

CONTINUING REARRANGEMENT BUT ABSENCE OF
SOMATIC HYPERMUTATION IN IMMUNOGLOBULIN GENES
OF HUMAN B CELL PRECURSOR LEUKEMIA

BY JEFFREY BIRD, NAOMI GALILI, MICHAEL LINK,*
DANIEL STITES,[†] AND JEFFREY SKLAR

*From the Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305; the *Department of Pediatrics, Stanford University School of Medicine and the Division of Hematology/Oncology, Children's Hospital at Stanford, Palo Alto, California 94304; and the [†]Department of Laboratory Medicine, University of California, San Francisco, San Francisco, California 94143*

B-lineage lymphoid neoplasms, like other tumors, are generally assumed to represent monoclonal expansions from a single transformed parent cell. These neoplasms should therefore contain no more than two configurations of rearranged DNA for each Ig gene, that is, one rearrangement for each of two alleles (1, 2). However, exceptions to the latter rule appear to exist. Using hybridization probes for Ig DNA in Southern blot analyses, a number of B lymphoid tumors have been shown to contain more than two rearranged bands for each Ig gene (3, 5). One explanation for these findings is based on recent studies that have demonstrated that the Ig genes of some B cell neoplasms undergo extensive somatic hypermutation (6, 7). Occasionally these point mutations affect restriction enzyme cleavage sites in or around rearranged Ig genes, giving rise to additional nongermline bands in Southern blot analyses of Ig DNA (6-8). An alternative explanation for excess rearranged bands in B-lineage neoplasms is that 5' V regions may replace previously rearranged V regions at the VD joint, as noted in certain murine pre-B cell lines (9, 10). Still another explanation is that such tumors may arise from stem cells with unrearranged germline configurations of Ig genes that subsequently rearrange independently in clonal descendants of the original transformed cell. Finally, it could be that B-lineage neoplasms may occasionally develop from two or more separate cells.

In this report, we describe our analysis of these issues in human B-lineage acute lymphoblastic leukemia (ALL).¹ This leukemia, the most common cancer of children, is usually composed of neoplastic B cell precursors possessing rearranged Ig genes but lacking surface Ig (11, 12). Our investigation indicates that cases of B-lineage ALL show greater than two clonal, rearranged Ig heavy chain gene bands far more frequently than any other B-lineage neoplasm yet described. To determine the origin

This work was supported by grant CA-34233 from the National Institutes of Health. J. W. Bird is a trainee of the Program in Cancer Biology and was supported by U.S. Public Health Service grant number CA-09302, National Cancer Institute/Dept. of Health and Human Services. J. Sklar is the recipient of a Research Career Development Award from NIH.

¹ *Abbreviations used in this paper:* ALL, acute lymphoblastic leukemia; CALLA, common acute lymphoblastic leukemia.

of these extra bands we have molecularly cloned and sequenced Ig heavy chain DNA from two such cases containing three and seven rearranged bands, respectively. Our data indicate that different bands are attributable to unique recombinations of specific V, D, and J sequences and generally do not seem to represent replacement V region rearrangements. V region segments from two particular V_H region families predominated in these rearrangements. In addition, by comparing multiple copies of the same gene rearrangement from different cells within the tumors, we have found that somatic hypermutation within the Ig heavy chain gene is exceedingly rare in the B cell precursors that compose these neoplasms. These studies suggest that Ig heavy chain genes continue to rearrange within the neoplastic cells of some cases of B-lineage ALL and that in such cases these tumors may arise from B cell precursors at a stem cell stage of differentiation.

Materials and Methods

Leukemic Cells. Malignant cells were obtained from patients with ALL at the time of initial presentation or relapse after informed consent was obtained. Specimens were obtained from bone marrow aspirates as well as peripheral blood, and only specimens containing >80% malignant cells were used in this study. The diagnosis of ALL in each case was established by standard morphologic and histochemical parameters based upon examination of smears of peripheral blood and bone marrow aspirates. The B lineage of these leukemias was confirmed by immunophenotyping studies. Blast cells from 37 of 38 cases expressed HLA-DR antigens and all failed to express cell surface Ig or a variety of T cell antigens (CD 3, 4, 5, 7, 8). In general, blast cells were found to lack CD 20, but all expressed the B-lineage antigens CD 19 and 24; 20 of 23 cases tested expressed CD 10, the common acute lymphocytic leukemia antigen (CALLA) (13). Five of 12 cases examined expressed cytoplasmic μ heavy chains. Cells were cryopreserved at -196°C in 10% DMSO and 10% FCS before analysis.

Southern Blot Analysis and Library Construction. High molecular weight genomic DNA was isolated and analyzed using Southern blot techniques that have been described (2). Hybridization probes used included human J_H , C_κ , C_λ (2), J_κ (14), C_β (15), and J_γ (15, 16). DNA inserts for specific libraries were prepared from Eco RI-digested leukemic DNA electrophoresed in 0.8% agarose gels. DNA fragments for cloning were electroeluted from gel slices which measured 1 cm on each side of the desired band as determined by analytical Southern blot. Eco RI inserts of <11 kb were ligated into the chromosomal arms of phage vector λ 607 and larger inserts were ligated into λ Embl 3. After in vitro packaging of ligated DNAs (Gigapack; Stratagene Cloning Systems, La Jolla, CA), 10^6 recombinant phages of the unamplified library were plated on *Escherichia coli* LE392 or on the selective *E. coli* strain Hfl and 10 plates were screened by hybridization with the J_H region probe (2), according to the method of Benton and Davis (17) using nylon membranes. For each library a minimum of six independent phages that hybridized to the probe were plaque purified by three successive platings and DNA from these independent phage isolates were then subcloned for DNA sequencing.

