# HIGH FREQUENCY OF CLONAL IMMUNOGLOBULIN OR T CELL RECEPTOR GENE REARRANGEMENTS IN ACUTE MYELOGENOUS LEUKEMIA EXPRESSING TERMINAL DEOXYRIBONUCLEOTIDYLTRANSFERASE

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The leukemias afford a unique opportunity to study the differentiation pathways of various hematopoietic lineages because neoplastic cells can be considered clonal counterparts of normal cells frozen at various stages of maturation. Monoclonal antibodies directed against a variety of differentiation-associated antigens have provided the means to identify specific molecules that reflect characteristic if not unique, phenotypic patterns in the various lymphoid and nonlymphoid lineages (1–4). More recently, there has been considerable progress in delineating certain early differentiation events in the lymphoid lineage through an analysis of the clonal somatic rearrangements of immunoglobulin and T-cell receptor genes, which provide irreversible markers of lineage, clonality, and stage of differentiation (5–12). However, insufficient information has been accumulated about the critical early events of hematopoietic differentiation, i.e., the mechanisms that determine the commitment of a cell to a particular lineage.

In this context, although neoplastic phenotypes are not always precise replicas of normal ones, lineage specificity is, in general, faithfully conserved in leukemias and lymphomas, as shown by conservation of lineage-specific cell surface markers in both myeloid and lymphoid leukemias (4, 13) and by the presence of B or T cell–specific gene rearrangements in lymphoid leukemias and lymphomas (5–12). However, a simple theory for lineage commitment stating the irreversible entrance of cells into one of the differentiation lineages is questioned by findings of apparently anomalous expression of myeloid or lymphoid markers outside of their respective lineages (13). These phenomena, which include the presence of

nuclear terminal deoxyribonucleotidyltransferase (TdT)[1] in leukemic myeloblasts (14–16), and the inappropriate rearrangement of an immunoglobulin (Ig) or T cell receptor (TCR) gene in lymphoid or myeloid lineage cells (17–20), have been collectively referred to as "lineage infidelity" (21). This concept, however, implies a somewhat anomalous or pathologic nature of these events, while it remains entirely possible that they reflect normal stages of differentiation that are frozen or selected by the leukemic transformation. Reflecting this alternative explanation and the unresolved nature of the controversy, "lineage infidelity" has been recently renamed "lineage promiscuity" to symbolize the possible physiologic rather than pathologic nature of these events (13).

A potentially informative approach to this problem is to determine whether the unfaithful or promiscuous expression of a given marker represents the isolated, sporadic activation of a single gene or is part of a more complex differentiation program involving the coordinated expression of multiple genes and their products. Of considerable interest in this respect are leukemia cases that consistently display anomalous markers and in which the original lineage derivation can be studied by analyzing Ig or $T_\beta$ gene rearrangements as irreversible immunogenotypic footprints of previous "commitments." Such an opportunity is afforded by the subgroup of acute myelogenous leukemias (AML) accounting for approximately one-tenth of AMLs, which are characterized by myelomonocytic cytochemistries and cell surface antigens; by the concomitant expression of TdT, a lymphoid lineage–associated marker; and by poor prognosis (14–16). Because the nuclear enzyme TdT has been postulated to play a role in the generation of Ig and T cell receptor diversity (22, 23), we investigated whether, in myeloid leukemias, these two uncommon events, gene rearrangement and TdT expression, might be associated and represent activation, albeit abortive or transient, of a program of lymphoid initiation expressed before irreversible commitment.

Our results demonstrate that TdT expression and Ig and $T_\beta$ gene rearrangement are strongly associated. Rearrangements were observed in >60% of the $TdT^+$ AMLs and in all of the $TdT^+$ undifferentiated leukemias we studied. The implications of these findings for both normal and neoplastic cell differentiation and for the relationship between TdT function and Ig and $T_\beta$ gene rearrangement are discussed.

