Molecular Analysis of Acute Undifferentiated Leukemia: Two Distinct Subgroups at the DNA and RNA Levels

By Junichi Hara, Keiko Yumura-Yagi, Akio Tawa, Shigehiko Ishihara, Mitsunori Murata, Naohiro Terada, Yutaka Izumi, Eric Champagne, Yoshihiro Takihara, Tak W. Mak, Mark Minden, and Keisei Kawa-Ha

On the basis of negativity for myeloperoxidase (MPO) and absence of lineage-associated antigens on the cell surface, 11 children were diagnosed as having acute undifferentiated leukemia. To analyze the molecular events associated with hematopoietic cell differentiation, we analyzed the configuration of the immunoglobulin (Ig) and T-cell receptor (TCR) δ, α, γ, and β genes in these patients. In parallel, transcription of the genes for MPO, terminal deoxynucleotidyltransferase (TdT), CD3-γ, Ig-μ, TCR-γ, and β was also examined. Six patients showed rearrangements of both the Ig heavy (H) and TCR-δ genes, frequently accompanied with Ig-κ, TCR-α, γ, and β gene rearrangements. These findings indicated that the leukemic cells from the six patients had been committed to the lymphoid lineage. This concept was supported by the presence of TdT transcripts in three analyzed specimens from these patients. In contrast, the remaining five patients did not display rearrangements of the lg or TCR genes, and TdT transcripts were undetectable in two patients tested. MPO transcripts were not detected in four patients analyzed, thus providing no evidence of myeloid differentiation. After hybridization with the CD3-γ gene, three of six patients showed transcription of the CD3-γ gene. In addition to CD3-γ transcripts, one patient with rearrangements of the Ig-H, TCR-δ, α, γ, and β genes also had full-length TCR-β and γ transcripts, indicating a T-precursor–cell origin of the leukemic cells from this patient. The Ig and TCR genes were in the germline configuration in the other two patients with CD3-γ transcripts. One of them did not express the CD7 antigen but did express the CD33 antigen on the cell surface, suggesting that CD3-γ transcription may not always be an event restricted to cells differentiating along the T-cell lineage.

PHE NOTYPIC ANALYSES using monoclonal antibodies (MoAbs) combined with immunoglobulin (Ig) and T-cell receptor (TCR) gene analyses have made it possible to classify a majority of acute leukemias into acute nonlymphocytic leukemia (ANLL), T-cell acute lymphoblastic leukemia (T-ALL), B-cell ALL (B-ALL), and B-cell precursor ALL (B-precursor ALL) based on their cellular origins. In a small population of acute leukemias, however, the cellular origin has remained obscure because of the lack of lineage-associated antigens on the cell surface as well as undifferentiated morphological and cytochemical features. These leukemias are referred to as acute undifferentiated leukemia (AUL), which is presumed to result from clonal expansion of poorly differentiated hematopoietic cells. Delineation of the cellular origin and characterization of the features of AUL would be useful not only for furthering our understanding of the developmental processes of hematopoietic cells but also for establishment of appropriate treatment strategies. We previously showed heterogeneity of AUL at the Ig and TCR-β gene levels using Southern blot analyses. To gain further insights into the developmental processes of hematopoietic cells, we applied newly developed TCR-δ probes in addition to Ig heavy (H), light (L), and TCR-γ and β probes to the leukemic cells from 11 patients diagnosed as having AUL according to our criteria. Northern blot analyses were also performed using myeloperoxidase (MPO), terminal deoxynucleotidyltransferase (TdT), Ig and TCR probes.

MATERIALS AND METHODS

Patients and cell samples. Among patients with ALL (L1 and L2 morphology in the French-American-British [FAB] classification), 11 patients were diagnosed as having AUL based on negativity for MPO staining (less than 3% positive cells) and the absence of reactivity with lineage-associated MoAbs. Patients with myelofibrosis, hypoplastic leukemia, and Down’s syndrome were excluded from this study. Mononuclear cells were obtained from bone marrow (BM) by Ficoll-Hypaque (FH) centrifugation at the time of diagnosis. The evaluated samples contained greater than 90% malignant cells. The investigation was approved by the institution’s clinical trials review committee. Patients were informed that a part of BM samples would be used for research purposes and that their privacy would be protected.

