Predominance of Fetal Type DJ_H Joining in Young Children with B Precursor Lymphoblastic Leukemia as Evidence for an In Utero Transforming Event

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Summary

The presence of N sequences in the complementarity determining region 3 (CDR3) of the rearranged immunoglobulin H chain is developmentally regulated: N regions are generally present in the DJ_H joinings of adult B cells but are often absent in fetal B cells. Analysis of the CDR3 in 61 B precursor acute lymphoblastic leukemias indicated that 87.5% of the leukemias obtained from children ≤3 yr old lacked N regions at the DJ_H junction. In contrast, in children >3 yr old, only 11.1% of the leukemias lacked N regions at this junction, a frequency similar to what we have observed in B cells from children and adults. These findings suggest that the majority of leukemias presenting within the first 3 yr of age arise from an in utero transforming event.

Hypervariability within the CDR3 of the human Ig H chain is initially generated at the time of VDJ joining by the recombination of multiple V_H, D, and J_H gene segments (1–3). This VDJ recombination process is dependent upon two recombinase enzymes, RAG1 and RAG2, since mice deficient in either gene fail to produce lymphocytes with rearrangements in their Ig or TCR loci (4–8). Exonucleolytic activity produces joinings in which germline nucleotides are lost from the ends of the joined segments (1–4). Variability is increased when nontemplate-derived nucleotides (N regions) are added between joined gene segments through the action of another enzyme, terminal deoxynucleotidyl transferase (Tdt)[1] (1–4). In both IgH and TCR rearrangements, palindromic (P) mono- or dinucleotides may be found adjacent to a recombined gene gene segment when the segment is present in its entirety. These germline-encoded nucleotides arise from a flip-over mechanism of the 5′ end of one strand of the joining segment (9).

The developmental regulation of N region addition has been demonstrated in both mice and humans (10–13). DJ_H joinings that lack N regions are found more frequently at the fetal stage of development. No more than 5% of the DJ_H junction sequences of B lymphocytes present in murine fetal liver contained an N region, whereas in newborn mouse spleen and liver, 5–23% of the DJ_H junctions had N regions (10–12). In contrast, a significantly higher percentage of DJ_H junctions with N regions (64–73%) were found in B cells from adult (4–8 wk) murine spleen (10–12). A similar trend was found in human B cells; evaluation of >500 DJ_H joining DNA sequences obtained from human fetal, neonatal, and adult lymphoid tissue revealed N regions at the DJ_H junction at frequencies of 68%, 86%, and 91–100%, respectively (13, 14). Together, these observations suggest that CDR3 sequences lacking N regions at the DJ_H junction are representative of a DJ_H recombination event that occurred during the time of fetal development when TdT activity may have been absent. Indeed, in the murine system, TdT levels rise slowly in the developing thymus, which correlates with the absence of N regions in fetal TCR-γ/δ rearrangements (15, 16). Similarly, in the murine B lymphoid system, TdT was not detected in fetal liver but was demonstrated in adult bone marrow B lineage cells (16).

The functional significance of the absence of N regions in the early stage of development is not clear. Gu et al. (12) speculate that the absence of N regions implies predominant expression of germline-encoded specificities. Thus, idiotypic interactions in a germline-encoded network might play a functional role in the development of the antibody repertoire early in ontogeny. In newborns, Feeney (10) found a higher percentage of N regions in productive vs. nonproductive rearrangements and speculated that this increase suggests a preferential activation of B cells whose IgH sequences contain N regions by antigens or cellular interactions. Alternatively, IgH sequences with N regions might have an advan-

[1] *Abbreviation used in this paper:* Tdt, terminal deoxynucleotidyl transferase.

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tage in the transition from pre-B cell to B cell, perhaps through enhanced binding to surrogate L chains.

B lineage acute lymphoblastic leukemia (ALL) of childhood results from the transformation of B precursor cells and their clonal expansion (17–19). We reasoned that if lack of N regions at the DJ_H junction was a marker for fetal-derived B cells, then leukemias arising from an in utero transforming event should show a bias for DJ_H joinings that lack N regions. Furthermore, the age distribution of leukemias lacking N regions at their DJ_H joinings might provide insight into the length of time required to develop clinical disease from the time of the transforming event.