DNA Sequencing. Eco RI inserts were removed from phage DNA using Eco RI plus Hind III and fragments that contained the J_H region by Southern blot analysis were isolated and further digested with Bst EII, which cleaves within each J_H region except J_4 . Fragment ends were blunted using T4 DNA polymerase and the rearranged fragment not corresponding to any Bst EII fragment from germline J_H region DNA was isolated and ligated into M13 mp18 phage DNA vectors. DNA sequences of both strands of the M13 inserts were determined by the method of Sanger et al. (18).

Identification of Clonal Origins of Isolated V Regions. DNA sequences upstream from the various V or D regions cloned in Eco RI libraries from the TD leukemia were used to probe genomic Southern blots of Hind III-digested DNA to correlate Eco RI and Hind III bands for each rearrangement. Hind III-Bst EII fragments from Eco RI inserts were blunted using T4 DNA polymerase, cloned into the Hinc II site of the plasmid pUC18 and amplified. Frag-

ments cut from the 5' noncoding region of Hind III-Bst EII fragments for each VDJ or DJ rearrangement were individually labeled, and used as probes on Southern blots of Hind III-digested human sperm DNA to identify nonrepetitive sequence probes that hybridized to one or a few germline fragments. These probes were then used to examine Hind III-digested DNA from the TD leukemia. Southern blots hybridized with specific 5' V region probes were later washed to remove the hybridization signal and then were rehybridized with the J_H probe to assign each V region to a rearrangement on the J_H-probed blot.

Results

Multiple Ig Heavy Chain Gene Rearrangement Patterns in ALL. Tissues from 38 cases of B-lineage ALL were analyzed for rearrangements of Ig heavy chain DNA. Genomic DNA isolated from bone marrow and blood samples were digested separately with Eco RI and Bam HI restriction enzymes, and also with Hind III restriction enzyme in 11 cases. Ig DNA bands were detected on Southern blot analysis of these digests by hybridization either with a 6.5-kb Bam HI-Hind III DNA fragment containing the entire human J_H region (2) or a 0.7-kb Bgl II-Hind III DNA fragment containing the human heavy chain enhancer region (4). 11 of 38 cases (29%) showed more than two rearranged Ig heavy chain bands in analyses with at least one enzyme. Of the remaining cases, 11 showed only one rearranged heavy chain band, 16 showed two rearranged bands, and nearly all samples contained considerably less intense bands corresponding to Ig heavy chain genes in the germline configuration. Those samples with more than two rearranged heavy chain bands showed no obvious association with the type of specimen (peripheral blood or bone marrow), time of biopsy (diagnosis or relapse), immunophenotypic features, or clinical course compared with all other samples analyzed.

To distinguish between somatic point mutation, separate VDJ joining events, and other possible explanations for finding more than two rearranged Ig heavy chain gene bands in these specimens, two patient samples (AW and TD) were selected for more intensive study, including Ig DNA cloning and nucleotide sequence analysis. The leukemic cells from patient AW were isolated from peripheral blood samples taken at relapse. Immunophenotypically, these cells expressed cytoplasmic μ heavy chain protein, characteristic of pre-B cells (19). Southern blot analysis showed three apparent rearranged Ig heavy chain bands (Fig. 1). The combination of germline hybridization patterns for DNA probed with C _{κ} (2) and a rearrangement detected by a J _{κ} probe (5, 14) suggested that a κ allele in this patient had aberrantly rearranged and subsequently deleted its C _{κ} gene region (20) in some portion of the tumor population. For the λ light chain analysis, the high molecular weight band present in Eco RI-digested DNA from AW but not in germline DNA from an unrelated individual was probably a frequently observed Eco RI polymorphism rather than a rearranged λ chain gene. Pst I-digested DNA from AW confirmed that the λ chain genes were in the germline configuration.

Cells from patient TD were taken from the bone marrow at the time of initial diagnosis. These cells lacked cytoplasmic μ heavy chain protein. Analyses of DNA from these cells showed four apparent Ig heavy chain gene rearrangements using Eco RI digestion, while analysis using Hind III detected a total of seven rearrangements. DNA probed with both C _{κ} and J _{κ} probes showed germline bands of decreased intensity compared with the bands detected in equal amounts of DNA from

