Immunoglobulin heavy-chain gene rearrangement in adult acute lymphoblastic leukemia reveals preferential usage of J_H-proximal variable gene segments

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The aim of this study was to characterize individual-segment and overall patterns of V_H gene usage in adult B-lineage acute lymphoblastic leukemia (ALL). Theoretical values of V_H segment usage were calculated with the assumption that all V_H segments capable of undergoing rearrangement have an equal probability of selection for recombination. Leukemic clones from 127 patients with adult B-lineage acute leukemias were studied by fingerprinting by means of primers for the framework 1 and joining segments. Clones from early preimmune B cells (245 alleles identified) show a predominance of V_H6 family rearrangements and, consequently, do not conform to this hypothesis. However, profiles of V_H gene family usage in mature B cells, as investigated in peripheral blood (6 samples), B-cell lymphomas (36 clones) and chronic lymphocytic leukemia (56 clones), are in agreement with this theoretical profile. Sequence analyses of 64 V_H clones in adult ALL revealed that the rate of V_H usage is proportional to the proximity of the V_H gene to the J_H locus and that the relationship can be mathematically defined. Except for V_H6, no other V_H gene is excessively used in adult ALL. V_H pseudogenes are rarely used (n = 2), which implies the existence of early mechanisms in the pathway to B-cell maturation to reduce wasteful V_H-(D_H)-J_H recombination. Finally, similar to early immunoglobulin-H rearrangement patterns in the mouse, B cells of ALL derive from a pool of cells more immature than the cells in chronic lymphoid B-cell malignancies. (Blood. 2001; 97:2716-2726)

Introduction

Immunoglobulin heavy chain gene rearrangement involves the joining of 1 variable (V_H), 1 diversity (D), and 1 joining segment (J_H) and precedes light chain gene rearrangement in a hierarchical pattern. This process is essential for the progression of early B cells to maturity and functional antibody assembly.[1,2] A total of 123 V_H segments[3,4] (depending on haplotype), 26 D segments,[4,5] and 9 J_H segments[5] are organized in a telomeric-to-centromeric orientation on chromosome 14 band q32.2 V_H segments are classified into 7 families (V_H1 through V_H7) on the basis of amino acid sequence homology[6] (Table 1). V_H3 is the largest family, followed by V_H4 and V_H1 (64, 32, and 19 members, respectively), whereas V_H2, V_H5, and V_H6 contain only 4, 2, and 1 member, respectively. The unique V_H6 is the most 3′ element and is closest to the D and J_H loci.[4]

Only half of all V_H genes carry intact recombination signal sequences (RSS), which are important *cis*-acting elements required for V_H-(D)-J_H recombination.[7] The remaining are consequently "nonfunctional," as are V_H genes with point mutations resulting in frame shifts and stop codons (28 V_H genes). Forty-two V_H segments either are known to be functional (39 segments) or have been found transcribed (1 segment) or with open-reading frames (2 genes) but not yet in a rearranged form. A putative requirement for recombination is low-level tissue-specific transcription[8-10] from V_H promoters (one per segment), coordinated with chromatin accessibility to recombination enzymes,[11] in both human and murine B cells.[12-15] When these factors are taken into account, 55 out of 123 (44.7%) V_H segments are capable of undergoing rearrangement, but only 42 of 55 (76%) are potentially functional (Table 1). Under the assumption that 2 of 3 recombinations will be out of frame, the probability that any rearrangement will be functional is $1/3 \times (42/55) = 0.25$ or 25%.

The final immunoglobulin repertoire seen in B cells will reflect diversity generated through V_H-(D)-J_H recombinations, and any positive or negative selections throughout development and maturation, prior to contact with antigens.[5] Although it is clear that the repertoire shifts throughout ontogeny and maturation, the early controlling events are not clear. The final repertoire in mature adult B cells are unrestricted and unbiased, as shown by studies in peripheral blood, spleen B cells, or tonsil B cells.[16-21] Many groups have reported that mammalian fetal V_H-(D)-J_H repertoires (in liver, spleen, bone marrow, or peripheral blood) are highly restricted, and only a few rearranged V_H genes (V_H3 or V_H5 or V_H6) dominate.[16,17,22-28] Other researchers disagree.[29-31] Murine studies show a consistent overuse of J_H-proximal V_H segments in fetal[22-23,25-27] and adult[23,26-27,32] B-cell progenitor cells.

Few studies have been done in adult human preimmune B cells. Immature splenic B cells, but not mature peripheral B cells, have been shown to exhibit fetal-like V_H6 overusage.[17] In post–allogeneic marrow transplantation patients, it has been shown that the B cells repopulating the periphery early (at 2 to 5 months) have overexpression of V_H6 and a pattern of V_H usage similar to those of neonatal and infant blood cells. By 6 to 12 months, the pattern

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Table 1. V_H gene segments on chromosome 14q32.2

Type of V_H gene segment	No. segments in V_H families						Total
	V_H1/V_H7	V_H2	V_H3	V_H4	V_H5	V_H6	
Total germline segments (rows A, B, C)	19	4	65	32	2	1	123
(%)	(15.4)	(3.3)	(52.8)	(26.0)	(1.6)	(0.8)	
Rearrangable (rows A + B)	14	4	27	8	1	1	55
(%)	(25.5)	(7.3)	(49.1)	(14.5)	(1.8)	(1.8)	
A) Functional	10	3	20	7	1	1	42
(%)	(23.8)	(7.1)	(47.6)	(16.7)	(2.4)	(2.4)	
Functional protein equivalent found	9	3	19	6	1	1	
Transcribed mRNA equivalent found	—	—	—	1	—	—	
Other V_H with ORF, intact promoter, and RSS	1	—	1	—	—	—	
B) Pseudogenes with intact promoter and RSS	4	1	7	1	—	—	13
C) Nonrearrangeable pseudogenes	5	—	38	24	1	—	68

Based on Matsuda et al.[4]
mRNA indicates messenger RNA; ORF, open-reading frames; RSS, recombination signal sequence.

becomes less marked.[33] Others studies disagree[34] and suggest a limited clonal diversity at 6 to 10 weeks and a normal repertoire by 6 to 9 months. This was supported by a group who showed that V_H3 is the predominant family in the periphery at 6 months.[35] It has been suggested that B-cell development during reconstitution of the periphery might follow a wavelike shifting pattern.[36]

Acute lymphoblastic leukemia (ALL) is a good model for the study of V_H-(D)-J_H rearrangements, as it allows examination of individual recombination events from clonally expanded pre-immune B-cell populations. Previous work[37,38] including our own,[39] demonstrated essentially normal V_H usage profiles in B-lineage ALL in both adults and children, but with a noticeable increase in V_H6 usage (between 8.5% and 12%). These studies were carried out on mixed age groups with limited numbers, a situation that restricts firm statistical comparisons.

Here we expand our preliminary study[39] and report on the immunoglobulin IGH gene analysis carried out on 127 adult ALL patients (245 alleles) with the use of IGH fingerprinting to define the V_H gene pattern of rearrangement. Our aim was to investigate the pattern of V_H usage in ALL patients, free of antigen-driven selection pressure, and test the hypothesis that preimmune B cells (as in adult B-lineage ALL clones) undergo random, nonbiased, nonrestricted V_H segment usage.

