Changing Antigen Receptor Gene Rearrangements in a Case of Early Pre-B Cell Leukemia: Evidence for a Tumor Progenitor Cell With Stem Cell Features and Implications for Monitoring Residual Disease

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A case of acute lymphoblastic leukemia (ALL) was encountered in which the two clonal \( \gamma \)-T-cell receptor gene (TCR\( \gamma \)) rearrangements found in bone marrow (BM) samples at relapse both differed from the single clonal TCR\( \gamma \) rearrangement present in BM obtained at diagnosis 5 years previously. In contrast, two clonal Ig heavy chain genes (IgH) rearrangements present at relapse were identical to those present at diagnosis. Comparison of the DNA sequences of the relapse TCR\( \gamma \) rearrangements with that of the diagnostic TCR\( \gamma \) rearrangement indicated that they must have been generated de novo from TCR\( \gamma \) loci in germline configuration. By polymerase chain reaction using clonotypic N-region oligonucleotide primers (N-PCR), cells bearing the diagnosis or relapse TCR\( \gamma \) rearrangements were undetectable in the sample from the opposite time point. Two BM samples obtained at different times in clinical remission were both devoid of detectable residual tumor when analyzed by N-PCR, indicating a depth of remission of less than 1 tumor cell per 4 \( \times 10^6 \) BM mononuclear cells. The tumor cells expressed a primitive phenotype: T-cell antigen-negative, CALLA/CD10-negative, CD20-negative, CD19-positive, and positive for the myeloid marker My9. This case, which appears to represent a tumor arising from a progenitor cell with both early B-lineage and certain stem cell features, has implications for monitoring residual ALL and possibly also for treatment of the disease.

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analyzed and percent positive cells staining above the negative control was determined.

**Preparation of DNA.** DNA was purified from aspirates or cell pellets by lysis in sodium dodecyl sulfate (SDS)-containing buffer followed by proteinase K digestion and phenol extraction. 1

**Southern blotting.** The Southern analysis of BM DNA was performed by standard procedures as described. 1 Blots were hybridized with Jp or Ju probes that had been labeled with 32P by hexamer priming. 1

**PCR amplification of tumor-specific antigen receptor gene rearrangements.** The N-PCR procedure is outlined in Tycko et al. 2 The procedure consists of two applications of PCR. In the first step, TCRγ or IgH gene rearrangements present in diagnostic or relapse BM DNA are amplified using conserved V- and J-region primers and the PCR products are sequenced. In the second step, oligonucleotide primers are constructed to match the clonotypic junctional sequences (including N-region sequences) and used in a second application of PCR to assay for the tumor-specific gene rearrangements in BM samples. 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 specifications of the manufacturer. Each reaction contained 2 μg of starting DNA and 300 ng of each of the two oligonucleotide primers. Thermal cycling was as follows: denaturation for 1 minute at 94°C with an initial denaturation of 3.5 minutes; primer annealing at a temperature ranging from 55°C to 60°C and optimized for each set of oligonucleotide primers; chain elongation at 72°C for 1.5 minutes. The number of cycles was 30 in PCR runs in which a single set of primers was used and 40 (20 + 20) in runs in which two sets of nested primers were used sequentially. The sequences of the upstream Vy9 primers were: (outer primer) GGAATTCCAAAT-

**Analysis of PCR products.** PCR products were analyzed by electrophoresis on 1.3% to 1.5% agarose gels, followed by photographic documentation of ethidium bromide staining and, in the IgH PCR analysis, Southern transfer with blot hybridization to a DNA probe labeled with 32P. The probe consisted of gel-isolated PCR product generated from a relapse tumor sample using the consensus IgH primers. Blots were hybridized and washed at high stringency essentially as described and exposed from 2 to 48 hours on Kodak XAR5 film (Eastman Kodak, Rochester, NY).

**DNA sequencing.** The PCR products generated from diagnostic and relapse BM DNA using the Vy9/Jy and conserved Vy/Ju primer pairs were digested with restriction enzymes (EcoRI and Sal I for TCRγ products and Pst I and Sal I for the IgH products) and ligated into M13 sequencing vector. Multiple M13 clones were isolated and sequenced. Sequences that were found more than once in a set of clones were provisionally considered to represent clonal gene rearrangements. This finding was confirmed by the results of subsequent N-PCR using primers constructed to match these sequences.