Immunologic phenotype. A million cells were incubated for 30 minutes with each MoAb at concentration recommended by each supplier, washed twice with phosphate-buffered saline (PBS) containing 0.05% sodium azide, and then incubated for 30 minutes with fluorescent-conjugated goat antimouse Ig (Becton Dickinson, Mountain View, CA). For surface Ig (sIg) detection, cells were stained with goat anti-human Ig antibody labeled with fluorescent isothiocyanate (Tago, Burlingame, CA). Cells were washed twice with PBS and examined by fluorescence microscopy, and 200 cells were counted for each assay. Samples with greater than 25% cells demonstrating reactivity with the antibody were considered positive. MoAbs used in this study included T11(CD2), T3(CD3), J5(CD10), B4(CD19), B1(CD20), My7(CD13), Mo2(CD14), My9(CD33), PLT(CD41a), I(HLA-DR; Coulter Immunology, Hialeah, FL), Leu-1(CDS) and Leu-9(CD7) (Becton Dickinson). The background fluorescence was determined by incubating cells with isotypic negative control antibodies (Coulter Immunology) in place of the specific antibodies, and the background staining was less than 5% in each assay.
MOLECULAR ANALYSIS OF AUL

Table 1. Clinical Data

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<th>Patient No.</th>
<th>Age (yr)</th>
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<th>PAS*</th>
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<td>-</td>
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Abbreviations: ND, not done; CCR, continuous complete remission. * >3% positive cells.

Southern blot hybridization. High mol wt DNA was extracted from mononuclear cells and digested with restriction endonucleases. The digested DNA was electrophoresed through 0.8% agarose gels and transferred to nylon membranes. Blots were hybridized to probes labeled with 32P by the random primer method.15 The Ig gene probes used in this study were the Ig probe (3-kb EcoRI-HindIII genomic fragment provided by Dr P. Leder)13 and the C probe (2.5-kb EcoRI genomic fragment provided by Dr P. Leder).14 The TCR gene probes used in this study included the J, probe (0.7-kb EcoRI-HindIII genomic fragment provided by Dr T.H. Rabbitts)15 and the C probe (0.8-kb BglII-EcoRV fragment of cDNA YT35).16 For analysis of the TCR-δ and α gene configuration, the M probe (4.5-kb EcoRI genomic fragment), the H5 probe (1.5-kb XbaI genomic fragment), the C probe (0.5-kb EcoRI-HincII fragment of genomic DNA CTH2) and the C probe (0.35-kb Sau3A-HindIII fragment provided by Dr J. Kappler)17 were used.

Northern blot hybridization. Cytoplasmic RNA was extracted after lysis with Nonidet P-40 in the presence of 10 mol/L vanadyl-ribonucleoside complex and removal of nuclei from mononuclear cells.4 Ten micrograms of RNA were verified by ethidium bromide staining of gels (CD2 and CD5), B-cell–associated antigens (CD10, CD19, CD20, and slg), and myeloid-associated antigen (CD13). Eight patients were HLA-DR+, CD3, CD7, CD14, and CD41w were also analyzed in eight, four, five and three patients, respectively, and were negative. Three patients (patients 5, 6, and 11) were analyzed for expression of the TCR-δ, HGPO3 cDNA EcoRI fragment20; C, mentioned above, and C, 1.3-kb genomic EcoRI fragment provided by Dr T.H. Rabbitts.21

RESULTS

Clinical characteristics. The characteristics of the 11 patients are shown in Table 1. The age range was from 7 months to 11 years. The initial leukocyte count ranged from 3.2 to 152.0 x 10⁹/L. All were morphologically diagnosed as having ALL (L1 or L2) based on the lack of morphologic signs of myeloid differentiation and negative staining for MPO (<3% positive cells). In three of the ten tested patients, more than 3% leukemic cells were positive for periodic acid-Schiff (PAS) staining. Eight patients achieved complete remission, but three did not. Among the eight patients who achieved complete remission, however, six patients relapsed and died.

Phenotypic analysis. Table 2 shows the reactivity of malignant cells with our panel of MoAbs. According to our phenotypic criteria for AUL, 11 patients were diagnosed as AUL; their cells failed to express T-cell–associated antigens (CD2 and CD5), B-cell–associated antigens (CD10, CD19, CD20, and slg), and myeloid-associated antigen (CD13). Eight patients were HLA-DR+. CD3, CD7, CD14, and CD41w were also analyzed in eight, four, five and three patients, respectively, and were negative. Three patients (patients 5, 6, and 11) were analyzed for expression of the

Table 2. Summary of Phenotypic Analysis

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<th>CD20</th>
<th>CD13</th>
<th>CD14</th>
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<th>CD41w</th>
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<td>0</td>
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<td>26</td>
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Abbreviation: ND, not done.

Numbers are specific percentages of positive cells. The background staining was not subtracted.
CD33 antigen, and the leukemic cells from patient 11 were positive.