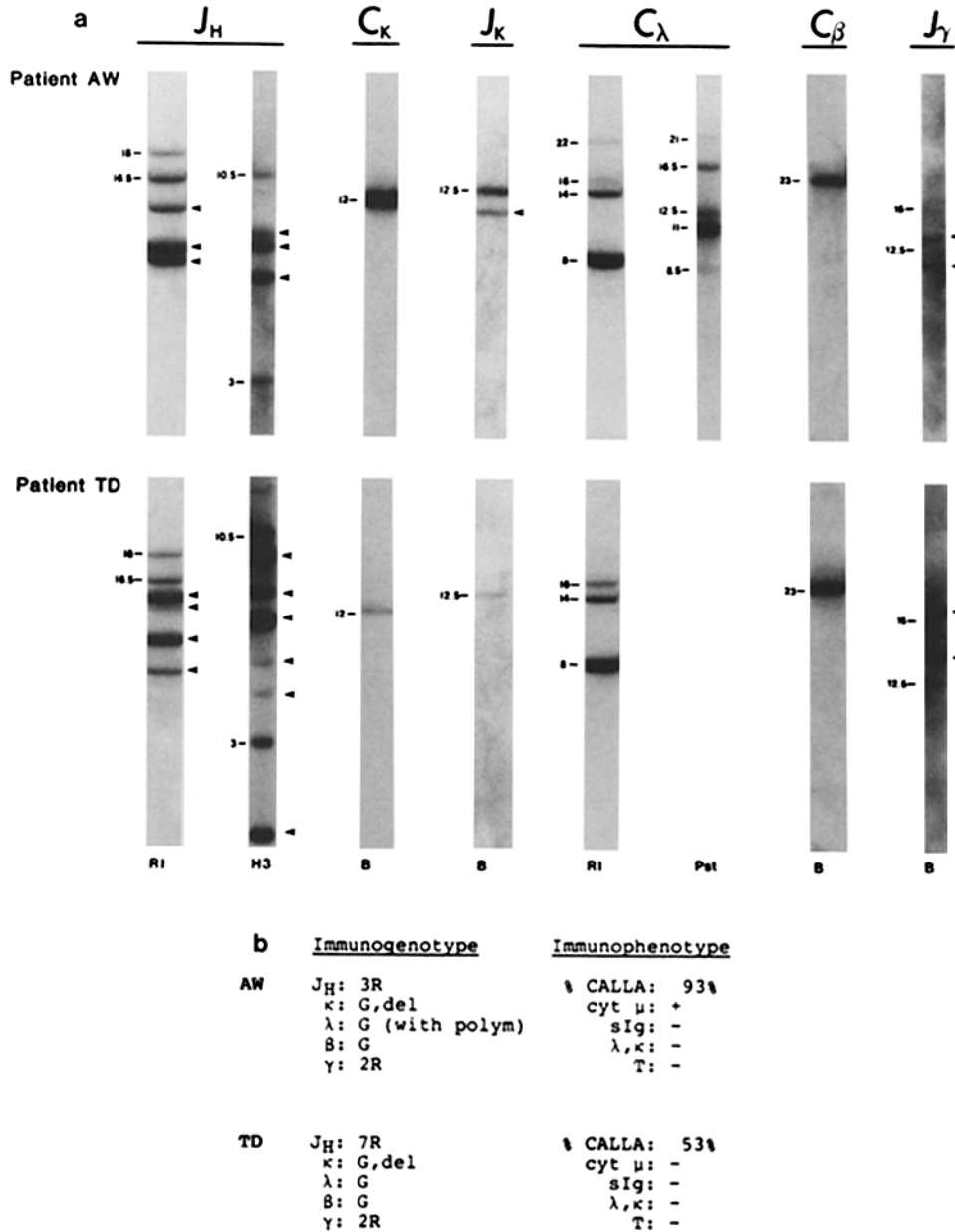


FIGURE 1. Southern blot analyses of leukemic DNAs from patients AW and TD. (a) Genomic DNAs were digested with the restriction enzymes indicated at the bottom of each lane; RI, Eco RI; H3, Hind III; B, Bam HI; and Pst, Pst I. Hybridization probes used for Southern blot analyses are indicated above the lanes. In all cases, germline DNA samples extracted from human sperm were digested and analyzed in adjacent lanes to verify the positions of unrearranged bands (data not shown). The sizes of unrearranged, germline bands are shown in kilobases. Arrows appear alongside clonally rearranged bands. (b) Summary of Southern blot and immunophenotype analysis. Calla, common ALL antigen (CD10); cyt μ = cytoplasmic μ heavy chain protein as indicated by immunophenotyping; sig = cell surface μ Ig; λ , κ , surface Ig light chains; T, T cell surface antigens; R, rearrangements.

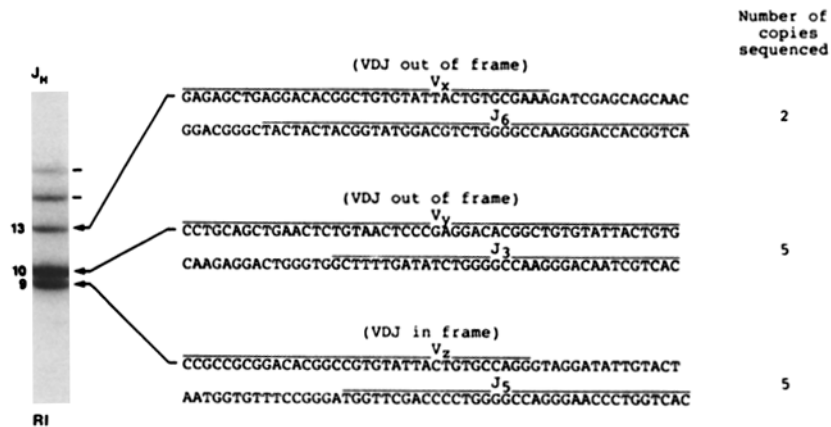


FIGURE 2. DNA sequences of VDJ rearrangements from AW leukemic DNA. At the left is shown a Southern blot autoradiogram of AW leukemic DNA digested with Eco RI restriction enzyme and hybridized with the J_H probe. The sizes of rearranged bands are given in kilobases. Sequences were cloned as two size-fractionated libraries (13 kb and 9-10 kb) from Eco RI-digested AW leukemic DNA. DNA sequences of the three different rearrangements from AW leukemic DNA are shown in the middle of the figure. The number of copies sequenced in each case, indicated at the right, denotes the number of individual clones from the library which yielded identical sequences. Different V region sequences are labeled x, y, and z and J region sequences are numbered based on previously identified sequences (21). Frameshift rearrangements were identified by translation of the DNA sequence in all three frames and alignment with known V and J region amino acid sequences.

other patient samples. This result could reflect deletion of κ alleles, or possibly the loss of chromosome 2 in some of the tumor cells. λ chain genes in the leukemic DNA of TD were in the germline configuration. DNAs from both patient samples contained germline β T cell receptor genes and two clonal rearrangements each for the γ T cell receptor genes.