Patients, materials, and methods

Patient and control samples

Bone marrow (BM) specimens from 159 adults (aged 15 to 55) with B-lineage ALL were investigated as part of the UKALL XII adult leukemia trial. Between 1992 and 1999, samples were collected from patients at presentation (147 cases) or first relapse (12 cases). The majority of cases were common ALL (cALL) (93 of 159; 58%) or pre-B ALL (48 of 159; 30%); fewer had null ALL (14 of 159; 9%) or L3 B ALL (4 of 159; 2.5%).[40]

Control samples used for comparison consisted of 6 BM (BM1 through BM6) and 6 peripheral blood (PB) (PB1 through PB6) samples from healthy donors, PB from 56 patients with chronic lymphocytic leukemia (CLL), diagnostic biopsies from 36 B-cell lymphoma patients, and BM from 63 childhood ALL patients.

DNA extraction procedures

Where fresh material was available (including all normal BM and PB, approximately 60% of adult B-lineage ALL, and all CLL and B-cell lymphoma samples), mononuclear cell separation, DNA extraction, and qualitative assessment of DNA prior to polymerase chain reaction (PCR) amplification were carried out as previously described.[41] Where fresh material was not available (about 40% of adult B-lineage ALL), DNA was extracted from archival material (stained or unstained slides) as follows. Slides were washed twice, briefly, with sterile water. Cells were scraped off damp slides into 0.5 mL Dexpat suspension (Biowhittaker, Workingham, Berkshire, United Kingdom) and boiled for 10 minutes in a dry heating block. After this was allowed to cool to room temperature, a suspension was spun in a microfuge for 10 minutes at 4°C. Supernatant was then transferred to a new tube and extracted twice with an equal volume of phenol/chloroform/isopropanol (25:24:1 vol/vol), followed by one chloroform/isopropanol (24:1 vol/vol) extraction. DNA was then recovered by ethanol precipitation. Qualitative assessment of DNA prior to PCR amplification was carried out as before.[41]

PCR assay of V_H-(D)-J_H rearrangement

For material from B-cell lymphoma, CLL, and childhood ALL, assessment of IgH rearrangement (fingerprint analysis) was carried out by PCR as previously described.[39] In patients with less than 5 μg DNA total (fewer than 30% of adult ALL patients) the IgH pattern was analyzed by radionucleotide-incorporated PCR.[41] In brief, 6 PCR reactions, spiked with the radionucleotide α^{32}P–deoxycytidine triphosphate, were set up per sample, by means of 1 each of 6 sense family-specific (V_H1 through V_H6) framework 1 primers in combination with an antisense J_H primer, as previously described.[41] The V_H1 forward primer was designed to also amplify V_H7 sequences. The B-cell lymphoma samples were assayed with sense family-specific (V_H1 through V_H6) primers for the leader sequences[42] and the J_H primer described above. CLL samples were amplified as described for the adult ALL, as were samples from childhood ALL.

Cloning and DNA sequencing

After agarose or polyacrylamide gel separation, the DNA bands were excised and purified through Sephadex G50-medium columns or Jetsorb gel extraction kit (Genomed, Bad Oeynhausen, Germany), respectively. DNA was digested with the restriction enzymes included in the designed primers (*Hind*III and *Eco*RI) (Biolabs, Hitchin, Herts, United Kingdom) and then cloned into Bluescript plasmid KS+ (Stratagene, Amsterdam, The Netherlands), and DNA from colonies was prepared by means of the QIAprep Spin Plasmid Kit (Qiagen, Crawley, West Sussex, United Kingdom), following the manufacturer's recommendations. The relevant DNA fragments were sequenced by means of an automated sequencer (ABI PRISM 377, Warrington, Cheshire, United Kingdom) according to the manufacturer's specifications.

Densitometry

Normal BM, normal PB, and a small proportion of adult B-lineage ALL PCR products were quantitated by densitometry. Visible PCR bands on photosensitive films were scanned on an imaging densitometer (Biorad

GS-700, Hemel Hempstead, Herts, United Kingdom) and analyzed for optical density (Quantity One version 4.0).

Sequence analysis

The V_H-(D)-J_H sequences were analyzed on the Internet by the Immunogenetics database (http://imgt.cines.fr: 8104),[43] which identified the closest matching functional V_H and J_H segment. Those sequences that were not successfully matched were sent to BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) on the Internet for possible matches to pseudogenes. The human V_H locus as published by Matsuda et al[4] was taken to be the main reference for comparison of sequences.

Statistical analysis

Standard statistical tests were carried out (χ^2 contingency tests, parametric and nonparametric *t* tests, and parametric and nonparametric correlation) by means of the statistical programs Graphpad Prism (Software Inc, San Diego, CA) and Statistical Product and Service Solutions (SPSS) 10.0 for Windows. Poisson regression statistics for analyzing rare frequencies were calculated on SAS, and nonlinear regression curve fitting was carried out on GraphPad Prism.

Results

V_H-(D)-J_H rearrangement pattern in normal BM and normal PB

We used 6 normal adult BM samples and 6 normal adult PB samples for the amplification of V_H-(D)-J_H rearrangements by radiolabeled PCR followed by high-resolution polyacrylamide gel electrophoresis (PAGE). Amplified DNA products were found to be highly polyclonal, and evenly distributed DNA bands or ladders (average of 14 visible bands per ladder) were observed in both normal BM and PB (Figure 1), with each band within a ladder differing from the next by an estimated 1 base pair (bp). The total number of V_H-(D_H)-J_H rearrangements were estimated to be equal to or greater than the total number of PCR bands observed, since each band can be expected to carry more than one clone (total estimate: greater than 472 for normal BM and greater than 525 for normal PB specimens). Bands were quantitated by densitometry, and data were pooled for every ladder to obtain the relative frequency of usage for each V_H family (Table 2; Figure 2A-B). The intensity of bands for V_H3 and V_H4 families was always higher than those of other V_H amplifications (particularly in the BM), accounting for 50% to 70% of all V_H usage. V_H1 followed with bands of intermediate to strong intensity (12% of all products), except for PB6 (less than 4%). In contrast, V_H2, V_H5, and V_H6 bands were consistently weak (6%, 6%, and 2.6% of all products, respectively) and were often visualized only after prolonged exposure. In normal BM, the latter V_H families always ranked in the following order: V_H2 was greater than V_H5, which was greater than V_H6, and V_H5 was sometimes slightly stronger in PB (PB1 and PB5), compared with V_H2 and V_H6. V_H6 usage showed the faintest signals in all controls. The mean profiles are shown in Figure 2D. The overall ranking order of V_H family usage for normal BM and PB was in broad agreement (V_H3 was greater than V_H4, which was greater than V_H1, which was greater than V_H2, which was greater than V_H5, which was greater than V_H6), with expected theoretical profiles calculated from germline segments (rearrangeable or functional) (Table 1; Figure 2D) that have the same ranking order, except for V_H1, which is expected to be stronger than V_H4.

To compare V_H family usage profiles with expected theoretical profiles, the former were expressed as a ratio relative to the latter, with ratios near to 1 indicating similarity (Figure 3A-B). Normal BM had smaller differences from germline V_H (rearrangeable or functional) for V_H families V_H1 through V_H5, with differences not exceeding 2.2-fold in either direction. *V_H6* usage was 3- to 4-fold lower than expected although original values for actual and expected usage were low (less than 2.5%). Normal PB matched germline V_H (functional) well, with the largest deviation from the expected occurring at V_H5 (3.2-fold) although all original values were small (less than 5%). Normal BM and normal PB were also compared with each other, and profiles were found to be similar, with the largest deviations occurring at V_H6 (BM samples were 8.3-fold lower) although again all original values were low at less than 5%.