Materials and Methods

Source of Cells. Bone marrow samples with >70% lymphoblast replacement were obtained at diagnosis from 63 patients (6 mo

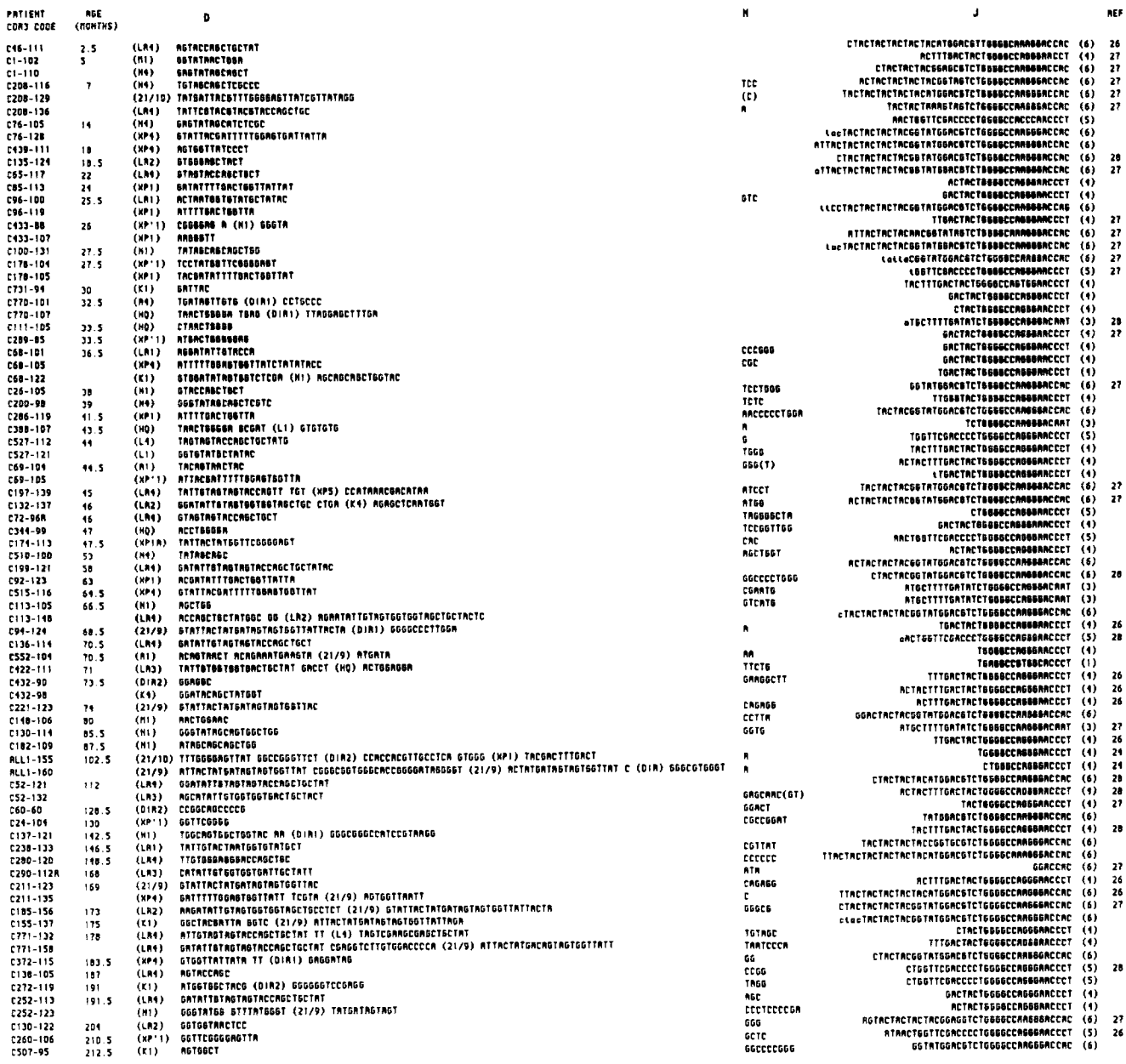

Figure 1. DNA sequence of the DJ_H region from 61 cases of B lineage lymphoblastic leukemias. The first column indicates the sequence code (case number and the length of the CDR3, including the V_H and J_H primers). Each sequence is subdivided into D (and when applicable multiple D with intervening N regions), N, and J_H (5′ end) regions. Germline D and J_H segments with maximum homology to the segments used in the CDR3 sequences are shown to the left and right of these segments, respectively. P nucleotides are enclosed by parentheses. 5′ ends of J_H segments sharing homology with the 3′ end of the D segment are indicated by lower-case letters. References are shown where the indicated sequences have been previously published.

to 17 yr) with ALL. Morphologically normal bone marrow samples from three children (21–26 mo) with solid tumors obtained on routine staging were used as a source of normal marrow B cells. Consent was given by the patients or their parents and by the Committee for Protection of Human Subjects at the Children's Hospital of Philadelphia.

Diagnosis of ALL in each patient was based on standard morphologic and histochemical parameters of the leukemic cells. The B cell precursor lineage of these leukemias was confirmed by immunophenotyping in which all the leukemias expressed the B lineage antigens CD19 or CD10 and were negative for T cell antigens (20).

DNA Amplification, Cloning, and Sequencing of CDR3. Marrow cells were fractionated on a Ficoll-Hypaque gradient (d = 1.077) and genomic DNA was isolated from mononuclear cells by established procedures (21) or by rapid cell lysis using nonionic detergents (22).

