-renewal. Evidence to support this concept comes from several different lines of research, including glucose-6-phosphate dehydrogenase (G6PD) enzyme studies, recombinant DNA analysis based on restriction fragment length polymorphisms (RFLPs), determination of T cell receptor gene or immunoglobulin gene rearrangements, demonstration of immunoglobulin idotypes and light-chain types in B cell malignancies, and karyotype analysis (see reference 1 for review).

In G6PD studies, the unicellular development of neoplasias can be demonstrated by finding a single type of G6PD in

the malignant cells of heterozygous patients who have a double-enzyme pattern in their normal tissues (1). However, only cells from female patients heterozygous for the X chromosome-linked G6PD gene can be analyzed. The largest single study of this type in childhood acute lymphoblastic leukemia (ALL) consisted of 19 girls heterozygous for G6PD (2). Determination of the methylation patterns of X-linked RFLPs in heterogeneous females is another sensitive method of analysis based on the same principle (3). From differences in the arrangement of DNA in immunoglobulin genes in two subpopulations of cells, Sklar and associates (4) identified four cases of "biclonal" B cell lymphoma. Similarly, Weiss and co-workers (5) suggested that some patients with lymphomatoid papulosis, a clinically benign but histologically malignant skin disease, have a "multiclonal" disorder. This idea was based on the patterns of T cell receptor gene rearrangement found in biopsy specimens from different sites in a single patient and on the presence of three rearranged bands identified by analysis of T cell receptor genes from other patients. Kitchingman et al. (6), using a Cμ heavy-chain immunoglobulin gene probe, found more than two rearranged bands in 18 of 93 cases of B cell precursor ALL. However, these results may not indicate bi-clonality but rather somatic hypermutation in the rearranged immunoglobulin genes (7), replacement of rearranged V regions at the VD joint by 5’ V regions (8, 9), or clonal evolution involving continued rearrangement of the immunoglobulin or T cell receptor genes after malignant transformation (10-12).

Detection of completely unrelated stem lines in the analysis of leukemic cell karyotypes has been regarded as evidence of biclonal or multiclonal disease. At the Fourth International Workshop on Chromosomes in Leukemia (13), stem lines were considered related when they contained at least one change (numerical or structural) in common, and unrelated when not even one chromosomal change was common to all the lines. Although multiple leukemic lines have been found in approximately one-fourth of cases of ALL at diagnosis (14-16), only rarely have they appeared to represent "independent" lines (15). The presumed biclonal nature of cases with seemingly independent stem lines has not been substantiated by other genetic or molecular approaches.

With improved banding techniques, > 90% of newly diagnosed cases of ALL can be identified as having clonal chromosome abnormalities (17, 18). Using such methods, we were able to detect two apparently independent stem lines coexisting in newly diagnosed cases of ALL. Immunoglobulin and T cell receptor gene rearrangement and RFLP analyses in these cases provided evidence for multistep leukemogenesis and suggested that both stem lines were derived from a single progenitor cell as a late event of malignant transformation.

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1. Abbreviations used in this paper: ALL, acute lymphoblastic leukemia; FAB, French-American-British (criteria); G6PD, glucose-6-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; PGK, phosphoglycerate kinase; RFLPs, restriction fragment length polymorphisms.

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Methods

From November 1978 to July 1987, bone marrow leukemic cells from 463 children with newly diagnosed ALL were adequately banded for karyotype analysis. Informed consent was obtained from all patients or their parents, and the investigation was approved by the institution’s Clinical Trials Review Committee.

Chromosome analysis. Bone marrow samples were processed immediately after collection according to the method of Williams et al. (19). Metaphase preparations were G-banded with trypsin and Wright’s stain. Chromosome abnormalities were classified according to the International System of Human Cytogenetic Nomenclature (1985) (20). A leukemia line is defined by the presence of at least two cells with the same extra chromosome or the same structural change, or at least three cells with the same missing chromosome. The term stem line indicates the most frequent chromosome constitution at any given time; other related lines are termed side lines or sublines. Two abnormal lines with no chromosomal feature in common are “independent” stem lines.