## Materials and Methods

*Specimens.* Representative samples of peripheral blood were collected during the course of standard diagnostic procedures. The diagnosis of each myeloid leukemia was established by conventional (FAB) diagnostic criteria (24). Specifically, Jenner-Giemsa, Sudan black B (SBB), myeloperoxidase (MP), periodic acid–Schiff (PAS), acetyl-butyrate esterase (ABE), and α-napthyl acetate esterase (ANAE) were performed, and the leukemias classified as myeloid were scored as positive with SBB/MP and/or the esterases and were scored as negative with PAS. The leukemias considered to be undifferentiated failed to react with any of these reagents. A mononuclear cell suspension of >95% viability was prepared from each specimen by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and where necessary, Percoll (Pharmacia Fine Chemicals) density gradient centrifuga-

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[1] *Abbreviations used in this paper:* ABE, acetyl-butyrate esterase; AML, acute myelogenous leukemia; MP, myeloperoxidase; PAS, periodic acid–Schiff; SBB, Sudan black B; TdT, terminal deoxyribonucleotidyltransferase.

tion. The vast majority, >85%, of the mononuclear cells isolated from each specimen were cytomorphologically neoplastic.

*Cell Marker Analysis.* The presence of cell surface Ig (sIg), cytoplasmic heavy chains (Cμ), sheep erythrocyte (E) rosette formation, HLA-DR antigens, and TdT were determined as previously described (14, 25). The expression of myeloid and lymphoid differentiation antigens was determined by indirect immunofluorescence and flow cytometry using a panel of mAB (OKT3 [CD3], OKT4 [CD4], OKT6 [CD1], OKT8 [CD8], OKT11 [CD2], Leu-1 [CD5], B1 [CD20], B2 [CD21], B4 [CD19], BA-1 [CD24], BA-2 [CD-9], anti-CALLA [CD10], Leu-9/3Al [CD7], Mo-1 [CD11], MoU26, MY9 [gp67], MoU48/My7 [CD13], Leu-M3 [CD14]) the characteristics and reactivity of which have been previously determined (1, 2, 3). A cell population was considered positive if >25% of the neoplastic cells reacted with the mAB.

*DNA Extraction and Southern Blot Analysis.* DNA was prepared by cell lysis, proteinase K digestion, extraction with phenol, and precipitation with ethanol (26). 15 μg of DNA were digested with Eco RI or Bam HI restriction endonucleases, electrophoresed in a 0.8% agarose gel, denatured, neutralized, transferred to a nitrocellulose filter, and hybridized according to Southern (27). Filters were washed in 0.2× standard sodium citrate, 0.5% SDS, pH 7, at 60° for 2 h (28).

*DNA Probes.* The $T_\beta$ probe used in this study was derived from a human cDNA $T_\beta$ clone (YTJ-2) isolated from the Jurkat-2 T lymphoma cell line (a gift from Dr. T. Mak, Ontario Cancer Institute, Toronto, Canada). This clone hybridizes to both alleles ($C\beta1$ and $C\beta2$) of the constant ($T_\beta C$) region, and to one or more alleles of the variable (V) regions (10, 29). To generate a probe representative for the $T_\beta C$ region, the insert of the YTJ-2 plasmid was digested with Hinc II restriction endonuclease, and the 0.8 kilobase (kb) $T_\beta$ C-specific fragment was purified by preparative agarose gel electrophoresis. The Hinc II restriction separated the V from the C regions by interrupting the joining (J) region (8). The probe for the Ig locus, representative of the J region ($J_H$) of the heavy chain (IgH) locus, was obtained from Dr. Stanley Korsmeyer (National Institutes of Health, Bethesda, MD) and has already been described (5, 6). DNA fragments were ^{32}P-labelled by nick translation for use as probes (27).

# Results

*TdT⁻ AML Display Infrequent $T_\beta$ and $Ig_H$ Gene Rearrangements.* We investigated the occurrence of $T_\beta$ and Ig rearrangements in a panel of 25 leukemias defined as classical TdT⁻ AML (FAB M1–M5) on the basis of morphology and cytoenzymatic analysis. Cell surface antigens other than those characteristic of myeloid differentiation were not detected in any of these leukemic samples. In particular, the CD7 antigen was not expressed by any of these leukemias.