Cloning of Ig Heavy Chain Gene Rearrangements. Size-selected genomic DNA libraries were made for Ig heavy chain gene rearrangements detected by Southern blot analysis using Eco RI-digested leukemic DNA and the J_H probe. DNA fragments corresponding to each rearranged J_H band were cloned in individual libraries, except for the two lower bands from AW and the two higher bands from TD, for which mixed libraries were made. Recombinant phage libraries were plated and screened using the J_H probe without prior amplification of the phage, so that multiple, unrelated isolates of Ig DNA representing the same rearrangement from different lymphocytes could be analyzed in most cases.

Restriction site analysis and subcloning of DNA representing the three heavy chain bands from AW and the two lower bands from TD were straightforward, yielding homogeneously sized DNA fragments for each band. 13 independently isolated phages were analyzed from the mixed library representing the higher two Eco RI bands of TD. Combined Eco RI plus Hind III digestion of insert DNAs from this library revealed three rather than the two expected size classes of insert DNAs hybridizing to the J_H probe. Further restriction analysis with Bst EII endonuclease, which cleaves within each J_H region except J_4 and liberates a 5' rearrangement-specific fragment,

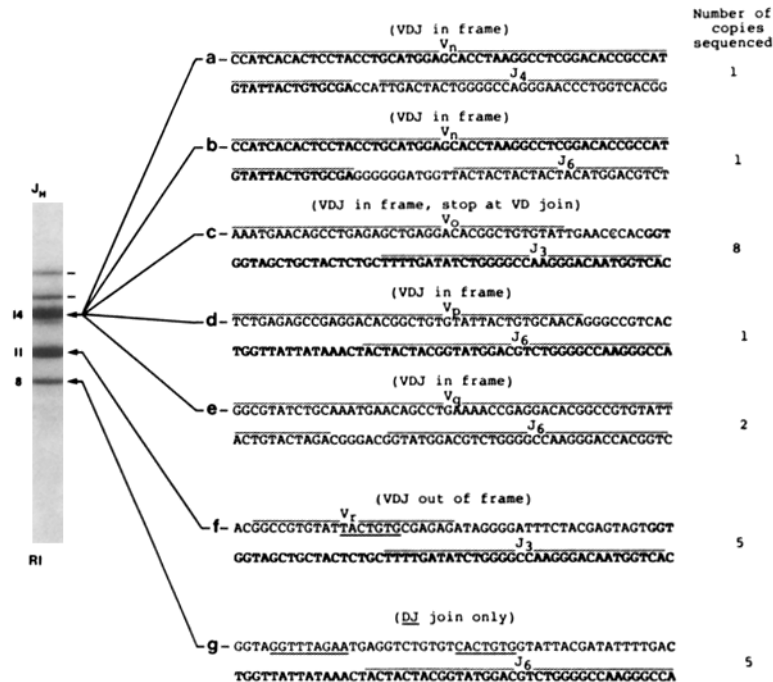


FIGURE 4. DNA sequences of VDJ rearrangements from TD leukemic DNA. Sequences were cloned as three size-fractionated Eco RI libraries (14, 11, and 8 kb). Rearrangements *a-e* were isolated from the library that contained ~14-kb fragments, rearrangement *f* from the 11-kb library, and rearrangement *g* from the 8-kb library. Different V and J region sequences are indicated using subscripts and rearrangements are labeled *a-g*. Frameshift rearrangements were identified by translation of the DNA sequence in all three frames and alignment with known V and J region amino acid sequences. Nucleotides are highlighted in bold letters where there is apparent sequence relatedness between different rearrangements (see Results). In rearrangement *g*, nonamer and heptamer sequences separated by 12 nucleotides, consistent with signals for recombinase action in VDJ rearrangement are underlined. The heptamer TACTGTG has been underlined in rearrangement *f*.

Nucleotide Sequence Analysis of Ig Heavy Chain Genes from Patient TD. In patient TD, the rearrangement corresponding to the lowest (~8 kb) band from the Eco RI genomic Southern blot appeared to have resulted from a DJ₆ joining event. This rearrangement is labeled *g* in Fig. 4. Nucleotide sequence upstream of this DJ gene segment did not share amino acid coding homology with V region sequences but matched germline sequence previously described 5' of a human D region (23).

The middle (11 kb) Eco RI band of TD resulted from a VDJ₃ rearrangement, as determined from the homology of the deduced amino acid sequence to known V regions (Fig. 3) and the published nucleotide sequence of the J₃ region. This rearrangement is labeled *f* in Fig. 4. 42 nucleotides including a presumed D region plus N sequences lay between the V and J₃ regions. This sequence shifts the amino acid sequence into a nonfunctional reading frame between the V and J₃ regions. Sequence from five isolates of this rearrangement were identical.