Blast cell phenotyping. Cases were classified according to French-American-British (FAB) criteria (21), based on bone marrow cell morphologic and cytochemical staining characteristics. Bone marrow cells were separated on a Ficoll-Hypaque gradient. Cell surface antigens were detected by a standard indirect immunofluorescence assay with monoclonal antibodies to lymphoid-associated antigens, including CD2, CD3, CD5, CD7, CD10, CD19, CD20, and CD22 as well as myeloid-associated antigens including CD11, CD13, CD14, CD15, and CD33 (22). Blast cells were also tested for surface and cytoplasmic immunoglobulin and rosette formation with sheep erythrocytes. Depending on the pattern of reactivity, the cells were classified as T, B, pre-B, CALLA’ early pre-B (common) or CALLA’ early pre-B, as previously described (23).

Immunoglobulin and T-cell receptor gene analysis. Probing of high-molecular-weight DNA was performed as described previously (6). Bam HI, Eco RI, and Hind III digestions were used to analyze immunoglobulin heavy-chain genes; the probe consisted of a 3.4-kb Eco RI/Hind III fragment of the joining region (JH) that detects 18-kb Bam HI, 17-kb Eco RI, and 12-kb Hind III germ-line fragments. Bam HI digestions were also probed with a 2.5-kb α light-chain gene fragment (25) that recognizes a 12-kb germ-line fragment. T-cell receptor β chain gene rearrangements were analyzed after Bam HI, Eco RI, or Hind III digestion of high-molecular-weight DNA by probing with pB400, a 0.4-kb cDNA containing sequences from Cβ2 (26). DNA from patients 1, 2, and 4 were analyzed with all three enzymes for the JH and T-cell receptor β chain genes. In patient 5, Bam HI and Hind III were used for JH analysis, and Bam HI for T-cell receptor analysis. DNAs were labeled by the oligolabeling procedure (27) and generally had specific activities of 5×10⁶ cpm/μg. High stringency conditions were used for hybridization and washing. The filters were exposed to XAR film (Eastman Kodak Co., Rochester, NY) in the presence of intensifying screens. All experiments included control DNA containing germ-line arrangements of the immunoglobulin and T-cell receptor genes.

X-chromosome inactivation analysis. Mononuclear and polymorphonuclear cell fractions were isolated by sedimentation through a discontinuous Histopaque gradient (specific gravities, 1.077 and 1.119). High-molecular-weight DNA was extracted (28) and digested with restriction endonucleases using reaction conditions suggested by the enzyme suppliers (Boehringer Mannheim Diagnostics, Inc., Houston, TX; New England Biolabs, Cambridge, MA; and Pharmacia Fine Chemicals, Piscataway, NJ). Southern blot analyses of phosphoglycerate kinase (PGK) and hypoxanthine phosphoribosyl transferase (HPRT) restriction fragment alleles were performed by a modification of the methods described by Vogelstein et al. (29). Formamide was not used. Hybridizations and washes were performed at 68°C and 65°C, respectively. A 0.812-kb Eco RI–Bam HI fragment from the 5' region of the PGK locus (30) and a 1.7-kb Pst I–Bam HI fragment from the 5' region of the HPRT locus (31) were used as hybridization probes.

Results

The five girls with two “independent” leukemic stem lines at diagnosis were 3–11 yr old, and had presenting leukocyte counts of 3.1–246×10⁹/liter, hemoglobin levels of 3.8–10.4 g/dl, and platelet counts of 8–85×10⁹/liter (Table I). Patient 1 presented with an anterior mediastinal mass; none of the patients had initial central nervous system leukemia. The blast cells in four cases were classified as FAB L1, and in one case as FAB L2 (Table I). Immunologic subtypes of ALL were T-cell in patient 1, pre-B cell in patient 4, and common in patients 2, 3, and 5. All cases lacked cytochemical evidence of myeloid differentiation at diagnosis. When cases were examined for reactivity with monoclonal antibodies against myeloid-associated differentiation markers, 70% of the blast cells from patient 3 expressed MY9 without other myeloid markers. By dual immunofluorescence study, the blast cells from patient 3 were demonstrated to coexpress B4 and MY9 antigens (Fig. 1). No leukemic cells were stored for patient 1.

Fig. 2 depicts the partial karyotypes of both stem lines from each patient, representing analysis of 11–60 marrow metaphase preparations per case. The second stem-line accounted for 15–50% of the completely analyzed abnormal metaphases in these cases.