Clonality of V_H-(D)-J_H rearrangements in adult B-lineage ALL

We assayed 159 adult patients with B-lineage ALL for the presence of V_H-(D)-J_H rearrangements as described above. We found 127 patients to have one or more discrete clonal V_H-(D)-J_H rearrangements (Figure 1D), unlike the normal BM ladders previously

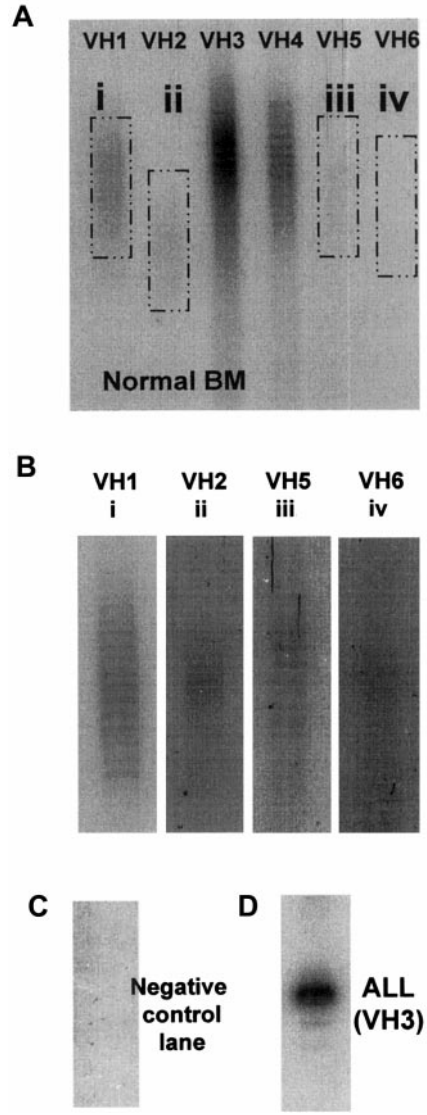

Figure 1. Typical PAGE fingerprints showing usage of V_H families in V_H-(D)-J_H rearrangements. (A) Full fingerprint of normal BM; film exposed for 24 hours. (B) Darkened sections from panel A for visualization of faint bands. (C) Film exposed for 10 days and showing blank control. (D) Adult ALL consisting of a major V_H3-positive clonal population; 24 hours' exposure.

Table 2. V_H segment usage in normal bone marrow and normal peripheral blood

	V_H families						Total V_H
	V_H1/V_H7	V_H2	V_H3	V_H4	V_H5	V_H6	
Normal BM (6 donors)							
Total reflected density of PCR ladders	575.3	210.2	2072.6	962.3	162.6	23.0	4005.9
Mean reflected density per donor	95.9	35.0	345.4	160.4	27.1	3.8	667.6
Mean relative frequencies (%)	(14.4)	(5.2)	(51.7)	(24)	(4.1)	(0.6)	
SD (CV %)	51 (53)	24 (68)	22 (6)	29 (18)	17 (61)	3 (78)	
Total no. PCR-amplified bands	90	60	126	116	60	20	472
Normal PB (6 donors)							
Total reflected density of PCR ladders	980.8	550.7	3336.1	2501.0	644.0	390.8	8403.4
Mean reflected density per donor	163.5	91.8	556.0	416.8	107.3	65.1	1400.6
Mean relative frequencies (%)	(11.7)	(6.6)	(39.7)	(29.8)	(7.7)	(4.7)	
SD (CV %)	59 (36)	38 (41)	247 (44)	197 (47)	41 (38)	48 (74)	
Total no. PCR-amplified bands	85	60	137	129	69	45	525

BM indicates bone marrow; PCR, polymerase chain reaction; CV, coefficient of variation; PB, peripheral blood.

described. In 32 ALL patients (20%), no discrete bands were detected. A clonal pattern was highest in the subtypes L3 B ALL (4 of 4; 100%), followed by pre-B ALL (43 of 48; 91%), null ALL (12 of 14; 86%), and finally cALL (68 of 93; 74%).

Of the 127 clonal patients, 60 had a single clone (47.5%), 37 had 2 clones (29%), and 30 had 3 to 6 clones (23.5%), with an average of 1.9 clones per patient (excluding those with no clones). Among patients with 2 or more discrete bands, 58 out of 67 (86.5%) had signals of different intensities (greater than 4-fold differences by densitometry), suggestive of oligoclonality, as recently described.[44] In the remaining 9 patients, 2 or more bands with similar densities were observed, and bi-allelic rearrangement could not be ruled out. Of the 245 total clones, 60 (24%) were monoclonal, 74 (30%) were biclonal, and 111 (45%) were from multiclonal cell populations. Each discrete band was taken to represent an independent clone of leukemia and was allocated to a V_H family and scored as 1.

V_H family usage in adult B-lineage ALL

As expected from the germline configuration and patterns seen in normal BM and PB, the largest number of clones identified were V_H3 clones (88 of 245; 36%), followed by V_H1 (50 of 245; 20.4%) and V_H4 (46 of 245; 18.8%) (Table 3). However, we observed that there were considerably more V_H6 clones (41 of 245; 16.7%) than V_H2 (13 of 245; 5.3%) and V_H5 (7 of 245; 2.9%), contrary to a theoretical expectation of only 0.6% to 1.6%. Information on 102 alleles (in adults) and 91 alleles in children had been previously reported in a preliminary study.[39] Except for V_H6, the profile appears to be similar to the germline V_H (rearrangable) profile.

The largest immunophenotypic sub-group of adult B-lineage ALL in this study was represented by cALL (135 of 245; 55%). V_H family usage for this group almost exactly mirrored the profile observed in ALL patients as a whole. V_H family usage in pre-B ALL (82 of 245; 33%) showed a smaller proportional representation of V_H1 (12 of 82; 14.6%) and an even greater bias toward V_H6 usage (18 of 82; 22%). Comparable patterns were observed in null ALL (20 of 245; 8%) and L3 B ALL (8 of 245; 3%).

Statistical analyses were carried out (χ^2 contingency test) comparing the actual frequencies of V_H family usage in adult B-lineage ALL with expected frequencies. The expected frequencies were calculated by redistributing the total number of ALL clones in the same proportions as either (1) mean normal BM or (2) germline V_H (rearrangeable). In both comparisons, adult B-lineage ALL was found to have a significantly different overall profile from what was expected ($\chi^2 = 50$, $P < .0001$; $\chi^2 = 39$, $P < .0001$, respectively), with deviation from the expected V_H6 usage being the greatest contributory factor for both. The profile for adult B-lineage ALL was plotted as a ratio relative to mean normal BM or germline V_H (rearrangeable) (Figure 3C). V_H6 usage was higher than expected in both comparisons (29.2-fold and 9.2-fold, respectively). This observation remained true in the cALL and pre-B ALL sub-groups (Figure 3D-E), in which the latter showed greater deviation in V_H6 usage from the expected (38.3-fold and 12.07-fold higher, respectively) than did the former (23.27-fold and 7.33-fold higher, respectively).