**Materials.** Restriction endonucleases and M13 sequencing vectors were obtained from Bethesda Research Laboratories (BRL; Gaithersburg, MD). The Random Primers Labeling System (BRL) was used for generating 32P-labeled DNA probes. Sequencing reagents (Sequenase Kit) were obtained from United States Biochemical Corporation (USB; Cleveland, OH). Reagents for PCR (Gene Amp Kit) were obtained from Perkin-Elmer Cetus Corporation (Norwalk, CT).

**RESULTS**

**Southern blots of tumor DNA at diagnosis and relapse show changing TCRγ rearrangements.** To investigate antigen receptor gene rearrangements in the patient's tumor cells, DNA isolated from sequential BM aspirates spanning a 7-year clinical course was subjected to Southern blot analysis using TCRγ and IgH J-region probes. Five aspirates were analyzed: one obtained at diagnosis, two obtained at first relapse, one obtained at second relapse, and a third obtained 3 months before the patient's death.

When Southern blots of these DNAs were hybridized with the TCRγ J-segment probe, a single rearranged band detected in the sample obtained at diagnosis was absent from both the first and second relapse samples, which instead showed a rearranged band at a slightly lower position (Fig 2A). Although a single rearranged band was...
seen in relapse samples on Southern analysis, the greater intensity of this band relative to the germline band suggested that it might consist of two distinct but comigrating gene rearrangements derived from the two TCRγ alleles. This was in fact confirmed by subsequent DNA cloning and sequencing of TCRγ rearrangements in one of the relapse samples (see below).

The Southern blots used to prepare Fig 2A were then stripped and rehybridized with an IgH J-segment probe. The resulting autoradiogram showed two rearranged bands in similar positions in both the diagnostic and two relapse samples (Fig 2B). These rearrangements were not detected in the two remission samples.

Southern blot analyses for TCRβ gene rearrangements were also performed on each sample. No clonal rearrangements of this locus could be detected at any time during the clinical course (data not shown).

Amplification and sequence analysis of TCRγ rearrangements at diagnosis and relapse. The TCRγ rearrangements present in diagnostic and second relapse samples were first amplified by PCR using a downstream oligonucleotide primer specific for the conserved 3' end of the Jγ1 and Jγ2 segments and upstream primers specific for either the conserved 5' end of the Vγ1 cluster or for the 5' end of the Vγ9 region. DNA from the diagnostic sample amplified more intensely with the Vγ1/Jγ primer pair, while the second relapse sample amplified more intensely with the Vγ9/Jγ primer pair (not shown). This finding was consistent with the band locations of the TCRγ rearrangements on Southern analysis with HindIII digestion, which also suggested that the relapse samples might contain one or more Vγ9-Jγ rearrangements. The diagnostic Vγ1/Jγ PCR product and the second relapse sample Vγ9/Jγ PCR product were then cloned in M13 phage for DNA sequencing.

Four clones derived from the diagnostic sample were identical and showed Vγ4 joined to Jγ (Fig 1A). The junctional sequence showed small exonucleolytic deletions into the 3' end of Vγ4 and the 5' end of Jγ and an interposed G/C-rich stretch of random nucleotides (N-
insertion). The clones derived from the relapse sample all showed Vγ9 joined to Jy but fell into two groups with different junctional sequences (Fig 1A). The differences were seen both in the extent of deletions into Vγ9 and Jy and in the sequence of the N-insertion. This finding indicated that, as suspected from the relative band intensity, the single rearranged band seen on Southern analysis of the relapse samples actually consisted of two distinct but comigrating Vγ9-Jy rearrangements.

*N-PCR analysis of the BM samples.* We have previously reported a method for measuring minimal residual disease in patients with ALL in remission. This method uses PCR amplification of DNA within antigen receptor genes with clonotypic junctional, or “N-region,” primers. The procedure, which we refer to as N-PCR, has been shown to reliably detect as little as one tumor cell in $4 \times 10^5$ normal BM mononuclear cells.

To investigate the time of appearance of each of the TCRγ gene rearrangements and evaluate the depth of the remissions, N-PCR was performed on each of the DNA samples. Clonotypic N-region primers were constructed based on the sequences in Fig 1A. A clonotypic primer for the IgH rearrangement was constructed based on sequence determined from multiple M13 clones containing PCR product generated from the DNA of the diagnostic sample using consensus VH and JH primers (Fig 1B). N-PCR using the primer specific for the diagnostic TCRγ rearrangement gave a product of the anticipated size in the diagnostic sample but gave no detectable product either in the remission or in the relapse samples (Fig 3A). Conversely, N-PCR using a clonotypic primer specific for one of the relapse TCRγ rearrangements yielded a product in both of the relapse samples but gave no detectable product in either the remission of the diagnostic samples (Fig 3B). As expected, normal BM controls subjected to N-PCR did not yield detectable amplified product.