**TCR and Ig gene analyses.** For analysis of the TCR-δ and α gene configuration, the MH6 and H5 probes were used. As shown in Fig 1, the MH6 and H5 probes detected rearrangements in J61 following BamHI or HindIII digestion and in J62 and J63 following EcoRI digestion, respectively. In cases of deletion of the MH6, H5, and C6 genes, it was considered that rearrangements to Jα genes had taken place because the Jα loci are located between C6 and Cα. Representative Southern blots and the results are shown in Figs 2 and 3 and in Table 3. The MH6 probe revealed bi-allelic rearrangements in patients 4 and 6. Three patients (patients 1 through 3) displayed single rearranged bands without retention of the germline configuration, and patient 5 showed no hybridized band with the MH6 probe, indicating that rearrangements to Jα genes had taken place (Fig 2). After hybridization with the H5 probe, a rearranged band was shown in patient 5 alone without a germline band (Fig 3). These findings suggest that rearrangements had occurred in the Jα locus on the other allele in patients 1, 2, 3, and 5. Rearrangements of the TCR-α gene in four patients and the germline configuration in the remaining seven patients were confirmed by comparing the intensity of bands corresponding to the Cα and Cα probes on the same blots (data not shown).

Rearrangements of the TCR-γ and β genes were demonstrated in two (patients 1 and 2) and four patients (patients 1 through 4), respectively. Ig-H gene rearrangements were detected in six patients (patients 1 through 6) without retention of the germline configuration, and one of these patients (patient 6) also showed Ig-κ gene rearrangement (Table 4).

Among these six patients who showed rearrangements of both the Ig-H and TCR-δ genes, TCR-α, γ, and β gene rearrangements were also observed in two patients (patients 1 and 2), and rearrangements of the TCR-α and β genes were shown in one patient (patient 3). Three patients displayed rearrangements of the Ig-κ (patient 6), TCR-β (patient 4), and TCR-α (patient 5) genes. Overall, among 12 alleles of examined six rearranging genes, six to nine alleles rearranged in five patients. In one patient five of ten alleles were involved. In contrast, the remaining five patients (patients 7 through 11) showed germline configurations for the Ig and TCR genes. These results are summarized in Table 4.

**RNA analyses.** The results for RNA samples are summarized in Table 4, and representative northern blots are
As markers of lymphoid differentiation, transcription of the Ig-μ TCR-γ, β, and CD3-γ genes was analyzed. Hybridization of RNA samples from three patients (patients 3, 4, and 10) with a Cα probe showed low levels of Ig-μ transcripts whose size did not match that of full-length transcripts containing a VH gene (Fig 4). Among these three patients, two patients (patients 3 and 4) showed rearrangements of the Ig-H gene, but the remaining patient (patient 10) did not. Ig-μ transcripts were not detected in the other five analyzed patients (patients 1, 5, 7, 9, and 11). As shown in Fig 5, full-length 1.7-kb TCR-γ transcripts were observed in only one patient (patient 1). This patient also had full-length 1.3-kb and immature 1.0-kb TCR-β transcripts with rearrangements of both the TCR-γ and β genes. In the remaining patients, neither TCR-γ nor full-length TCR-β transcripts were detected. Low levels of immature 1.2-kb and 1.0-kb TCR-β transcripts were detected in patients 3 and 11, respectively. It is likely that these truncated TCR-β transcripts may be derived from recombined DJβ and Cβ genes or the Cβ gene alone in the germline configuration. TCR-β transcripts could not be detected in five patients (patients 4, 5, 7, 9, and 10). Expression of the CD3-γ gene was displayed in three patients (patients 1, 10, and 11; Fig 5).

**DISCUSSION**

The criteria for diagnosis of AUL are various and differ with each institution. In this study diagnosis of AUL was made on the basis of undifferentiated morphological and cytochemical features and an immunological phenotype incompatible with the B, T, or myeloid lineage. The CD7 antigen is generally used to identify T lineage cells. However, we applied the diagnosis of AUL to acute leukemias solely expressing CD7, since these leukemias usually have no rearrangements of the TCR genes, indicating the undifferentiated nature of these leukemic cells. Indeed, the CD7 antigen is occasionally expressed on the cell surface of myeloblasts and thus is not a lineage-specific antigen indicating the T lineage. The CD33 antigen is a marker of primitive myeloid cells. Acute mixed-lineage leukemia, presumed to be derived from pluripotent hematopoietic cells, frequently expresses CD33 with concomitant expression of lymphocyte-associated antigens. Therefore, to analyze the molecular events during the process of early hematopoietic differentiation, acute leukemias solely expressing the CD7 or CD33 antigen were not excluded from the present study unless other lineage-associated markers were expressed.