The VDJ rearrangements found in 8 of 13 isolates examined from the highest (~14 kb) group of rearranged Eco RI bands were also identical. This rearrangement, la-

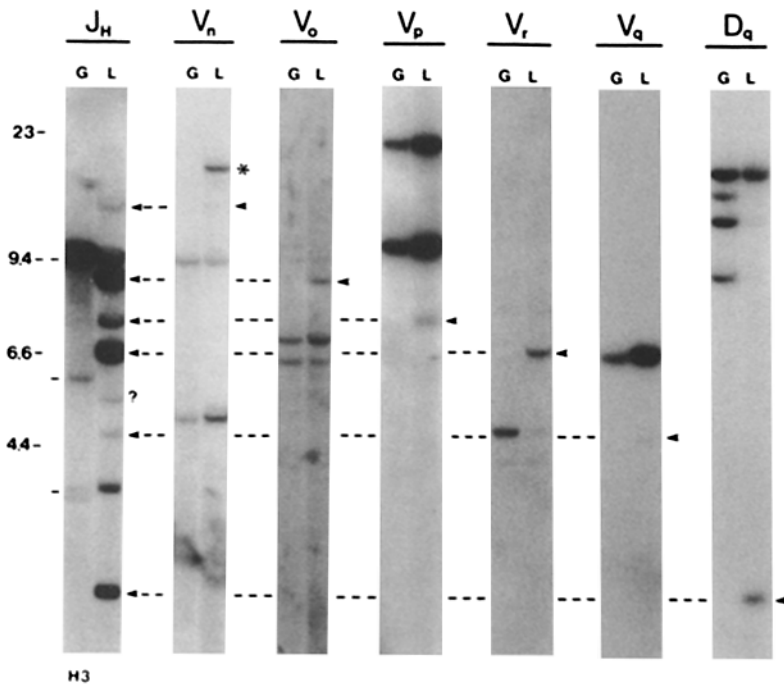


FIGURE 5. Southern blot analysis of TD leukemic DNA using specific V region probes. Genomic DNAs were cut with Hind III and run equal lengths on 0.8 agarose gels for Southern transfer to nylon membranes. Strips of membrane were then hybridized with different upstream 5' V or D region probes (denoted at the top of each lane) and autoradiographed. The hybridization signal was then removed and the same strips were rehybridized with J_H probe (as in far left lane) to identify corresponding bands. Germline hybridization patterns are shown in lanes marked G and rearrangements in TD leukemic DNA are shown in lanes marked L. V region designations match those from Fig. 4; the D region probe D_q was isolated from rearrangement *g*. The ? symbol indicates a rearrangement not cloned or sequenced. The germline DNA used in this analysis contains a commonly detected polymorphic doublet of bands at ~ 3.5 kb in the J_H autoradiogram at the left. The asterisk in lane V_n denotes a probable polymorphic germline band.

beled *c*, used the same apparent DJ_3 joint as rearrangement *f*, although the two rearrangements contain different V regions. Matching nucleotide sequences extended 18 nucleotides upstream of a common DJ_3 joint in both rearrangements *f* and *c*. In addition, the V region from rearrangement *c* appeared to be truncated at the 3' end by 12 nucleotides compared with most cloned V regions, resulting in the creation of an in-frame termination codon adjacent to the VD joint.

DNA of five other isolates from the library containing the 14-kb Eco RI group of rearranged bands were sequenced and these were found to have resulted from four different VDJ gene arrangements. One isolate, labeled rearrangement *d*, contained a VDJ rearrangement that used the same apparent DJ_6 joint used in rearrangement *g*, which lacked a V region. 15 identical nucleotides extended upstream from the common DJ_6 joint. Three other isolates from the 14-kb group contained rearrangements that also involved J_6 ; however, they were distinct from rearrangements *d* and *g* in that they used different V and D regions and had different points of recombination into the J_6 sequence. Two of these three isolates, designated as

the single rearrangement *e* in Fig. 4, were identical to one another throughout the entire VDJ region; the third is labeled rearrangement *b*. The last isolate, designated rearrangement *a*, contained a VDJ₄ rearrangement that used the same V region as rearrangement *b*, but included different D and J region sequences. None of the rearrangements *a*, *b*, *d*, or *e* appeared to have non-sense codons that would prevent translation of functional VDJ protein, although *a*, *b*, and *e* had unusually short D regions of only one to three amino acids. Possibly mutations outside of the regions of 400 nucleotides that have been sequenced may have accounted for the apparent lack of μ heavy chain production by this patient's cells. Suppression of structurally normal heavy chain Ig genes could also explain the lack of observed cytoplasmic Ig. Alternatively, leukemic populations carrying these less abundant rearrangements may have constituted too small a fraction of the cells in this neoplasm to be detected by the relatively insensitive assays commonly used to assess cytoplasmic Ig. The predominant 8-kb, 11-kb, and the major 14-kb rearranged Eco RI Ig heavy chain gene fragments in this patient's leukemic DNA (rearrangement *c*, *f*, and *g*) all had non-sense codons consistent with the immunophenotypic data.

All Cloned Ig Genes from TD Represent Clonal Rearrangements from Leukemic Cells. Because several of the Ig gene rearrangements cloned from the 14-kb region of the gel were not found repetitively in multiple copies as other rearrangements had been, the question arose as to whether or not these rearrangements had derived from non-leukemic B lymphocytes contaminating the leukemic cells. To ensure that the less abundant 14-kb rearrangements had actually derived from leukemic cells, the following strategy was adopted. As seen in Fig. 1, a Hind III digest of TD DNA displays many more rearranged Ig heavy chain gene bands on Southern blot analysis than the Eco RI digest showed. Each band, even relatively less intense ones, presumably arose from clonally expanded leukemic cells representing at least 1% of the total cells in the specimen (1% being the threshold of detection for a clonal population as a rearranged band in a Southern blot autoradiogram [2]). Unique sequence hybridization probes were generated from the noncoding-region DNA 5' of each cloned V or D region (the latter in the case of rearrangement *g*). These probes were used to analyze Hind III digests of unrelated germline DNA and TD leukemic DNA to correlate each cloned Eco RI fragment with a clonal rearrangement detected with a J_H probe in the Hind III Southern blot of TD leukemic DNA (Figs. 5 and 6). In each case, probes for the cloned Eco RI fragments hybridized to separate Hind III bands except for 5' probes from rearrangements *a* and *b*, which used the same V region gene and hybridize to the same rearranged band in TD. One minor rearranged Hind III band detected with the J_H probe failed to hybridize with any of the probes generated from the clonal Eco RI fragments and consequently could not be assigned to an Eco RI fragment or cloned rearrangement.