V_H family usage in other lymphoid malignancies

For comparison, previous data on V_H family usage in childhood B-lineage ALL (91 clones),[39] B-cell lymphoma (36 clones), and CLL (56 clones) have been displayed in Table 4 and Figure 2C. The childhood B-lineage ALL profile appears to be most similar to the adult pre-B ALL profile, with the exception that *V_H6*, though overrepresented (10 of 91; 10%), is not as greatly elevated as in the adult ALL group. Both the lymphoma and CLL profiles closely resembled the germline V_H functional profile (Figure 2D), with very low usage of *V_H6*, in B-cell lymphoma (0 of 36; 0%), compared with an expected frequency of 2.4%, whereas VH6 usage in CLL was 3.6% (2 of 56). As with adult B-lineage ALL, all 3 profiles were plotted as ratios relative to the most appropriate expected profiles (Figure 3F-H). The childhood B-lineage ALL profile had higher-than-expected *V_H6* (19.17 relative to mean normal BM; 6.04 relative to germline rearrangeable V_H) although, owing to lack of a large enough data set, statistical analysis could not be carried out. Both B-cell lymphoma and CLL had ratios close to 1 for all V_H families.

Sequence analysis of V_H-(D)-J_H rearrangements in adult B-lineage ALL

We further analyzed 77 of 254 adult B-lineage ALL clones by DNA sequencing (Table 5). The germline V_H segment counterpart of the V_H rearranged genes were identified in 65 clones. The remaining 12 could not be identified precisely, though they were clearly true V_H-(D)-J_H rearrangements (listed at bottom of Table 5). This was because the clone either (1) matched more than 3 known V_H segments equally well, so no single segment could be assigned, or (2) did not match any of the published V_H segments well (less than 90% homology) and thus could have been a derivative of an unpublished polymorphic variable. Out of the 65 identified clones, 1 (clone 17) was allocated to the polymorphic variant *V_H (5-a)*,

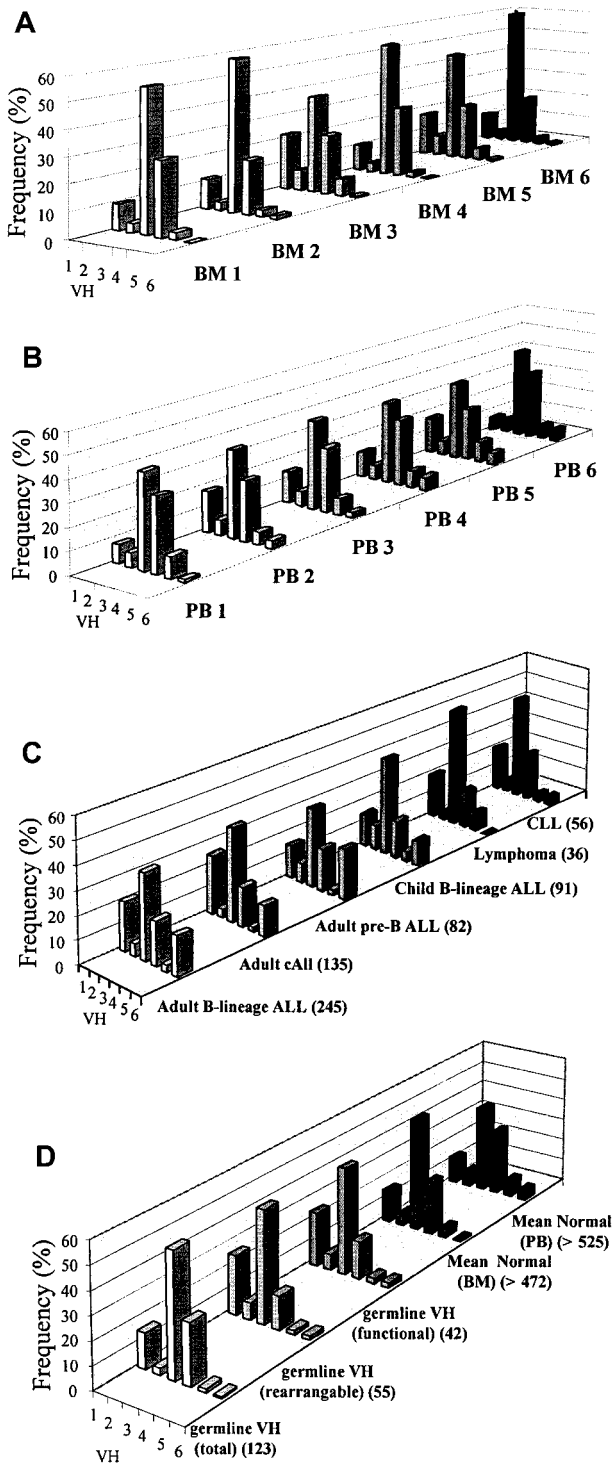

Figure 2. V_H family usage profiles. (A) Six normal adult BM samples. (B) Six normal adult PB samples. (C) Adult B-lineage ALL and other lymphoid malignancies (total alleles analyzed in brackets). (D) Expected usage (germline segments in brackets) and mean usage in normal BM and PB (estimated minimum number of clones in brackets).