The genomic organization of the $T_\beta$ and $Ig_H$ loci were studied in these AMLs by Southern blot hybridization using the $T_\beta C$ probe and $J_H$ probe respectively. Based on previous observations, Eco RI and Bam HI digestion were used to identify DNA rearrangements of the $T_\beta$ locus (10), and Eco RI digestion was used to identify the rearrangements of the $Ig_H$ locus (6). The results are summarized in Table I. 23 of the 25 classical TdT⁻ AMLs did not exhibit rearrangement of either the $T_\beta$ or the $Ig_H$ locus. One FAB-M2 leukemia displayed rearrangement at the $T_\beta$ locus, and one FAB-M3 leukemia displayed rearrangement at the $Ig_H$ locus.

*TdT Expression Identifies a Subset of AML with a High Frequency of Clonal Rearrangements of $Ig_H$ or $T_\beta$ Loci.* We investigated the occurrence of $T_\beta$ and $Ig_H$ rearrangements in a panel of 13 acute leukemias defined as TdT⁺ AMLs. All 13 cases had in common the diagnostic features of AML, including positive cytochemical staining for myeloperoxidase and the expression of myeloid lineage–

TABLE I

A Summary of Immunogenotypic Analysis of TdT⁻ Myeloid Leukemias

| FAB classification | n | Cases showing rearrangements: | |
| --- | --- | --- | --- |
| | | IgH locus | T$_\beta$ locus |
| M1 | 4 | 0 | 0 |
| M2 | 8 | 0 | 1 |
| M3 | 9 | 1 | 0 |
| M4 | 2 | 0 | 0 |
| M5 | 2 | 0 | 0 |

TABLE II

TdT⁺ Myeloid Leukemias: Diagnostic, Immunophenotypic, and Immunogenotypic Features

| Case | FAB | Myeloid | | B lymphoid | | T lymphoid* | | TdT | IgH‡ | T$_\beta$C§ |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | Early U26 MY9 | Late CD13/ CD14 | Early CD19/ CD10 | Late C$\mu$ | Early CD7‡ | Late T3, T4, T8 | | | |
| 1 | M1 | + | +/ND | +/+ǁ | − | − | − | + | R | G |
| 2 | M1 | + | +/ND | +/+ǁ | − | − | − | + | R | G |
| 3 | M1 | + | +/ND | −/+ǁ | − | + | − | + | G | R |
| 4 | M1 | + | +/− | −/− | − | − | − | + | R | R |
| 5 | M1 | + | +/− | −/− | − | + | − | + | G | G |
| 6 | M2 | + | +/− | −/− | − | + | − | + | G | R |
| 7 | M2 | + | +/+ | −/− | − | + | − | + | G | R |
| 8 | M2 | + | +/− | −/− | − | + | − | + | G | R |
| 9 | M2 | + | +/+ | −/− | − | − | − | + | G | G |
| 10 | M2 | + | +/− | −/− | − | + | − | + | G | G |
| 11 | M2 | + | +/− | −/− | − | + | − | + | G | G |
| 12 | M2 | + | +/− | −/− | − | + | − | + | G | G |
| 13 | M4 | + | +/+ | −/− | − | + | − | + | R | G |

\* T1, T6, and T11 are negative in all cases.
‡ In all CD7⁺ cases, double immunofluorescent staining demonstrated coexpression of CD13 and CD7 antigens.
§ R, rearranged; G, germline.
ǁ Double immunofluorescent staining demonstrated coexpression of CD13 and CD10 in cases 1, 2, and 3.

restricted cell-surface antigens, but in addition, expressed intranuclear TdT positivity (Table II). In the absence of TdT expression, all 13 cases would fit the conventional diagnostic criteria of FAB M1–M4 leukemias (24). Coexpression of the 3A1 (CD7) antigen and myeloid antigens was noted in 9 of the 13 AMLs. CALLA (CD10) antigen was coexpressed with myeloid antigens in 3 of the 13 AML cases.