DNA samples from patients 2–5 were examined for arrangement of the immunoglobulin and T-cell receptor β chain genes by Southern blot analysis (Fig. 3). DNA digests from patient 3 showed four rearranged bands hybridizing with a JH probe after Bam HI or Hind III digestion. When digested with Eco RI, Bam HI, or Hind III and hybridized with a JH probe,
Table II. Leukemic Cell Phenotypes

<table>
<thead>
<tr>
<th>Patient</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
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<tr>
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<td>90</td>
<td>100</td>
<td>100</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>E rosette</td>
<td>+</td>
<td>–</td>
<td>ND</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>TdT</td>
<td>ND</td>
<td>0</td>
<td>100</td>
<td>66</td>
<td>100</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>7</td>
<td>92</td>
<td>85.3</td>
<td>92.2</td>
<td>85</td>
</tr>
<tr>
<td>CD10 (CALLA)</td>
<td>2</td>
<td>90</td>
<td>85.7</td>
<td>90.4</td>
<td>88</td>
</tr>
<tr>
<td>CD19 (B4)</td>
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<td>90</td>
<td>86</td>
<td>89.2</td>
<td>93</td>
</tr>
<tr>
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<td>10</td>
<td>6</td>
<td>80.5</td>
<td>21</td>
</tr>
<tr>
<td>CD22 (Leu14)</td>
<td>ND</td>
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<td>22</td>
<td>78.3</td>
<td>20</td>
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<tr>
<td>Clg</td>
<td>ND</td>
<td>0</td>
<td>9</td>
<td>88</td>
<td>0</td>
</tr>
<tr>
<td>Slg</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>ND</td>
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<td>1.4</td>
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<tr>
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<td>18</td>
<td>9.9</td>
<td>1.5</td>
<td>10</td>
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<tr>
<td>CD2 (T11)</td>
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<td>14.6</td>
<td>1.1</td>
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<td>ND</td>
<td>70</td>
<td>0</td>
<td>ND</td>
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<td>CD13 (MY7)</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
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<td>5</td>
<td>12.9</td>
<td>7.5</td>
<td>0</td>
</tr>
<tr>
<td>CD14 (MY4)</td>
<td>ND</td>
<td>ND</td>
<td>6.2</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

Immunophenotype T cell Common Pre-B Common
FAB Subtype L1 L1 L1 L2 L1

Abbreviation: ND, not done.
* Results given as percent positive cells in samples after separation on a Ficoll-Hypaque gradient.

DNA from patients 2, 4, and 5 had two, one, and one rearranged bands, respectively. The second heavy-chain gene was in the germ-line configuration in patients 4 and 5; patient 2 also had a germ-line band of the heavy-chain gene, most probably from residual normal cells. DNA from patients 3 and 4 demonstrated germ-line \( \kappa \) light-chain gene configurations, while that from patient 2 had two rearranged \( \kappa \) light-chain genes (data not shown).

Analysis of the \( \beta \) chain gene of the T cell receptor after \textit{Bam} HI digestion disclosed the presence of two rearranged bands in cases 2 and 5. The T cell receptor constant region probe hybridized to four rearranged and one germ-line band in case 3. Despite the finding of only one rearranged heavy-chain allele in case 4, the T cell receptor constant region probe hybridized to three rearranged and one germ-line band.

Two of four patients from whom DNA was available were found to be heterogeneous for a PGK. \textit{Bst} XI RFLP (patients 2 and 4); a third was heterozygous for a HPRT \textit{Bam} HI RFLP (patient 5). Results obtained with the PGK probe are shown in Fig. 4. DNAs from patients 2 and 4 were run in lanes 1–6 and 7–12, respectively. DNA in each lane was digested with \textit{Bst} XI and \textit{Pst} I to distinguish the alleles; DNAs in lanes 2, 4, 6, 8, and 10 were also digested with \textit{Hpa} II, which cleaves the active allele(s) into smaller fragments. Analysis of mononuclear cells (lanes 1 and 2, and 7 and 8) and polymorphonuclear cells (lanes 3 and 4, and 9 and 10) obtained while patients were in complete remission disclosed a monoclonal pattern; that is, the intensity of each band was reduced but both bands remained after \textit{Hpa} II digestion. Blast cells (lanes 5 and 6 and 11 and 12) showed a single 1.05-kb inactive PGK allele; the active 0.9-kb allele was entirely digested in each case.