Discussion

Our data demonstrate that more than two clonal rearrangements of heavy chain Ig bands can be detected in human B-lineage ALL cells in roughly 30% of cases when analyses are performed using several restriction enzymes. Others have recently made similar observations (24, 25). Occasionally extra rearranged immunoglobulin bands can be found in other human B cell tumors; however, the incidence of such extra bands is significantly lower than that seen in B-lineage ALL (roughly 1-5%

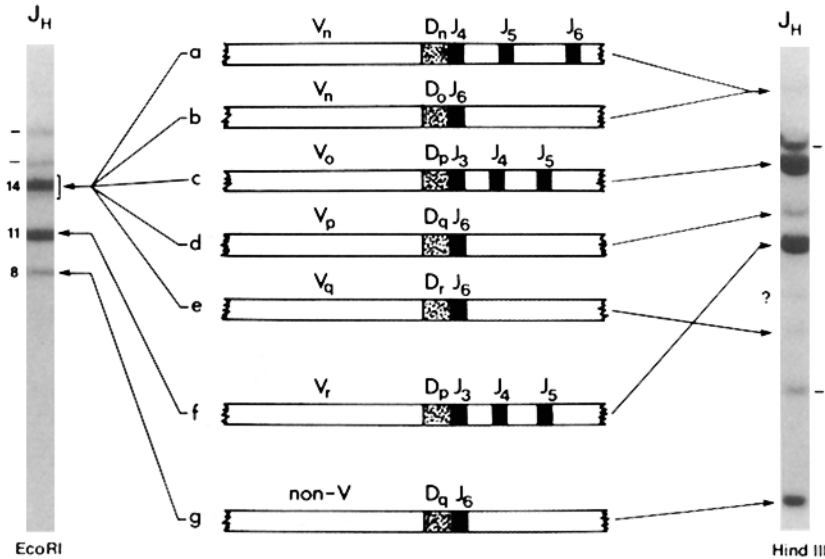


FIGURE 6. Summary of VDJ rearrangements from TD leukemic DNA. Genomic Southern blots of TD leukemic DNA digested with Eco RI (*left*) and Hind III (*right*) were probed with the J_H probe. DNA sequences cloned in three size-fractionated Eco RI libraries were later correlated with bands on the Hind III-digested Southern blot by probing with specific V region probes from different clones (see Results and Fig. 4). Rearrangements are lettered *a-g*, different V and D regions are denoted using subscripts *n-r*, and J regions are denoted using subscripts *1-5* as in Fig. 4. J regions are numbered based on homology to previously identified sequences (21). (-) Germline bands, (?) rearrangement not cloned or sequenced.

[Sklar J., et al., unpublished observations]). At least a portion of these extra bands in mature B cell tumors (those containing rearrangements of both heavy and light chain genes) probably represent somatic mutation events in which a postrearrangement mutation changes the position of a rearranged band in some subpopulation of the tumor cells (6-8). By cloning and sequencing DNA contained within rearranged bands of two B-lineage ALL cases in this study, we have shown that this is probably not the case in B-lineage ALL, and that the multiple rearrangements detected in these neoplasms result from unique combinations of V, D, and J elements.

Several different mechanisms could account for the presence of multiple Ig heavy chain rearrangements within B-lineage ALL. One possibility is that the cells within some fraction of the neoplastic population are polysomic for chromosome 14. Karyotype analyses were performed on cells of patient AW and several of the interpretable chromosome spreads were in fact trisomic for chromosome 14, suggesting that each cloned rearrangement in AW may have been present on a different chromosome 14 allele in a single trisomic line of tumor cells. On the other hand, Southern blot analysis and genomic DNA cloning indicated that Ig heavy chain DNA in the germline configuration was particularly abundant in AW leukemic DNA, which was not found to be the case in TD and most other cases examined by Southern blot analysis. Additionally, blood samples drawn from AW were unusually rich in leukemic cells, containing 93% CD10⁺ cells among all peripheral blood leukocytes. This suggested that

a chromosome 14 with a heavy chain Ig gene in the germline configuration may have been present in the leukemic cells of AW. If this were the case, the presence of a total of four leukemia-related heavy chain alleles would suggest the existence of two distinct malignant subpopulations. The actual number of populations possessing different Ig heavy chain gene rearrangements cannot be determined on the basis of the available data and would probably require cloning of individual tumor cells either directly or as hybrids with other cells. Cells of B-lineage ALL are difficult to maintain in culture and attempts to grow cells of AW and TD before and after fusion to mouse myeloma cells were unsuccessful. In any event, assuming that a single trisomic population of cells existed in AW, chromosome duplication (leading to trisomy 14) must have occurred before heavy chain gene rearrangement because each of three rearranged genes in AW used different V, D, and J elements. Since chromosomal aneuploidy is usually thought to occur only after malignant transformation, these results suggest that gene rearrangement continues to take place in the transformed B cell precursors.