PCR was carried out as described (23) using an annealing step at 55°C for 1 min, an elongation step at 72°C for 30 s, and a denaturation step at 92°C for 1 min (14, 24). The V_H and J_H nucleotide consensus primers used in the PCR and the J_H consensus (J_HC) probes used for screening have been described (14, 24). These consensus primers do not preferentially amplify CDR3 sequences containing specific J_H segments (14). Restriction endonuclease digestion of the PCR-amplified DNA, electrophoresis in a 4% Nusieve agarose gel (FMC Bioproducts, Rockland, ME), purification, and cloning of CDR3-containing bands were performed as described (14, 24). Positive clones were picked up randomly (10–20/sample), and a double-stranded DNA template was prepared and screened by T lane sequencing. In each leukemia, from one to three different T lane sequences were identified that were present in multiple copies and were fully sequenced using the dideoxy method (25). In each normal marrow all the T lanes contained different sequences and ~20 sequences were obtained from each marrow.

DNA Sequence Analysis. DNA sequencing data were analyzed for utilization of D and J segments using the sequence analysis software pack (Release 5; Genetic Computer Corp., Madison, WI) at the University of Wisconsin and a Micro Vax II computer (Digital Equipment Corp., Marlboro, MA), according to described criteria (14).

Results and Discussion

80 DNA sequences of VDJ joinings from 61 B lineage ALLs were analyzed for the presence of N regions at the DJ_H junction (Fig. 1). Overall, 31/80 (38.8%) DJ_H junctions lacked N regions, a frequency higher than that reported for human adult tissues and similar to that found in human fetal tissue (13). When the percentage of leukemias lacking N regions was analyzed as a function of age at diagnosis, a striking pattern emerged: in patients ≤3 yr old, 87.5% (14/16) of the leukemias were comprised entirely of CDR3 sequences lacking N regions; whereas in children >3 yr old, only 11.1% (5/45) of the leukemias met this criterion.