We studied the panel of 13 DNAs extracted from these leukemias by Southern blot hybridization using T$_\beta$ and J$_H$ probes and the appropriate restriction enzymes as described above. The complete analysis of eight representative cases is illustrated in Fig. 1, and data relative to analysis of the remaining cases are summarized in Table II. In total, 8 of the 13 DNAs analyzed displayed detectable rearrangements of the Ig$_H$ and/or the T$_\beta$ loci. 4 of the 13 cases displayed
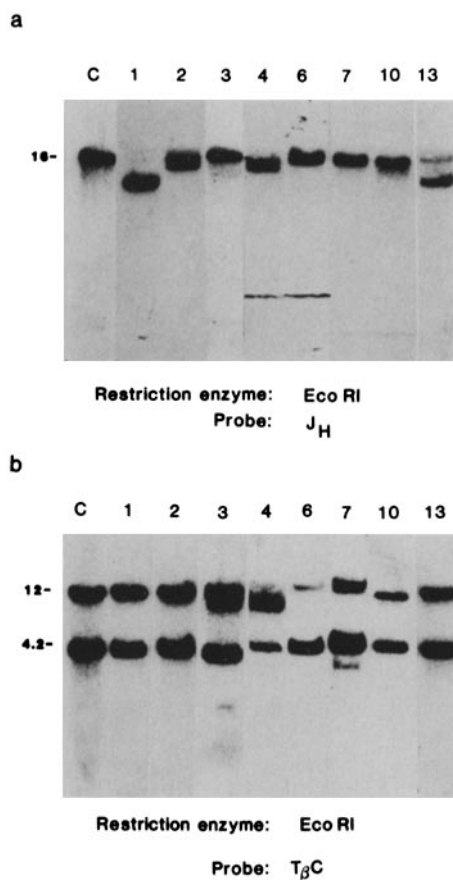
a



Restriction enzyme: Eco RI
Probe: $J_H$

b



Restriction enzyme: Eco RI
Probe: $T_\beta C$

FIGURE 1. Ig and $T_\beta$ rearrangements in TdT$^+$ AML. Eco RI–digested DNA from normal human fibroblasts (C) and from leukemic blasts (lanes 1–4, 6, 7, 10, 13 correspond to DNA from cases so numbered in Table II) were analyzed for gene rearrangements using the $J_H$ probe (a) or the $T_\beta$ probe (b).

rearrangements at the Ig$_H$ locus, and 5 of the 13 displayed $T_\beta$ rearrangements. Case number three displayed rearrangements at both loci.

With respect to other lymphoid cell surface markers, we noted that all three FAB-M1-TdT$^+$ leukemias expressing the CALLA (CD10) antigen also displayed Ig$_H$ or $T_\beta$ gene rearrangements (Ig$_H$ locus in two cases and $T_\beta$ locus in one case), while five of the nine TdT$^+$ AMLs expressing the CD7 antigen also displayed rearrangements.

*TdT$^+$ Undifferentiated Leukemias Display Rearrangements at Both Ig$_H$ and $T_\beta$ Loci.* Using the scheme of restriction enzyme digestion and probes described above, we studied DNAs from four cases of undifferentiated acute leukemia that were unclassifiable by conventional morphologic and cytoenzymatic criteria (Table III). Here cases were PAS$^-$, MP$^-$, and ABE$^-$. The B4(CD19) antigen was detectable in case 1, and the MY9, CD13, and CD10 (CALLA) antigens were detectable in case 3. The results of Southern blot hybridization are shown in Fig. 2 and summarized in Table III. Rearrangements at both the $T_\beta$ locus and the Ig$_H$ locus were clearly detected in all four cases.

TABLE III

Undifferentiated Acute Leukemias: Immunophenotypic and Immunogenotypic Analysis

| Case | FAB | Myeloid | | B lymphoid | | T lymphoid | | TdT | IgH* | T$_\beta$* |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | Early U26 MY9 | Late CD13 | Early CD19/ CD10 | Late C$\mu$ | Early CD7 | Late T3, T4, T8 | | | |
| 1 | U‡ | − | − | +/− | − | − | − | + | R | R |
| 2 | U | − | − | −/− | − | − | − | + | R | R |
| 3 | U | + | + | −/+ | − | − | − | + | R | R |
| 4 | U | − | − | −/− | − | − | − | + | R | R |