Karyotypic information is not available on leukemic cells of TD because metaphase chromosomes could not be obtained. However, the presence of seven clonal rearranged bands within Southern blots carried out with Hind III digestion of DNA from this leukemia, together with the confirmation of the origin of these bands from separate VDJ rearrangements, suggests that polysomy for chromosome 14 is an unlikely explanation for the presence of the extra rearranged heavy chain Ig bands. In addition, densitometric analyses of Southern blot autoradiograms (data not shown) prepared from TD and human sperm DNA hybridized with probes for chromosome 14 (Ig C_μ and C_ε), chromosome 2 (Ig C_κ), chromosome 9 (*tcl-3*) (26), chromosome 18 (*bcl-2*) (27), and chromosome 22 (*bcr*) (28), suggested that chromosome 14 was present on average in fewer than three copies per cell in the TD leukemia.

Another formal mechanism that could possibly account for the multiplicity of rearranged Ig heavy chain bands in B-lineage ALL is the development of these leukemias from more than one B cell precursor. Recent data on glucose-6-phosphate dehydrogenase (G6PD) isotypes in 12 cases of B-lineage ALL from female patients heterozygous for isotypic forms of this X-linked enzyme indicate that the great majority, if not all cases of ALL, are monoclonal and arise from a single parent cell (29). Since the incidence of more than two rearranged bands is as high as 30%, these G6PD clonality studies would have been expected to show two clones with reasonably high probability (>85%) in some cases of ALL if our results are due to origin from more than one parent cell. Alternatively, a multistep transformation (30, 31) is possible in which a premalignant event occurs in a common progenitor that lacks Ig gene rearrangements. This partially transformed cell may divide and subsequently rearrange its Ig heavy chain genes, followed by a second independent transformation step which gives rise to fully neoplastic cells. In either scenario, evolution of these tumors still involves a single common parent cell.

Recent data have suggested that VDJ rearrangements are not fixed in all mammalian B cells. In vitro analysis of some murine pre-B cell lines has shown that unrearranged V region elements may replace V elements previously joined to DJ rearrangements, apparently by using the conserved heptamer sequence TACTGTG found within the 3' end of most V region elements (9, 10). Such substitution rearrangements could have occurred in the TD leukemia that we have studied, involving the

two rearrangements c and f of TD, which showed different V region sequences both joined to identical D region sequences and J₃. If rearrangements c and f from TD were related by sequential V region recombinants then rearrangement f must have preceded rearrangement c because the latter lacked the required heptamer signal sequence.

Substitution rearrangements cannot, on the other hand, explain most of the multiple rearranged alleles within B-lineage ALL tumors because there are more than two unique DJ rearrangements in both of the tumors that we studied. The more likely explanation is that some number of B-lineage ALL tumors probably arise from cells at an earlier stage of B cell development than previously thought. This stage probably represents a stem cell committed to the B-lineage, but lacking rearranged Ig genes. Our evidence supporting this conclusion is indirect; however, that B-lineage ALL may differentiate from cells at least earlier than pre-B cell stage with two complete heavy chain gene rearrangements is suggested by the cloned and sequenced alleles of TD. Two of these (rearrangements d and g) shared the same DJ joint, but one had apparent germline sequence 5' of D and the other had a rearranged V element at this site. Possibly the former allele was a precursor of the latter. It may also be that in the case of the related rearrangements c and f, a chromosome with only a DJ rearrangement replicated and diverged to use two different V regions (rather than having undergone V region replacement). Both these possibilities further support the existence of ongoing rearrangements during B-lineage ALL development.

Data from both mice and humans have indicated that individual V region segments from certain V_H region families appear more frequently than other V_H region segments in the rearranged Ig genes of fetal B lymphocytes (32, 33). The majority of V_H regions present in the heavy chain Ig gene of the AW and TD tumors seem to come from the same pool of V_H regions as used in human fetal lymphocytes (33). For instance, in Fig. 3, the V_x, V_o, V_p, V_q, and V_r regions are closely related to the V_{HIII} family; V_z appears related to the V_{HII} family; and V_y appears related to the V region segment 15P1. Only the V_n region lacks homology to V_H region segments not previously isolated from fetal B cells. These findings underscore the general conclusion that Ig heavy chain gene rearrangements in neoplastic pre-B cells resemble normal VDJ rearrangements in B lineage cells of young individuals and that malignant transformation cannot apparently be correlated with unusual or aberrant rearrangements of Ig heavy chain genes.

Cells of B-lineage ALL are often described as being frozen at a pre-B cell stage of development since they do not progress to become B-cell leukemias. While it is true that these cells apparently lack the capacity to functionally rearrange light chain genes and to produce cell surface Ig, they are not blocked with respect to heavy chain gene rearrangements since our data are most compatible with continuing rearrangements of heavy chain loci for at least some interval after transformation. Perhaps retention of the capacity to rearrange heavy chain Ig genes plays some part in the observation that samples of B-lineage ALL often contain rearrangements of TCR genes as well as heavy chain Ig rearrangements (34). Since the same recombination apparatus appears to be involved in rearrangements of both types of loci (35), it seems reasonable to expect that TCR gene rearrangements could occur at some point during growth of these leukemias.