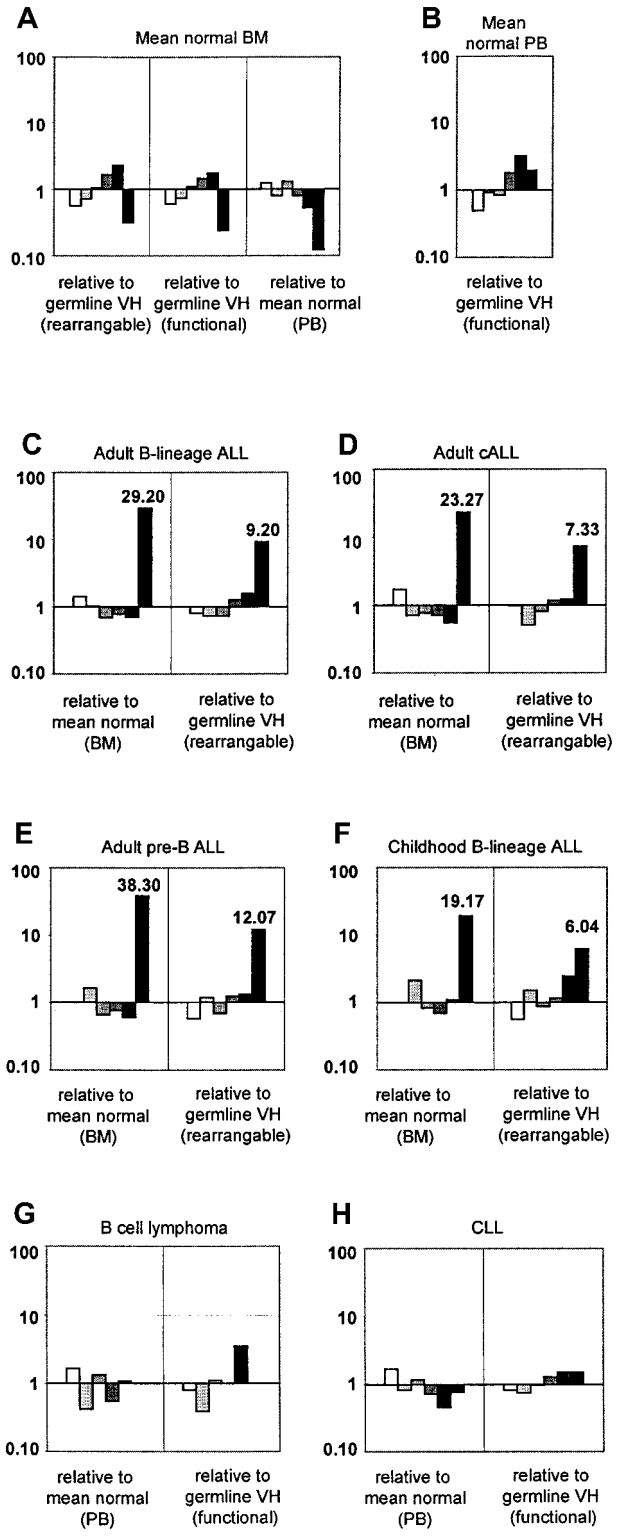

Figure 3. Relative V_H family usage profiles (ratios). Log-scale used throughout. (A) Mean normal BM relative to germline V_H (rearrangeable), germline V_H (functional), or mean normal PB. (B) Mean normal PB relative to germline V_H (functional). (C) Adult B-lineage ALL relative to mean normal BM or germline V_H (rearrangeable). (D) Adult cALL relative to mean normal BM or germline V_H (rearrangeable). (E) Adult pre-B ALL relative to mean normal BM or germline V_H (rearrangeable). (F) Childhood B-lineage ALL relative to mean normal BM or germline V_H (rearrangeable). (G) B-cell lymphoma relative to mean normal PB and germline V_H (functional). (H) CLL relative to mean normal PB and germline V_H (functional). □, VH1; ▒, VH2; ▒, VH3; ▒, VH4; ▒, VH5; ■, VH6.

which has yet to be mapped, leaving 64 clones whose matching V_H segments have been precisely mapped on the human V_H locus. Five of these matched 2 to 3 different segments equally, and all possible matches have been listed. Three clones (323, 243c, 362b) matched polymorphic segments that occur in an as-yet-unsized proportion of the human population. Where this polymorphism occurs, the locus carries 5 extra V_H segments between segments 3-30 and 4-31,

Table 3. V_H segment usage in adult B-lineage acute lymphoblastic leukemia

	No. patients with clones	No. clones in V_H families						Total no. clones
		V_H1/V_H7	V_H2	V_H3	V_H4	V_H5	*V_H6*	
Adult B-lineage ALL	127	50	13	88	46	7	41	245
(%)		(20.4)	(5.3)	(36.0)	(18.8)	(2.9)	(16.7)	
Subtypes								
Null ALL	12	4	0	4	6	2	4	20
(%)		(20)	(0)	(20)	(30)	(10)	(20)	
Common ALL	68	33	5	53	23	3	18	135
(%)		(24.4)	(3.7)	(39.3)	(17)	(2.2)	(13.3)	
Pre-B ALL	43	12	7	28	15	2	18	82
(%)		(14.6)	(8.5)	(34.1)	(18.3)	(2.4)	(22)	
B ALL	4	1	1	3	2	0	1	8
(%)		(12.5)	(12.5)	(37.5)	(25)	(0)	(12.5)	

ALL indicates acute lymphoblastic leukemia.

widening the gap between the 2 by approximately 250 kilobases (kb). We found 2 clones that matched 2 rearrangeable pseudogenes (4-55 and 3-65).

J_H segment usage

We successfully assigned known J_H segments to 70 clones (Table 5; Figure 4). In 7 other clones, the rearrangement process resulted in the deletion of a sizable section of the J_H, making it difficult to precisely identify the germline sequence. J_H4 occurred most commonly (29 of 70; 41.4%), followed by J_H5 (20 of 70; 28.6%) and then J_H6 (16 of 70; 23.9%). J_H1, J_H2, and J_H3 rearrangements were rare at between 1.4% and 2.9% (2 of 70; 1 of 70; 2 of 70, respectively).

Positional analysis of V_H gene usage in adult B-lineage ALL

Frequency of usage of specific germline V_H segments by adult B-lineage ALL (total, 64 clones) was scored (Figure 5). Only segments capable of rearranging (including pseudogenes) were considered. These included V_H genes with intact RS and promoter sequences. Segments without these elements were never found rearranged and were therefore excluded from further analysis. Note that in addition to the 55 rearrangeable segments taken from Table 1, five polymorphic segments (not reported in Matsuda et al[4]) have also been included in Figure 5. Where a clone was assigned to 2 or 3 possible segments, each possibility was given a score of 0.5 or 0.33, accordingly. We observed that 29 out of 60 (48%) rearrangeable V_H segments were used at least 0.33 times. Nonused V_H segments were scattered across the locus, including a clump of 6 adjacent segments near the J_H locus. The most J_H-proximal segment *(V_H6)* was overused (14 of 64; 22%) with a considerably higher frequency than the second most common segment (3-13; 5 of 64; 7.8%). Segments with a frequency of more than 2 tended to occur near to the J_H locus, whereas those with frequencies of 2 or lower were more often found in the midsection and upstream.

We estimated that if each segment had an equal chance of being selected for rearrangement, the expected frequency would be around 1 per segment, which differs from our observation. The hypothesis that the frequency of usage of any V_H segment is a function of its distance from the J_H locus was tested and confirmed by Poisson regression analysis and found to be correct ($P = .0009$; Figure 6A). This finding remains true, even when the extreme *V_H6* data were excluded from the calculation ($P = .0017$; Figure 6B). For simplicity, polymorphic segments were excluded from these calculations, and noninteger frequencies were truncated to the nearest integer (total frequencies equal 58 or 44 without V_H6).

We attempted to fit a simple model of nonlinear regression to our data, using the principles of one-phase exponential decay (used for defining radioactive decay over time). This model is defined by the equation below and was found to represent our data reasonably well, with and without *V_H6* data (Figure 7A-B). The equation for the curve is $Y = \text{span} \cdot e^{-K \cdot X} + \text{plateau}$, where span is the highest possible frequency, plateau is the lowest frequency, K is a unique constant, and the half-life equals $0.69/K$. For the curve shown in Figure 7B, the span equals 21, the plateau equals 0.5, K equals 0.0154, and the frequency decreased by half (half-life) every 45 kb upstream from the first segment. Thus, it has been shown that the relationship between frequency of V_H gene segment usage and distance from J_H locus can be mathematically defined.

Discussion

Mature B-cell populations

Our study analyzed V_H gene segment usage during V_H-(D)-J_H rearrangement in B cells from adult and childhood B-lineage ALL populations. We also assessed the baseline patterns in normal BM and normal PB B-cell populations, which showed no difference

Table 4. V_H gene segment usage in other lymphoid malignancies

	No. patients with clones	No. clones in V_H families						Total no. clones
		V_H1/V_H7	V_H2	V_H3	V_H4	V_H5	V_H6	
Childhood B-lineage ALL	63	13	10	39	15	4	10	91
(%)		(14.3)	(11)	(42.9)	(16.5)	(4.4)	(11)	
B-cell lymphoma	36	7	1	19	6	3	0	36
(%)		(19.4)	(2.8)	(52.8)	(16.7)	(8.3)	(0)	
CLL	56	11	3	26	12	2	2	56
(%)		(19.6)	(5.4)	(46.4)	(21.4)	(3.6)	(3.6)	

ALL indicates acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia.