* R, rearranged; G, germline.
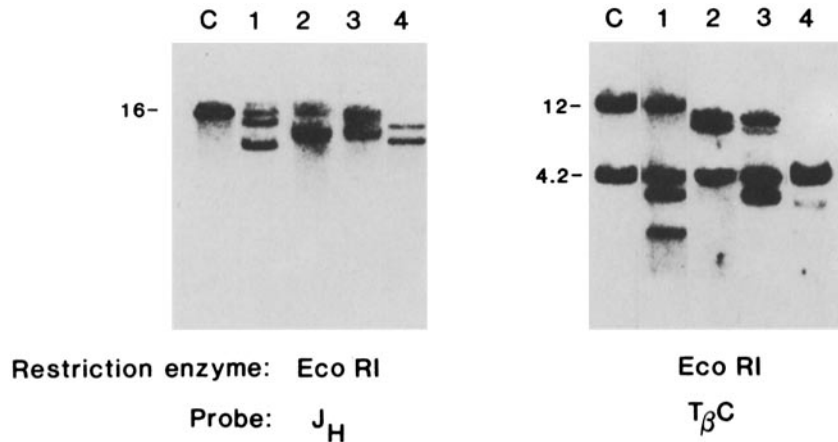‡ U, morphologically and cytoenzymatically undifferentiated, and therefore unclassifiable by FAB criteria.



FIGURE 2. Ig and T$_\beta$ gene rearrangements in TdT$^+$ undifferentiated acute leukemias. DNA from normal human fibroblasts (C) and from tumor cells (lanes 1–4 correspond to DNA from leukemias summarized in Table III) were analyzed for Ig$_H$ and T$_\beta$ gene rearrangement using the indicated restriction enzymes and probes.

## Discussion

The central observation of this study is the high frequency (>60%) of Ig and/or T$_\beta$ gene rearrangements in a subset of AML selected solely on the basis of their expression of the lymphoid marker TdT. Excepting TdT expression, the phenotype of the blast cells from all of these patients unequivocally fulfilled the morphological and cytochemical criteria of AML by the FAB classification and was in most respects analogous to TdT$^-$ AML, wherein Ig/T$_\beta$ gene rearrangements were found at a consistently lower frequency (<8%). These findings have important implications for understanding: (a) the role of TdT in gene rearrangements; (b) the relationship between lymphoid and myeloid lineages; and (c) the use of phenotypic and genotypic markers in the classification of leukemias.

*Relationship between TdT Expression and Ig or T$_\beta$ Gene Rearrangements.* The close association between TdT expression and Ig/T$_\beta$ gene rearrangements detectable in TdT$^+$ AML further suggests a role for TdT in the mechanism leading to the assembly of diverse Ig or T$_\beta$ genes (22, 23). TdT is a nuclear enzyme that

catalyzes the non–template-directed addition of deoxynucleoside triphosphates (31). Based on this biochemical function and on its presence in lymphoid precursors (32), it has been postulated that TdT may be responsible for the insertion of extra, random nucleotides (N regions) at the site of V-D-J recombinations during both Ig and $T_\beta$ gene rearrangements (22, 23), therefore changing the coding potential of these genes and contributing to the generation of antibody and T cell receptor diversity (N diversity) (23). The strong correlation between TdT expression and the presence of Ig or $T_\beta$ gene rearrangements in myeloid cells provides further circumstantial evidence for this hypothesis by showing that even outside the normal context of lymphoid differentiation, TdT expression and $Ig/T_\beta$ rearrangement act as coordinately controlled functions, most likely as parts of a functionally integrated program such as the assembly of Ig or $T_\beta$ genes.

*Implications for Lineage Commitment.* One important concept emerging from this study is that TdT expression in leukemic myeloblasts does not represent an isolated and bizarre episode of lineage infidelity but is rather part of a program of differentiation that involves TdT, Ig or $T_\beta$ gene rearrangement, and most likely CD7 gene expression. A critical question here is whether the activation of this program represents a pathologic phenomenon occurring in myeloid leukemias or is part of the normal differentiative repertoire of myeloid cells. One model that would be consistent with the former hypothesis is that leukemic transformation involves the structural or functional alteration of a regulatory gene that controls the activation of the TdT/CD7/gene rearrangement program. This hypothesis is difficult to test at present, because no evident and consistent genetic alteration such as, for example, a specific chromosomal translocation has been detected in $TdT^+$ AML.