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It is of interest that a high incidence of Ig and TCR gene rearrangements was observed in six patients in whom both the Ig-H and TCR-δ genes displayed rearrangements. In addition, these patients frequently showed rearrangements of the TCR-α, γ, and β genes, and none had only Ig nor TCR gene rearrangements. These findings strongly suggest that AUL with rearrangements of the Ig and TCR genes may represent cells that entered a pathway of differentiation along the lymphoid lineage. Indeed, the TdT gene, which is functionally associated with gene rearrangement, was expressed in all three examined patients, as will be discussed later. Thus the machinery-mediating gene rearrangements seemed to be highly activated in cells from those patients and
### Table 4. Summary of Southern and Northern Blot Analyses

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<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>0/12 - - + - -</td>
<td>CD3-γ</td>
</tr>
<tr>
<td>11</td>
<td>G</td>
<td>ND</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>0/10 - - - + + (1.0)</td>
<td>CD3-γ</td>
</tr>
</tbody>
</table>

**Abbreviations**: G, germline; R, rearranged; D, deleted; +, expression was detected; -, expression was undetected; ND, not done.

*Rearranged alleles.
†Examined alleles.

Not T- or B-lineage restricted. A high frequency of gene rearrangements in TCR gene loci has been demonstrated in B-precuror ALL. The frequency of TCR-δ and α gene rearrangements are particularly high in B-precuror ALL and approximately 90% of the tested B-precuror ALL showed rearrangements of the TCR-δ or α genes. Because Ig and TCR gene rearrangements seem to be mediated by common recombinases and the recombination system may be activated in B-cell precursors where recombination of VH to DJH and Vk to Jx genes occurs, leukemic cells from B-precuror ALL may display a high incidence of inappropriate gene rearrangements. In a similar way, it seems possible that common recombinases may be highly activated in very immature cells where the initial recombination events in the Ig-δ or TCR genes are initiated. Therefore leukemic cells arrested at an early differentiation stage corresponding to such immature lymphoid cells might undergo rearrangements of both the Ig and TCR genes not T- or B-lineage restricted.

**Fig 4.** Transcription of the MPO, TdT, and Ig-μ genes. Amounts and quality of loaded RNA samples were verified by ethidium bromide staining of gels prior to transfer (lower panel). C denotes positive controls (appropriate leukemic samples). The patient numbers are noted above each lane. Sizes of transcripts are indicated at the left of the blots.
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Fig 5. Transcription of the TCR-γ, β, and CD3-γ genes. Amounts and quality of loaded RNA samples were verified by ethidium bromide staining of gels prior to transfer (lower panel). C denotes positive controls (appropriate leukemic samples). The patient numbers are noted above each lane. Sizes of transcripts are indicated at the left of the blots.

during the development of leukemia. One or a few clones might subsequently become dominant in a similar way, as was recently shown for follicular lymphomas of B-cell origin.60

On the other hand, five patients showed no rearrangements of either the Ig or TCR gene. It is possible that these five patients were heterogeneous and consisted of leukemias such as acute leukemias derived from pluripotent stem cells, progenitor cells committed to the lymphoid lineage where the recombinational system was still inactive, and cells minimally differentiated along the myeloid lineage. This subgroup may also include acute leukemia whose leukemic cells show features of minimally differentiated megakaryocytes, ie, ultrastructural platelet peroxidase and platelet-associated glycoproteins.18 However, patients with myelofibrosis, low percentages of blasts, or Down's syndrome, which are common clinical features of megakaryocytic leukemia19 were excluded from this study, and all three tested patients showed no reactivity with PLT1 (CD41w), which presumably recognizes glycoproteins IIb/IIIa.41,42 Thus the frequency of minimally differentiated megakaryocytic leukemia in this subgroup seemed to be low.

With respect to the correlation between the ages of patients and gene rearrangements, the ages of patients without gene rearrangements (patients 7 through 11, median 1 year) were lower than those of patients with gene rearrangements (patients 1 through 6, median 7.5 years). Felix et al43 observed similar findings in B-precursor ALL that the leukemic cells from infants rarely displayed rearrangements of the TCR-γ or β gene and the Ig-H gene showed the germline configuration in some of them, in contrast to B-precursor ALL in children. Although phenotypes of our patients were different from those of the patients in their report, it is likely that acute leukemias in younger children might represent an earlier developmental stage of hematopoietic cells.

For further understanding of the cell origin of our patients, northern analyses were performed for eight patients. As a marker of myeloid differentiation, MPO gene expression was analyzed, but MPO transcripts could not be detected in any of the analyzed patients. MPO transcripts were reported to be detectable in cells with ultrastructural MPO.44 Thus all the tested patients, including four patients without rearrangement of the Ig or TCR gene, failed to yield evidence of myeloid differentiation.