Table 5. Properties of sequenced VDJ rearrangements from 78 adult B-lineage acute lymphoblastic leukemia clones

Patient (clone)	V_H family	Closest matching V_H segment*	Distance of V_H from J_H1 (kb)	Closest matching J_H segment	Age	Sex	Immunophenotype	Karyotype	WBC (log)	Time to first CR†	Clinical outcome‡
57	V_H6	*6-1*	74.31	*J_H1*	24	M	null ALL	t(4;11)	2.75	standard	CCR
355	V_H6	*6-1*	74.31	*J_H2*	40	F	null ALL	t(4;11)	2.38	standard	relapse
406	V_H6	*6-1*	74.31	*J_H4*	20	F	null ALL	t(4;11)	2.58	standard	CCR
5	V_H6	*6-1*	74.31	*J_H5*	17	M	CALL	other		standard	relapse
110	V_H6	*6-1*	74.31	*J_H4*	25	M	CALL	normal	0.23	standard	CCR
181	V_H6	*6-1*	74.31	Unclear§	29	M	CALL	normal	0.77	standard	CCR
243(a)	V_H6	*6-1*	74.31	*J_H4*	27	M	CALL	other	0.32	standard	relapse
373	V_H6	*6-1*	74.31	*J_H4*	48	F	CALL	t(1;19)			
378(a)	V_H6	*6-1*	74.31	*J_H5*	20	M	CALL	9p abn			relapse
80(a)	V_H6	*6-1*	74.31	Unclear§	31	F	pre-B ALL		1.03	standard	CCR
192(a)	V_H6	*6-1*	74.31	*J_H4*	20	F	pre-B ALL	t(4;11)	2.53	standard	CCR
192(b)	V_H6	*6-1*	74.31	*J_H5*	20	F	pre-B ALL	t(4;11)	2.53	standard	CCR
223	V_H6	*6-1*	74.31	*J_H4*	23	F	pre-B ALL	11q23 [non t(4;11)]	1.23	late	relapse
392	V_H6	*6-1*	74.31	*J_H4*	39	F	pre-B ALL	other		standard	CCR
263(a)	V_H1	*1-2*	121.36	*J_H4*	24	F	cALL		1.03	standard	CCR
321	V_H1	*1-2*	121.36	*J_H5*	18	M	cALL	HeH	1.34	standard	relapse
338	V_H1	*1-2*	121.36	*J_H4*	27	F	cALL	9p abn	0.93	standard	CCR
378(b)	V_H1	*1-2*	121.36	*J_H5*	20	M	cALL	9p abn			relapse
121	V_H1	*1-3*	139.94	*J_H4*	19	M	cALL	other	1.40	standard	relapse
123‖	V_H1	*1-3*‖	139.94	*J_H6*	16	F	pre-B ALL		0.36		relapse
126‖	V_H1	*1-3*‖	139.94	*J_H6*	17	F	pre-B ALL	9p abn	1.42	standard	relapse
362(a)	V_H4	*4-4*	146.80	*J_H5*	29	F	cALL	other	0.70	standard	relapse
26	V_H4	*4-4*	146.80	*J_H4*	25	M	pre-B ALL	t(12;21)	0.95	standard	relapse
410	V_H4	*4-4*	146.80	*J_H4*	35	F	B ALL		2.11		CCR
7(a)	V_H2	*2-5*	162.83	*J_H6*	31	M	pre-B ALL	t(9;22)	1.96	late	
222(a)	V_H2	*2-5*	162.83	*J_H4*	40	F	pre-B ALL	t(9;22)	2.05	standard	CCR
243(b)	V_H1	*1-8*	207.77	*J_H5*	27	M	cALL	other	0.32	standard	relapse
263(b)	V_H1	*1-8*	207.77	*J_H6*	24	F	cALL		1.03	standard	CCR
374	V_H3	*3-9*	220.00	*J_H4*	19	M	cALL				relapse
72	V_H3	*3-11*	221.00	*J_H4*	20	M	cALL				relapse
204	V_H3	*3-11*	221.00	*J_H6*	19	M	cALL	HeH	0.52	standard	CCR
283‖	V_H3	*3-13*‖	254.84	Unclear§	55	M	null ALL	normal	0.70	standard	CCR
76	V_H3	*3-13*	254.84	*J_H6*	21	M	cALL	normal	1.58	standard	CCR
255(a)	V_H3	*3-13*	254.84	*J_H4*	16	M	cALL				relapse
7(b)	V_H3	*3-13*	254.84	*J_H6*	31	M	pre-B ALL	t(9;22)	1.96	late	
401	V_H3	*3-13*	254.84	*J_H5*	49	F	pre-B ALL	t(9;22)		standard	relapse
112	V_H3	*3-23*	393.91	*J_H5*	22	M	pre-B ALL	t(1;19)	1.59	late	relapse
177	V_H3	*3-23*	393.91	*J_H5*	44	F	pre-B ALL	14q32 abn	0.85		
40	V_H3	*3-30*	459.71	*J_H6*	19	M	cALL		1.59	standard	
198	V_H3	*3-30*	459.71	*J_H3*	43	M	pre-B ALL	t(9;22)	0.61	standard	relapse
323	V_H4	*4-30-4* (poly)	See text	*J_H4*	20	M	cALL	other	1.74	standard	CCR
243(c)	V_H3	*3-30/3-30-5* (poly)	See text	*J_H1*	27	M	cALL	other	0.32	standard	relapse
389	V_H4	*4-31*	473.90	*J_H5*	22	F	null ALL	t(4;11)	2.30	standard	relapse
77(a)	V_H4	*4-31*	473.90	*J_H6*	24	F	pre-B ALL	6q del	1.19	standard	CCR
255(b)	V_H4	*4-39*	546.31	*J_H5*	16	M	cALL				relapse
272	V_H3	*3-43*	594.90	*J_H4*	28	F	cALL	6q del	1.65	standard	CCR
255(c)	V_H3	*3-48*	662.52	*J_H5*	16	M	cALL				relapse
408	V_H3	*3-53*	717.67	*J_H6*	18	M	cALL	other	1.99	late	relapse
299(a)	V_H3	*3-53*	717.67	Unclear§	18	F	pre-B ALL			standard	CCR
18	V_H4	*4-55* (Ps)	730.82	*J_H4*	16	F	pre-B ALL		1.36	standard	CCR
56	V_H4	*4-61*	763.82	*J_H5*	16	M	cALL	14q32 abn	0.79	standard	CCR
376(a)	V_H3	*3-64*	782.45	*J_H4*	50	F	cALL	t(9;22)	1.39	standard	relapse
327	V_H3	*3-65* (Ps)	790.80	*J_H4*	36	M	cALL		0.36	standard	CCR
289	V_H1	*1-69*	838.62	*J_H5*	20	M	cALL		1.08	late	CCR
376(b)	V_H1	*1-69*	838.62	*J_H4*	50	F	cALL	t(9;22)	1.39	standard	relapse
7(c)	V_H1	*1-69*	838.62	*J_H6*	31	M	pre-B ALL	t(9;22)	1.96	late	
296	V_H2	*2-70*	847.52	Unclear§	40	F	pre-B ALL	12p abn	1.05	late	CCR
11	V_H2	*2-70*	847.52	*J_H4*	19	M	B ALL		2.19	standard	relapse
134	V_H3	*3-73*	879.65	*J_H3*	48	M	B ALL	14q32 abn	2.74	late	relapse
47¶	V_H4	*4-4*¶	146.80	*J_H5*	26	M	cALL	other	1.01	late	CCR
		4-61¶	763.82								

Table 5. Properties of sequenced VDJ rearrangements from 78 adult B-lineage acute lymphoblastic leukemia clones (Cont'd)