In view of this hypothesis, the results of analyses for TCR genes were somewhat

surprising in the AW and TD cases. Both lacked detectable rearrangements of β TCR genes, but each showed two roughly equally intense rearranged bands for the TCR- γ locus together with little residual germline TCR- γ DNA. Although deletions of TCR- γ genes in some cells cannot be ruled out, these findings suggest that the AW and TD leukemic cells were homogeneous with respect to TCR- γ gene rearrangements. If this were true, then it would imply that the leukemic populations arose from a stem cell already containing two rearranged TCR- γ genes but no rearranged Ig genes at the time of transformation. The TCR- γ gene data also support the monoclonal origin of these two tumors.

Mouse pre-B cell tumors induced by Abelson murine leukemia virus are known to continue gene rearrangement during the course of their in vitro growth (36, 37). In that system, transformants established from bone marrow and late fetal liver (>14 d) most often contain two VDJ rearrangements and 60% of those cell lines produce cytoplasmic heavy chain. However, transformants established from early fetal liver represent less mature B cell precursors that rarely produce cytoplasmic heavy chains and that always contain DJ and sometimes VDJ rearrangements on each allele. These transformed lines are somewhat similar to cases of ALL discussed here in that many clonal transformants from early fetal liver continue to rearrange Ig heavy chain genes from DJ intermediates to yield many more than two heavy chain gene configurations. Our findings in human ALL cases contrast with these observations because the multiple heavy chain Ig gene rearrangements within ALLs generally appear to use many different DJ joints rather than the same DJ joints.

Of 38 cases of B-lineage ALL studied, 11 were found only to have one rearrangement involving the heavy chain J region. Others have similarly found that pre-B cell leukemias frequently contain only one heavy chain gene rearrangement (12, 38). Barring the possibility of comigrating rearranged fragments in these cases, the evidence from human leukemias suggests that DJ joining need not occur on both heavy chain alleles prior to VD joining as has been concluded from studies of transformed murine cell lines (36, 37).

Why only 30% of B-lineage ALL cases show greater than two rearranged heavy chain bands is not clear. Divergent clones (or subclones) that are not abundant within tumor cell populations may escape detection as rearranged bands in Southern blots. Several of the rearrangements identified in this study yielded superimposed bands in autoradiograms of several restriction enzyme digests, so that the full number of rearranged Ig genes in a leukemia may not be evident without extensive restriction enzyme analysis or even molecular cloning. A more fundamental explanation is that B-lineage ALL may be heterogeneous with respect to the stages of B cell development at which malignant transformation may occur. Cells transformed after heavy chain gene rearrangement may show only two heavy chain gene configurations unlike cells transformed before gene rearrangement, which may continue to undergo gene rearrangement during tumor growth.

Although somatic mutation appears to be a frequent event within rearranged Ig genes of more mature B cell tumors (such as follicular lymphoma), our data indicate that somatic mutation is rare in pre-B cells of B-lineage ALL. Others have reported an increased incidence of somatic mutation in the Ig genes of a single Abelson murine leukemia virus-transformed mouse pre-B cell line (39). By monitoring the reversion rate of a non-sense mutation that can be detected by production of a cytoplasmic

μ heavy chain protein, these workers have estimated the rate of mutation to be $\sim 3 \times 10^{-5}$ to 10^{-4} mutations/basepair/cell generation. In our studies we failed to find a single example of a point mutation among six rearranged Ig heavy chain alleles for which we sequenced multiple copies. Since >15,000 nucleotides have been sequenced without a single somatic mutation, prevalence of the mutations in these tumors is $< 6.7 \times 10^{-5}$ mutations/basepair. Calculation of the rate of mutation must also take into account that the alleles analyzed derive from actively proliferating cells separated by many divisions. Therefore the maximal rate of somatic mutation per generation is probably considerably lower than this.

These data suggest that somatic hypermutation occurs within normal B-lineage cells at some stage of development later than Ig heavy chain gene rearrangement. This interpretation is subject to the assumption that neoplastic cells retain biochemical and genetic features of their normal pre-B cell counterparts. We cannot judge the accuracy of this assumption concerning somatic hypermutation, but the results in this report imply that at least with respect to the capacity to rearrange V, D, and J region elements within Ig heavy chain genes this assumption is true.

Summary

Southern blot analyses revealed that cells from nearly 30% of childhood B cell precursor acute lymphoblastic leukemias (ALLs) contained more than two rearranged, nongermline bands for Ig heavy chain genes. DNA corresponding to these bands was molecularly cloned from two cases which showed three and seven rearranged bands, respectively. Nucleotide sequence analysis of the cloned DNA demonstrated that each band represented different VDJ or DJ rearrangements. While the same DJ joints were shared by several rearrangements, different DJ joints were found in the majority of rearrangements, precluding V region substitution as an explanation for the multiplicity of heavy chain rearrangements in these leukemias. Most of the V region segments involved in these rearrangements were restricted to V_H region families that have been shown previously to be preferentially rearranged in human fetal B lineage cells. Sequence analysis of multiple copies of the same VDJ rearrangements from different cells revealed no somatic mutation, a mechanism responsible for detection of extra rearranged Ig DNA bands in certain other B lineage tumors. The data suggest that in some cases of ALL Ig heavy chain genes begin and continue to rearrange *de novo* within the neoplastic B cell precursor populations derived from an original malignant cell transformed at a stem cell stage of differentiation.

We thank Dr. Steve Smith for attempts at tumor culturing; Dr. Roger Warnke for immunophenotype characterization; Courtney Moulds, Amy Paivanen, Diana Stocks, and Rosann Tung for technical assistance; Kathy Shaw for manuscript preparation; Pamfilo Verzola for photography; and Tom Reynolds for helpful discussions.

Received for publication 17 December 1987 and in revised form 11 April 1988.

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