The results of analyses of HPRT alleles in cells from patient 5 are shown in Fig. 5. Lanes 1 and 2 contain DNA from mononuclear cells obtained during remission; blast cell DNA was run in lanes 3 and 4. Bands at 18 and 12 kb represent \textit{Bam} HI/\textit{Pvu} II digestion (lanes 1 and 3). After \textit{Hpa} II digestion of remission cells (lane 2), the intensity of each band was reduced by \( \sim 50\% \) (a polyclonal result). \textit{Bam} HI/\textit{Pvu} II/\textit{Hpa} II treatment of blast cells (lane 4) produced a band at 11.2 kb, as a result of cleavage of the active 12-kb allele at one of the 5' \textit{Hpa} II sites (29); the 18-kb inactive allele was fully digested. The
lower-molecular-weight band in lanes 2 and 4 represents the cleavage products of the inactive alleles.

Four of the five patients remain in complete remission for 12+ to 47+ mo. Patient 1 had an apparent lineage switch (32) to acute myeloid leukemia after 22 mo of initial complete remission. At relapse, her blast cells were clearly myeloid, as evidenced by the presence of Auer rods as well as reactivity with myeloperoxidase, Sudan black B, and chloroacetate esterase. Of the 15 metaphase cells analyzed, eight were normal and seven revealed 46,XX,del(6)(q24), identical to the second stem line at diagnosis. This child attained a second remission after therapy for acute myeloid leukemia. She experienced another relapse 6 mo after completing all treatment, at which time the morphologic and phenotypic features of her blast cells were still indicative of myeloid leukemia. Of the 14 metaphase cells analyzed, seven were normal, five were 46,XX,del(6)(q24) and the remaining two were 47,XX,+21,del(6)(q24), indicative of clonal evolution.

Discussion

Of the 463 patients that we studied, 5 (1%) had two cytogenetically independent cell populations at diagnosis of ALL. These cases are readily distinguished from leukemias that show progressive karyotypic changes within the same clone (clonal evolution) (13) or bilinear features in the presence of a single karyotype. Clonal evolution, in which a leukemic sample contains two or more sublines with related chromosomal abnormalities, is a relatively common finding in ALL; almost one-

**Figure 2.** Partial G-banded karyotypes demonstrating two independent stem lines in patients 1–5. Arrows indicate the sites of breakpoints.

**Figure 3.** Southern blot analyses of immunoglobulin heavy-chain gene and T cell receptor (TCR) β chain gene rearrangements from leukemic blasts of patients 2–5. Probes and the restriction enzymes used are indicated below each lane. Rearranged bands are indicated by the arrows and germ line bands by lines. The lines with an asterisk represent a non–rearranging JH-related gene that is always seen after BamHI or HindIII digestion of DNA. This “phantom” JH band usually is seen as a single hybridizing band but occasionally splits into a doublet, as in patient 5. The sizes of the rearranged bands are given for patients 2–5. Patient 2: EcoRI JH, 16.0 and 7.3 kb; BamHI β-TCR, 25.5 and 13.6 kb. Patient 3: BamHI JH, 29.0, 24.5, 20.0, and 17.0 kb; BamHI β-TCR, 21.0, 19.0, 12.8, and 9.5 kb. Patient 4: BamHI JH, 29.0; BamHI β-TCR, 27.0, 20.0, and 14.5 kb. Patient 5: HindIII JH, 10.5 kb; BamHI β-TCR, 21.5 and 20.5 kb.
fourth of the cases tested have shown evidence of this process at diagnosis (14–16). Bilineal leukemia, in which populations of lymphoblasts and myeloblasts coexist within the same patient, is well documented but is far less frequent than cases showing clonal evolution (33–35). Leukemic transformation of a multipotential progenitor for both lymphoid and myeloid cells could explain the simultaneous occurrence of two phenotypically distinct but karyotypically similar cell populations (36). In this respect, Philadelphia chromosome-positive chronic myelogenous leukemia and some cases of acute myeloid leukemia and Philadelphia chromosome-positive ALL have been shown to involve multipotent stem cells capable of differentiation along either a myeloid or lymphoid pathway (37–39).