Patient (clone)	V_H family	Closest matching V_H segment*	Distance of V_H from J_H1 (kb)	Closest matching J_H segment	Age	Sex	Immunophenotype	Karyotype	WBC (log)	Time to first CR†	Clinical outcome‡
187¶	V_H2	*2-5*¶	162.83	*J_H4*	24	F	cALL				relapse
		2-26¶	426.35								
		2-70¶	847.52								
195¶	V_H4	*4-34*¶	498.28	*J_H6*	35	M	cALL	t(9;22)	1.60	never	
		4-39¶	546.31								
262¶	V_H4	*4-39*¶	546.31	*J_H5*	54	M	null ALL	t(4;11)	2.34	late	CCR
		4-59¶	751.94								
362(b)¶	V_H3	*3-30*¶	459.71	*J_H4*	29	F	cALL	other	0.70	standard	relapse
		3-30-3 (poly)¶	See text								
		3-30/3-30-5 (poly)¶	See text								
17	V_H5	*5-a*(poly)#	—	*J_H6*	16	F	pre-B ALL				relapse
71	V_H	Unclear**	—	*J_H4*	16	M	cALL		0.63	standard	CCR
241	V_H	Unclear**	—	Unclear¶	16	F	cALL	12p abn	1.91	standard	relapse
248	V_H4	Unclear**	—	*J_H4*	21	F	cALL	HeH	0.40	standard	CCR
347(a)	V_H1	Unclear**	—	*J_H5*	27	M	cALL	t(12;21)	1.60	standard	relapse
347(b)	V_H3	Unclear**	—	*J_H4*	27	M	cALL	t(12;21)	1.60	standard	relapse
77(b)	V_H3	Unclear**	—	*J_H6*	24	F	pre-B ALL	6q del	1.19	standard	CCR
80(b)	V_H1	Unclear**	—	*J_H5*	31	F	pre-B ALL		1.03	standard	CCR
130	V_H	Unclear**	—	*J_H5*	30	F	pre-B ALL	t(9;22)	1.89	late	CCR
149	V_H	Unclear**	—	Unclear¶	19	M	pre-B ALL	11q23 [non t(4;11)]	2.07	standard	relapse
222(b)	V_H3	Unclear**	—	*J_H4*	40	F	pre-B ALL	t(9;22)	2.05	standard	CCR
299(b)	V_H4	Unclear**	—	*J_H6*	18	F	pre-B ALL			standard	CCR
375	V_H4	Unclear**	—	*J_H6*	19	M	pre-B ALL	HeH	0.48	standard	relapse
244	False††	False††	—	False††	50	F	null ALL	t(4;11)	1.99	standard	CCR

ALL indicates acute lymphoblastic leukemia; cALL, common ALL.
*All named matches have less than 97% homology with no more than 3 mismatching base pairs, except those marked with ‖.
†Standard time to CR is up to 42 days.
‡Patients followed for at least 24 months or up to first relapse.
§Clones were found to match more than 1 J_H segment equally well; therefore, a J_H segment was not assigned.
‖These matches were relatively poor (90% to 95% homology) with between 8 and 10 mismatching base pairs.
¶Clones were found to match 2 to 3 V_H segments equally well; all possibilities are listed.
#This polymorphic V_H segment has not been accurately mapped at present.
**Clones were found to match more than 3 V_H segments equally well; therefore, a V_H segment was not assigned.

from the predicted profiles. The patterns in chronic lymphoid malignancies (in CLL) and lymphomas were also assessed and found to conform to profiles derived from mature post–antigen-stimulation B-cell profiles.[16-21,37,45-48]

By contrast, adult B-lineage ALL clones, taken as models of progenitors and precursors of B cells, showed a statistically significant deviation from the expected pattern, with overrepresentation of *V_H6* rearrangements and use of J_H-proximal segments. We showed that the relationship between frequency of usage and location on V_H locus can be mathematically defined by one-phase exponential decay whereby the frequency of usage halves with every 45-kb distance away from J_H proximity. It could be argued that the unexpected usage of individual V_H genes are a result of the leukemic process' driving an unnatural pattern of V_H rearrangement, rather than a reflection of truly normal preimmune B-cell events. We believe that our data reflect what could be a normal pattern of IGH rearrangements in pre–antigen-stimulation B cells, as the data do not differ from those in studies of fetal human and mouse B cells. The difference observed between BM or PB and the ALL group reflects a biological rather than a disease-related difference, as we first suggested in a preliminary study.[49] Comparing our results with previous reports, we did not find overuse of V_H5 (previously described as *V_H251*) in ALL[50] or V_H (4-34), V_H (4-39), V_H (4-59), V_H (3-23), and V_H (3-30) in fetal bone marrow.[28]

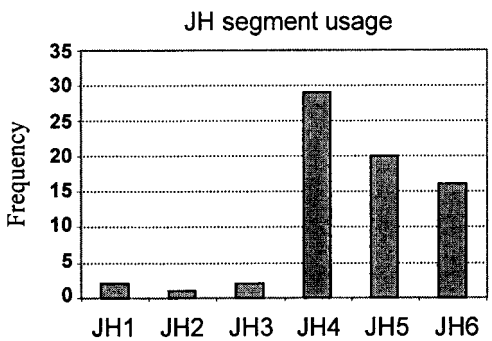

Figure 4. J_H gene segment usage in 70 sequenced adult B-lineage ALL clones. The J_H usage is expressed as the percentage of all alleles studied and is illustrated by vertical bars.

The V_H6 gene

The highly polymorphic human V_H region is thought to have arisen prior to racial divergence and has been stable for at least 30 000 years.[51] However, *V_H6* is unusual in being the sole member of its family with no polymorphic variables in different individuals[52] and racial groups.[51] Lack of genetic polymorphism in the 30-kb area around the *V_H6* segment is in sharp contrast to the expected occurrence of a restriction fragment length polymorphism every 100 to 300 bp.[53] A relative lack of repetitive elements in the same area is also unusual.[4,54] It has been suggested that *V_H6* is protected

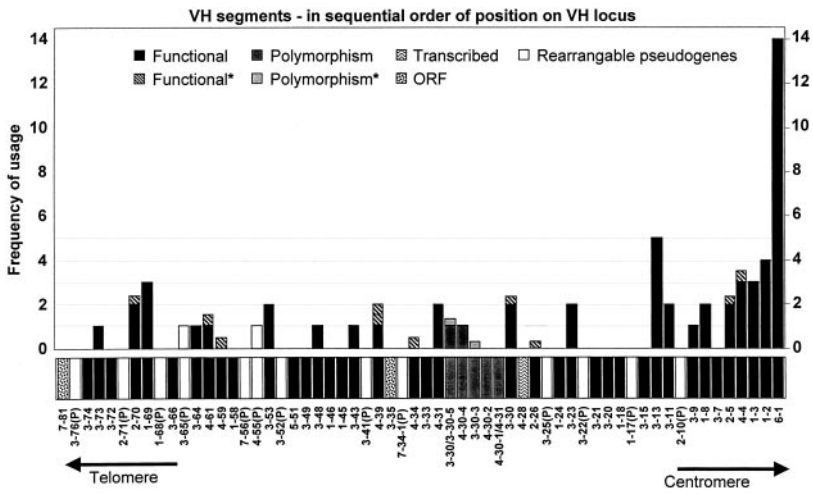

Figure 5. V_H gene segment usage in 63 sequenced adult B-lineage ALL clones. Only rearrangeable segments have been shown. Asterisk indicates that clone matched 2 to 3 segments in total, and each segment has been given a score of 0.33 or 0.5, accordingly.