![Fig 3. Detection of tumor cells bearing clonal TCRγ and IgH rearrangements in serial BM samples by PCR with clonotypic primers. (A) PCR performed with the primer pair specific for the diagnostic TCRγ rearrangement. A specific product is present only in the diagnostic sample (ethidium-stained gel). (B) PCR performed with the primer pair specific for one of the relapse TCRγ rearrangements (allele 2 in Fig 1). Specific products are present only in the relapse and second remission samples. Serial dilutions of tumor DNA from a relapse sample shows that the sensitivity of detection extends to $10^{-5}$, corresponding to approximately two tumor cells (ethidium-stained gel).](image-url)
Consistent with the stability of the IgH rearrangements seen on Southern analysis, both the diagnostic and the relapse samples showed an amplified product when subjected to N-PCR using clonotypic primers specific for one of the relapse rearrangements (Fig 3C). As with the TCRγ primers, the two first remission samples again showed no amplified product. In contrast, residual tumor was easily detectable in the third remission sample (May 19, 1987). To allow quantitation of the amount of residual tumor in remission samples, serial dilutions of tumor DNA were examined in the same experiment. This experiment confirmed that the sensitivity of N-PCR extended to the single cell level (Fig 3C). The absence of detectable amplified product in the first remission samples, in which a total of $4 \times 10^7$ cell-equivalents of DNA was examined, established an upper limit for the amount of residual tumor during first remission of less than one in $4 \times 10^7$ BM mononuclear cells.

**Immunophenotype analysis.** Cells obtained by BM aspiration at diagnosis and at first and second relapse were subjected to flow cytometric analysis using a panel of MoAbs and polyclonal antibodies specific for B-cell, T-cell, and myeloid marker antigens (Fig 4). The tumor cells were negative for T-cell markers A99 (polyclonal hetero T-cell) and CD2, but positive for the early B-cell marker CD19. However, the cells were also negative for CALLA/CD10 and CD20, and positive for the myeloid antigen My9/CD33. With the exception of an increasing degree of My9/CD33 reactivity between first and second relapse, there appeared to be no significant change in the phenotype over the clinical course.

**DISCUSSION**

In the course of studies of minimal residual disease in patients with ALL we encountered a case with several interesting features. First, the patient had a prolonged clinical course, including a 5-year first remission. Second, the tumor cells showed a primitive phenotype, with both early pre-B and myeloid antigens. Third, the tumor cells showed changes in antigen receptor gene rearrangements between diagnosis and relapse. These changes were apparent on Southern blot analysis, which suggested that the clonal TCRγ rearrangement present in the leukemic cells at diagnosis was replaced by a different rearrangement at first relapse and that this rearrangement persisted in the tumor cells at second relapse. This conclusion was confirmed by sequence analysis of PCR products amplified from the diagnostic and first relapse samples. The full extent of this replacement was indicated by N-PCR, which showed that the initial TCRγ gene rearrangement could not be detected in any of $2 \times 10^7$ nucleated BM cells obtained during the first or second relapse. N-PCR also failed to detect one of the two relapse TCRγ gene rearrangements in the diagnostic sample at a comparable level of sensitivity.
Theoretically, changes in antigen receptor gene rearrangements could occur either in the context of a true relapse or, alternatively, in the context of a second primary malignancy. The finding of stable IgH rearrangements, by Southern blot analysis and confirmed by N-PCR, excludes the latter possibility and indicates that tumor cells were clonally related throughout the clinical course. This conclusion is also supported by the immunophenotypic studies, which showed a virtually unchanged antigenic profile for the leukemic cells over the course of the disease.

In view of the evidence associating this patient’s clinical course with a single neoplasm, two alternative mechanisms with somewhat different implications might account for the changing TCRγ rearrangements. The first possibility is replacement of a pre-existing Vγ-Jγ rearrangement by recombination of an upstream unrearranged Vγ segment with either the 3’ end of the rearranged Vγ or with an unrearranged Jγ segment lying downstream. This type of V-segment replacement has been described previously in studies of murine IgH rearrangements. The second possibility is the generation of the relapse rearrangements de novo from TCRγ loci in germline configuration. Theoretically, changes in antigen receptor gene rearrangements over time could be expected to result in the deletion of the DNA containing Vγ9. Consequently, neither of the relapse Vγ9-Jγ rearrangements could have been derived from the original Vγ4-Jγ rearrangement by V-region replacement. Rather, both relapse rearrangements must have been derived de novo from TCRγ loci in germline configuration. This finding, in turn, suggests that the tumor cells present at relapse arose as the clonal progeny of a tumor stem cell that had both TCRγ alleles in germline configuration.