We analyzed expression of the TdT gene in five patients. Among them, three patients (patients 1, 3, and 5) with rearrangements of the Ig and TCR genes had TdT transcripts. In contrast, two patients (patients 10 and 11) with no rearrangement of the Ig or TCR gene did not express the TdT gene. Consistent with previous reports33,39 these findings suggest the relationship between the presence of TdT and the high frequency of Ig and TCR gene rearrangements and also support the notion of lymphoid lineage commitment of the cells from patients 1, 3, and 5, as mentioned above.

Transcription of the CD3-δ, ε, γ genes has been considered to be one of the earliest T-lineage cell-specific markers, and it has been observed that leukemic cells solely expressing the CD7 antigen also contain CD3-δ, ε, and γ transcripts despite having the germline configuration of the TCR genes.27,45,46
For these reasons RNA analysis of the CD3-γ gene was performed in this study. Among the six patients analyzed, CD3-γ transcripts were clearly detected in three patients (patients 1, 10, and 11). Patient 1 demonstrated rearrangements of the Ig and TCR-δ, γ, α, and β genes, as well as full-length TCR-β and γ transcripts. Taken together, these findings indicate that the leukemic cells from this patient may have been committed to the T-cell lineage. In spite of expression of the CD3-γ gene, patients 10 and 11 showed no rearrangements of the TCR genes or the Ig genes. Of particular interest is that leukemic cells from patient 11 did not express the CD7 antigen but did express the CD33 antigen on the cell surface. The CD33 antigen is presumed to be one of the earliest markers indicating myeloid differentiation.

To our knowledge this is the first report of a patient lacking T-cell–associated antigens who showed CD3-γ transcripts together with the expression of a myeloid-associated antigen. Leukemic cells from patient 11 showed characteristics of both T cells and myeloid cells, and thus these cells seem to be derived from bipotential hematopoietic cells that can differentiate along both lineages. Alternatively, these cells had been committed to the T-cell lineage, and the CD33 antigen was aberrantly expressed. Thus, taken together with recent findings by Cross et al, the finding in patient 11 suggests the possibility that CD3-γ transcripts may not be restricted to T-lineage cells. Irrespective of expression of the CD7 antigen, it is therefore still obscure whether the leukemic cells from patient 10 were derived from progenitor cells committed to the T-cell lineage.

Finally, expression of the Ig-μ, TCR-γ and β genes was examined using constant-region or full-length cDNA probes. Low levels of Ig-μ gene expression were detected in three patients (patients 3, 4, and 10). The sizes of Ig-μ transcripts resolved from leukemic cells did not match the size of mature Ig-μ transcripts containing Vμ transcripts. Except for patient 1, neither TCR-γ nor full-length TCR-β transcripts were detected, and only low levels of truncated TCR-β transcripts were demonstrated in two patients (patients 3 and 11). Most of such truncated transcripts have been reported to be heterogeneous germline transcripts consisting of several species derived from the constant region. It seems that germline transcription may be a reflection of an activated state of the Ig and TCR genes common to cells in the early stages of T, B, and myeloid development, and truncated transcripts may not indicate commitment to a specific lineage.

In the present study we demonstrated heterogeneity of AUL at the DNA and RNA levels. Overall, AUL could be divided into two major subgroups: one with rearrangements of both the Ig-H and TCR-γ genes frequently associated with Ig-κ, TCR-γ, α, and β gene rearrangements, and the other with the Ig and TCR genes in the germline configurations. A high frequency of rearrangements in the Ig and TCR gene loci appears to indicate commitment to the lymphoid lineage of leukemic cells from the patients classified in the first subgroup. In all four analyzed patients belonging to the second subgroup as well as the first subgroup, we did not detect transcripts of the MPO gene, thus providing no evidence of myeloid differentiation.

The majority of the studied patients had poor prognostic features (ie, an age <2 or >10 years, a high WBC count, and a FAB classification of L2). Although they were intensively treated with a protocol for high-risk ALL, including prednisolone, vincristine, L-asparaginase, adriamycin, and cyclophosphamide, only two patients remain in complete remission. Thus prognosis of patients belonging to either subgroup appears to be extremely poor, and specific treatment strategies are required.

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REFERENCES


Molecular analysis of acute undifferentiated leukemia: two distinct subgroups at the DNA and RNA levels

J Hara, K Yumura-Yagi, A Tawa, S Ishihara, M Murata, N Terada, Y Izumi, E Champagne, Y Takiara and TW Mak