Alternatively, these leukemias may represent a clonal expansion of a normal hematopoietic precursor the number of which is relatively low in normal hematopoiesis and becomes detectable only in the case of leukemic transformation. On this basis, at least two models for early differentiation events can be proposed. First, $TdT^+$ AMLs may reflect the existence of a stage in hematopoietic differentiation wherein concomitant activity of many lineage-associated genes or programs occur before commitment to a given lineage actually takes place. Thus, the myeloid cytoenzymes and immunophenotype might reflect final lineage commitments, whereas the gene rearrangement remains as an irreversible footprint of early precommitment lineage events. The $TdT^+$ AMLs that do not display Ig or $T_\beta$ gene rearrangements may represent cells that entered a myeloid lineage commitment pathway after TdT and in some cases CD7 antigen expression, and before gene rearrangement. Second, these myeloid leukemias may reflect the existence of a differentiative stage that marks lymphoid lineage initiation or availability but not irreversible commitment. This stage could include activation of a genetic program including TdT expression, gene rearrangement, and expression of one or more early lymphoid lineage antigens (e.g., CD7 or CD10 antigens); however, the activation of this program would not preclude a subsequent switch to the myeloid lineage.

Regardless of the exact pathway involved, the program involving previous TdT expression and gene rearrangements is neither frequent nor necessary for myeloid maturation, because most normal and leukemic myeloid cells are not

known to express TdT or display $Ig/T_\beta$ gene rearrangements. If, however, the frequency of $TdT^+$ AML (10%) reflects the approximate frequency of normal myeloid elements carrying Ig or $T_\beta$ gene rearrangements, the existence of these elements could be proven directly by analyzing Ig or $T_\beta$ gene rearrangement in mature myeloid elements.

Finally, the observation that all four undifferentiated leukemias we studied displayed rearrangements at both the Ig and $T_\beta$ loci is statistically provocative, yet difficult to interpret. On a pure speculative basis we could propose that $TdT^+$ undifferentiated leukemias may represent cases where leukemic transformation has frozen the target cell in the differentiation "window" where rearrangements actually occur, allowing for the accumulation of rearrangements of both Ig and TCR due to the continuous activity of a common recombinase (33).

*Implications for Classification and Diagnosis of Leukemias and Lymphomas.*   It has been proposed that Southern blot analysis and demonstration of Ig and $T_\beta$ gene rearrangements represents the definitive method for the identification of clonal lymphoid populations. However, it is clear from the data presented here that rearrangements are not strictly lineage specific and, furthermore, can be consistently found in the context of a well-characterized and relatively frequent subgroup of nonlymphoid malignancy. In agreement with previous reports (14–16), the $TdT^+$ AML patients studied here made a poor-prognosis subgroup, with infrequent and nondurable remissions. Thus, the example of $TdT^+$ AML further underscores the importance of a combined immunophenotypic and immunogenotypic approach for the classification of all hematopoietic neoplasms (25). This approach may lead not only to the discovery of novel differentiation pathways but also to the identification of new clinically and prognostically relevant subtypes of leukemias and lymphomas.

## Summary

Ig and T cell receptor rearrangements have been used as irreversible markers of lineage and clonality in the study of B- and T-lymphoid populations. We have addressed the issue of lymphoid lineage specificity of these rearrangements by analyzing a panel of 25 $TdT^-$ acute myelogenous leukemias, 13 $TdT^+$ AMLs, and 4 $TdT^+$ undifferentiated leukemias. We report that while gene rearrangements represent extremely rare events in classical $TdT^-$ AML (<8%), rearrangements of either the Ig or $T_\beta$ locus or both were detectable in the majority of the $TdT^+$ AMLs (>60%), and rearrangements of both loci were detectable in all of the $TdT^+$ undifferentiated leukemias. These data demonstrate a significant association between TdT expression and Ig or $T_\beta$ gene rearrangements even outside the lymphoid lineage, further supporting a role for TdT in Ig and T cell receptor gene assembly. These data also indicate that a coordinated program of lymphoid gene expression involving TdT-CD7-expression and $Ig/T_\beta$ rearrangements can be activated before myeloid commitment. Whether the activation of this program represents a normal, albeit rare, event in early myelopoiesis or a transformation-related event present only in leukemic cells remains to be determined.

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