The apparent stem cell features of the tumor progenitor cell in this case are consistent with the unusual immunophenotypic findings. These findings include presence of the early B-cell antigen CD19 in the absence of CALLA/CD10 and CD20—a phenotypic profile suggesting a pre-pre-B cell or early pre-B cell stage of differentiation. Furthermore, the tumor cells expressed the myeloid antigen My9/CD33. The combination of CALLA-negativity and myeloid antigen expression is somewhat unusual in childhood ALL and resembles phenotypes more often seen in infant null ALL, a neoplasm thought to derive from multipotent precursor cells. Similar evidence for an association between myeloid antigen expression and a stem cell etiology in ALL is provided by a recent study that described a higher incidence of coexisting B-lineage (IgH) and T-lineage (TCRβ) antigen receptor gene rearrangements in those cases that displayed myeloid antigen. Also, an apparently distinct subclass of acute myeloid leukemia (AML) has been documented in which the tumor cells express both myeloid antigens and the lymphoid marker terminal deoxynucleotidyl transferase (TdT) and in which clonal TCR and IgH gene rearrangements are usually present. Interestingly, adults with ALL appear to have a poorer prognosis if their tumor cells express myeloid antigens.

Development of lymphoid tumors from some type of stem cell may not be uncommon. Occasional cases of lymphoma and chronic lymphocytic leukemia show changing antigen receptor gene rearrangements over time. Furthermore, common ALLs often contain a multiplicity of...
IgH gene rearrangements differ from de novo V-D-J joinings, consistent with outgrowth of several subclones from a lymphocyte-committed stem cell.23 Indeed, it is conceivable that most cases of ALL actually originate from a transformed lymphoid stem cell, but that a dominant subclone consistently overgrows any competing subclone and consequently persists throughout the course of the disease. In the present case, the patient experienced a deep first remission (less than one leukemic cell in 400,000 total nucleated BM cells, as indicated by N-PCR of IgH genes in two separate bone marrow aspirates). These low levels of residual disease corresponded with reasonably prolonged remissions, especially between diagnosis and the first relapse, a correlation also noted in other cases of ALL that we have examined retrospectively.6 It is possible that the profound reduction of tumor cells during the first remission resulted in the eradication of the subclone containing the first TCRγ gene rearrangement while the transformed progenitor cell remained. Sometime during the first remission, this progenitor cell, which contained both TCRγ alleles in germline configuration but which continued to express recombinase activity, gave rise to a second subclone carrying new TCRγ rearrangements. The second remission was not as deep (reflected by the shorter remission) and the second subclone therefore survived through this remission to reemerge at the time of the subsequent relapse. This scenario would predict that the stem cell origin of certain tumors may be more likely to be shown in cases that relapse after more intensive therapy, greater cytoreduction, and possibly longer remissions.

The existence of tumors with certain stem cell features may have important implications for both the diagnosis and therapy of these neoplasms. With regard to diagnosis, the changes in antigen receptor gene rearrangements will obviously affect the ability to monitor residual disease by N-PCR or any related technique. In our experience with N-PCR primarily involving TCRγ genes, changes in gene rearrangements have been limited to the present case. In fact, stable TCRγ gene rearrangements have been noted in cases with numerous IgH rearrangements studied by Southern blotting. Nevertheless, it is clear from the present case that possible changes in gene rearrangements must be considered as a potential source of false-negative results in screening biopsy specimens for residual disease using these markers. Perhaps parallel analyses of remission samples for several antigen receptor gene rearrangements (eg, TCRγ and IgH) should be used for this reason.

Other implications of stem cell tumors pertain to therapy. Hematopoietic stem cells are thought to be relatively resistant to cytotoxic agents, as reflected in the rapid repopulation of the BM by normal elements after high-dose chemotherapy for leukemia. If tumors with stem cell features represent a distinct subgroup of lymphoid neoplasms, it may be that these tumors are the most likely to recur after conventional therapy and therefore require more intensive treatment to achieve a cure.

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