The percentage of leukemias without N regions in children ≤3 yr old was much higher than expected even when compared with data reported for normal human fetal liver or neonatal cord blood (13), whereas the frequency observed in children >3 yr old was close to that observed in adults (13, 14). To exclude the possibility that the paucity of N regions in these young patients could be due to an inherent abnormality in TdT activity, we examined the DJ_H joinings in lymphocytes obtained from the end of therapy marrows of three of these patients when residual leukemia was not detectable using PCR analysis (Fig. 2). N regions were present

PATIENT CDR3 CODE	D		N	J	
C100-91	(LR3)	GGAGGTGAT	C	TTGACAACTGGGGCCAGGGAACCCT	(4)
C100-91A	(LR2)	GGTAGC GA (HQ) TGGGGA		CTGGGGCCAGGGAACCCT	(5)
C100-94	(K4)	GCTATGG		ttTGACTACTGGGGCCAGGGACCCT	(4)
C100-102	(21/10)	GGGGAGCT (DF16) ACTACGGGGT	(T)	ACTACTTTGACTACTGGGGCCAGGGAATCCT	(4)
C100-103A	(HQ)	CTAACTGGGGA	AGATTT	TTTGACTACTGGGGCCAGGGAACCCT	(4)
C100-106	(21/9)	TAGTGGTTATACCAC (DIR1) CCACCAC		TGACTACTGGGGCCAGGGACCCT	(4)
C100-109	(LR4)	GTACCAGCTGCTATAT	CGATCACGGGG	GGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C100-109A	(H1)	GTATAGCAGTGGCTGGTAC (H1) CAGCCGGT		CTTTGACTACTGGGGCCAGGGAACCCT	(4)
C100-109B	(K1)	AGTGGCTACGA	CGGGCC	AATTGGTTCGACCCCTGGGGCCAGGGAACCCT	(5)
C100-115	(N1)	GGATAGCAGTGGCTGGT (DIR1) CCCACCC	C	CTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C100-115B	(XP4)	GAGTGG GAGC (XP4) TACTACGATGTT	ATCTC	ATTACTTTGACAACTGGGGCCAGGGAACCCT	(4)
C100-132	(XP'1)	AGGGTTCG (LR3) TGTTGATTG GGT (K1) TGGATATAGTGGCTACGAT	CC	ACAACTGGTTCGACCCCTGGGGCCAGGGAACCCT	(5)
C100-136	(N2)	TACGGGTTA GGACTT (LR5) TACTACTCT TCGGGACCC (DIR2) GGGCGGGG		CTACTATTATTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C178-97	(21/9)	ATTACTATGATA		TACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C178-103	(LR4)	GTTGGTACTA (DIR1) GGGGACGGGGT		TACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C178-117	(XP'1)	GTTCGGGGAGTT	CAC	AATACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C178-121	(LR4)	TCTAGGAGTACCAGCTGCTATG	AGA	ACTACTACTACTACGGTATGGACGCTCGGGGCCAAGGGACCAC	(6)
C178-122	(N1)	AGCAGCAGCTG	CCA	ACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C178-122B	(XP'1)	GTATTACTATGGTTCGGGGAGTTATT	TCC	CTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C178-135	(21/9)	TACTATGATAGTAGTGGTTATTACTAC (21/10) ATCATTAC	C	AATACTACTACTACTACGGTATGGGGCCAAGGGACCAC	(6)
C178-136	(DIR1)	GGTCCTCCAGG CACG (21/9) ATTACTATGATAGTAGTGGT	TCGTC	TTACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C178-136A	(LR4)	TAGTACGGCAGCTGCTA CA (N2) AACCGGGA	C	TACTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C111-103	(21/9)	AGTAAGTGGT	C	CTACTTCGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C111-106	(XP4)	GGTTTTTGGAGTGGTTATTACG	GG	GACTACTGGGGCCAGGGACCCT	(4)
C111-106B	(XP'1)	TTACTATGGTTCGGGGAG	CCGTGCGTCA	TTTGACTACTGGGGCCAGGGACCCT	(4)
C111-112	(K4)	GTGGATACAGCTATGG		AATACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)
C111-124	(21/10)	TTATGATTACGTTTGGGGGAGTATCGTTA	C(T)	ACAACTGGTTCGACCCCTGGGGCCAGGGAACCCT	(5)
C111-139	(21/10)	TGGGGGAGAGA ACCTTTGCAAAAGATTA (DIR2) TCGGGATTGGGCTT A (N1) ATAGCAGCAGCTGG		tTGACTACTGGGGCCAGGGAACCCT	(4)
C111-145	(LR2)	GGACATTGTAGTGGTGGTAGCTGCTAC	AAGATAGGGTT	TTTCTACTACTACTACGGTATGGACGTCTGGGGCCAAGGGACCAC	(6)