Although the coexistence of cyogenetically unrelated stem lines has been regarded as evidence of biclonal disease (13), this concept has not been substantiated by molecular clonal analyses. X chromosome–linked markers have been valuable in the study of clonal development of cancers (1–3, 29). As a result of random stable inactivation of one of the two X chromosomes in female somatic cells, normal tissues in women are mosaics, containing some cells with an active paternal X chromosome and other cells with an active maternal X chromosome. By contrast, proliferations that develop clonally are composed of cells expressing the same parental X chromosome. To evaluate the clonal or biclonal nature of leukemia with two cyogenetically independent stem lines, we studied four of our cases with X-inactivation markers. Leukemic cells from each of the three patients heterozygous for an X-linked RFLP showed a single active parental allele. It is possible that a cyogenetic abnormality common to all leukemic cells in each patient is not detectable by the techniques that we used or was present initially and subsequently lost from both sublines. It is theoretically possible that a subline diverged from the parent clone by complex rearrangements that recreated apparently normal chromosomes while producing additional karyotypic changes. However, the most likely explanation is that the patient’s leukemias developed clonally from cyogenetically normal but unstable progenitors and that the two apparently unrelated abnormal karyotypes arose by clonal evolution. A similar pathogenesis has been proposed for a patient with myelodysplastic syndrome (40). In this regard, clonal remissions, as indicated by the finding of one or predominantly one G6PD type, have been found in some cases of acute myeloid leukemia, despite evidence in one patient that a cyogenetically abnormal clone disappeared (41, 42). This finding suggests a multistep leukemogenesis with progression from a neoplastic, but cyogenetically normal, progenitor to a karyotypically abnormal, overtly leukemic clone. However, since for each case there is a 50% chance that two independent clones will express the same allele, there remains a 1 in 8 probability that these three cases are truly biclonal. Additional leukemias with more than one cyogenetically distinct line should be studied to address this issue with statistical confidence.

We have previously reported leukemias that had completely different karyotypes at diagnosis and at relapse (22). To explain this finding, we suggested either that two clonally distinct abnormal lines existed at diagnosis but were overlooked during cyogenetic studies or that a second leukemic transformation event had occurred. In light of results from the present study, we believe that some of these cases in fact arose from clonal evolution rather than transformation of separate lymphoid progenitors. Patient I in this study relapsed with acute myeloid leukemia, retaining only the second stem line seen at diagnosis. Had this abnormal line not been detected during the initial screening of blast cells, we would probably have classified this recurrence as a second malignancy. Although at diagnosis the second stem line represented 15% of the total abnormal population of metaphase cells, cyochemoical studies failed to disclose any evidence of myeloid differentiation. Conceivably, chemotherapy modified this minor leukemic clone by amplifying or suppressing differentiation programs, thereby causing a shift in the expression of phenotypic features. In this regard, progressive lineage conversion from ALL to acute myeloid leukemia has been reported in patients receiving 2'-deoxycoformycin (43, 44).

Multiple cyogenetically unrelated leukemic clones have been found in two patients with human T lymphotropic virus-positive adult T cell leukemia (45, 46). In one of the cases, the leukemic cells were monoclonal for the integration site of human T lymphotropic virus proviral DNA, a finding consistent with the hypothesis of multistep leukemogenesis (45). Our finding of a single active parental PGK allele in leukemic cell
populations characterized by two unrelated karyotypes and three rearrangements of the T cell receptor β chain gene (patient 4) is consistent with the concept that immune gene studies are not sufficient to disprove monoclonality. Conceivably, the "independent" stem lines in cases 2 and 5 developed after the rearrangement of immunoglobulin heavy-chain and T cell receptor β chain genes. Likewise, the immunoglobulin gene rearrangement probably preceded the cytogenetic changes and T cell receptor β chain gene rearrangement in case 4.

Although the biologic significance of expression of a myeloid marker, MY9, in the blast cells of patient 3 is undefined, it further illustrates the biologic heterogeneity of leukemia. Such mixed-lineage expression has been explained on the basis of (a) malignant transformation of a pluripotent stem cell or a rarely detected progenitor cell or (b) aberrant gene expression resulting from a leukemogenic event (47–50).

In summary, this study suggests that cytogenetically independent cell populations coexisting in individual patients with ALL arise from a cytogenetically normal progenitor and represent a form of clonal evolution. Our findings support the concept that leukemia involves multiple genetic events. They also suggest that some cases of so-called lineage switch in acute leukemia (32) may in fact represent subclonal selection and evolution due to the pressure of chemotherapy. Further studies of such cases may improve our understanding of leukemogenesis and clarify mechanism(s) of lineage conversion.

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