from mutations by strong selection pressure to maintain this area intact.[51] Compelling studies of the nature of the *V_H6* promoter have revealed that it has many unique features not shared by the other V_H segment promoters. Promoters of V_H genes contain one consistent transcription-factor–binding site, in addition to the TATA box. This octamer motif, with a consensus sequence ATGCAAAT, is found approximately 70 bases upstream of the transcription initiation site. The motif is perfectly conserved in all but 3 of the 42 functional V_H segments (octamers for the segments 3-20, 3-53, and *V_H6* have 1- to 2-bp variations on the consensus). The presence of an octamer is an absolute requirement for tissue-specific transcription of V_H segments in vivo and in vitro,[55-57] and a single base change prevents the binding of the Octamer-2 (a B-lineage–specific transcription factor).[58] Sun and Kitchingman[59-60] have shown that the imperfect *V_H6* octamer, with a single base-pair change (AgGCAAAT), lacks B-cell specificity and transcribes in an enhancer-independent fashion, has bidirectional transcriptional capabilities, and has greater transcriptional strength. Furthermore, non–B-cell silencer activity, as found in one of the V_H2 promoters, was not found in *V_H6*. These data suggest that the *V_H6* promoter is under a different transcriptional control compared with other V_H promoters. *V_H6* promoter is often the first V_H promoter activated during ontogeny and is also overrepresented in artificially activated resting small B cells.[19,61]

Our study describes, with statistical relevance, the overusage of the *V_H6* segment during V_H-(D)-J_H rearrangement, which we had previously noted in ALL.[39] Our work supports the idea that *V_H6* has special properties, such as marking the cells ready to undergo true semi-random rearrangement. Of note, there is a non-V_H gene (KIAA0125) near the 3′ side of *V_H6*[4] in the reverse orientation to *V_H6*, which has an extremely short coding region but extensive 5′ and 3′ untranslated regions and shows lymphoid-restricted expression,[62] with as-yet-unidentified function.

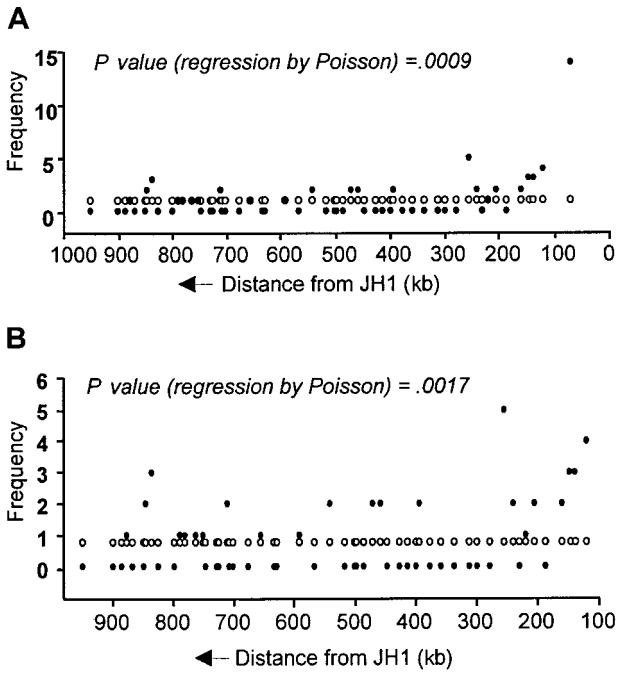

Figure 6. Comparison of expected and actual V_H gene segment usage in sequenced adult B-lineage ALL clones in relation to distance upstream from J_H locus. (A) All clones. (B) Excluding V_H6 clones. ●, actual frequency; ○, expected frequency.

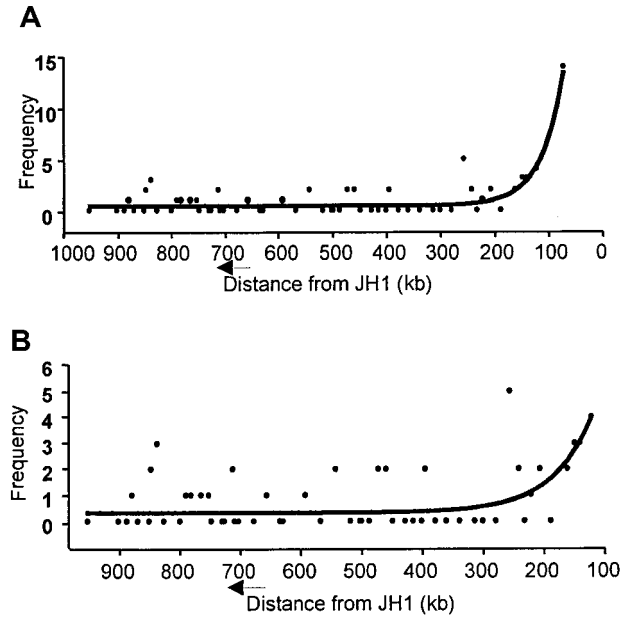

Figure 7. Nonlinear regression model of V_H usage across distance upstream of J_H1, using the principles of one-phase exponential decay. (A) Frequency of V_H gene usage including the V_H6. (B) Frequency of V_H gene usage excluding the V_H6 gene.

V_H pseudogenes

We were interested in analyzing how often pseudogenes, which retained both their abilities to transcribe and rearrange with intact promoters and RS sequences (13 of 81; 16%) but failed to yield productive proteins, were used. Although pseudogenes make up a sizable proportion of the total rearrangeable segments (13 of 55; 24%), only 2 (3%) of such pseudogenes were found rearranged in our study. From an examination of their locations in the V_H locus (Figures 5 and 6), it can be seen that the majority of the rearrangeable pseudogenes (9 of 13; 69%) lie at 500 kb or more from J_H1. This area corresponds to the plateau of the exponential-decay usage curve; therefore, a low rate of usage can be expected. Even so, pseudogene usage appeared to be lower than expected. Although all V_H segments with intact promoters and RS sequences can rearrange, restriction selection of wasteful V_H-(D)-J_H recombinations may occur.

J_H segment usage

We found that J_H4, J_H5, and J_H6 were greatly overrepresented, in keeping with most previous reports in childhood ALL[63-65] and adult PB,[66] with the exception that they all report a predominance order of J_H4 being greater than or equal to J_H6, which is greater than J_H5, whereas we find an order of J_H4 greater than J_H5, which is greater than J_H6. Preimmune cells from fetal liver use J_H3 and J_H4, with little J_H5 and very little J_H6 and J_H1.[29-30] Cuisiner and colleagues[24] revealed that although the overall order of preference in fetal liver was $J_H3 = J_H4$, which is greater than $(J_H1 = J_H2 = J_H5 = J_H6)$, on close inspection, J_H6 usage, which was otherwise rare, strongly associated with V_H6 usage. On the contrary, none of the J_H6-positive clones in adult B-lineage ALL were V_H6 positive (Table 5). This is good evidence that the increased V_H6 usage observed in adult preimmune cells is unrelated to that seen in fetal B cells and that separate mechanisms give rise to the observed repertoires.

In summary, we have shown that the profiles of V_H gene family usage in normal BM and PB are in broad agreement with theoretical values calculated under the assumption that all rearrangeable V_H segments have an equal probability of being selected. Furthermore, B-lineage cells from clonally expanded populations from B-cell lymphoma and CLL conform to this pattern. However, clones from early B-cell progenitors of precursor populations (from ALLs) do not conform to this pattern, so the assumption made does not hold true. Detailed analyses have revealed that rate of V_H usage is proportional to proximity to the J_H locus. It is plausible that most B cells first undergo rearrangement preferentially using J_H-proximal V_H, and that further rounds of V_H recombining with (D)-J_H occur later on, with the effect of normalizing the observed V_H repertoire. The final repertoire would appear unbiased with all V_H segments represented in their correct proportions, excluding a role for this phenomenon in leukemogenesis.

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