Figure 2. DNA sequences of the DJ_H regions derived from normal B cells obtained from the end of therapy remission marrow of three patients with B lineage lymphoblastic leukemia exhibiting fetal type DJ_H joinings. See Fig. 1 for further explanation.

in the majority of the joinings, indicating the presence of TdT activity. An alternative explanation for the lack of N regions is that bone marrow B cells (which give rise to ALL) might normally have a higher frequency of DJ_H junctions that lack N regions as compared with the other lymphoid tissues. N region frequency was calculated from the joining sequences obtained from normal bone marrow of three young children (21–26 mo old). N regions were present in 50/53 (94.3%) DJ_H junctions (data not shown), a frequency comparable with the 86% and 90–100% reported for newborns and adults, respectively (13, 14). A high frequency of multiple D regions was noted in the DJ_H junctions obtained from the normal B cells of the young leukemic patients, suggesting the possibility that abnormalities in the VDJ recombination process are present in these children.

To determine whether the DJ_H junctions without N regions in the 14 leukemias in children $\leq$3 yr old utilized specific D or J_H segments, we examined the frequency distribution of such segments. The D gene family is composed of $\sim$30 segments including multiple and duplicated loci, and thus patterns of utilization are difficult to analyze (1). XP was found to be the most commonly used D family in the leukemias, consistent with the report by Sanz (13) for fetal tissue. However, the leukemias showed a lower frequency of HQ52 utilization (9.1%) than that reported by Sanz (13) (14%) and Schroeder and Wang (29) (50%) for fetal tissue. J_H4 and J_H6 were the two predominant gene segments used in the leukemias comprising 80% of the sequences, whereas J_H1 and J_H2 usage was absent. In fetal tissue, Sanz (13) found J_H4 usage to be the most common followed by J_H3 and J_H6, whereas Schroeder and Wang (29) found J_H3 usage to be the most common followed by J_H4 and J_H5. These differences in J_H and HQ52 usage found in fetal liver tissue may reflect individual variation as samples were obtained from only one or two fetuses in each study (13, 29).

Consequently, developmental stage–specific trends in the usage of D and J_H gene segments in fetal B cells have been consistently demonstrated only for the D segment HQ52, and this usage varies widely (13, 29). While HQ52 usage appears to be a marker of fetal-derived B cells, its absence does not imply a more developmentally mature stage. Thus, the absence of N regions in the CDR 3 sequences appears to be a more useful marker of fetal origin. The vast majority (87.5%) of leukemias occurring in children $\leq$3 yr old lacked N regions at the DJ_H junction, suggesting that they arose from a transformation event in utero when the pool of fetal lymphoid cells is largest. Furthermore, the sharp decrease in the frequency of DJ_H junctions lacking N regions after 3 yr of age suggests that some leukemias initiated during fetal life require a maximum of 3 yr to become clinically evident. The absence of N regions observed in a small fraction of leukemic and normal B lymphocytes present later in life may represent a DJ_H joining event that occurred during fetal development in a long-lived B cell or may simply indicate that Tdt was inactive at the time of DJ_H joining in a developmentally mature lymphoid cell.

Greaves (30) has proposed a two-step mutation model in B lineage ALL in which the first event occurs in utero in association with an ontogenic drive to expand B cell precursors in liver and marrow. The second event produces clinically overt leukemia and is associated with the proliferative stress elicited in infants by the rapid onset of immune responses to exogenous antigens. Support for this latter event stems from the observation that the steep rise in the incidence rate of B precursor ALL follows shortly after serum antibody levels sharply rise toward adult levels during the first 2 yr of life (31). Our observation would be compatible with Greave's hypothesis but would limit the in utero transforming event to primarily those leukemias that occur during the first 3 yr of age.

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