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Rearrangement status of the malignant cell determines type of secondary IgH rearrangement (V-replacement or V to DJ joining) in childhood B precursor acute lymphoblastic leukemia

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Immunoglobulin heavy chain (IgH) oligoclonality in childhood B precursor acute lymphoblastic leukemia (ALL) as determined by Southern analysis is found in 30–50% of patients and has been shown to be the result of ongoing IgH rearrangement (mostly V_H-replacement and V_H to D–J_H joining) after malignant transformation. It is unknown however, what determines the type of secondary rearrangement. Also the biological basis of the variable degree of oligoclonality observed in childhood ALL is poorly understood. We analyzed in detail the IgH rearrangement status of the leukemic cells for a random panel of 18 childhood B precursor ALL patients by polymerase chain reaction (PCR)/sequencing analysis and by Southern analysis. By Southern analysis 10/18 (55.6%) patients were considered oligoclonal and 8/18 (44.4%) monoclonal. In contrast, by PCR minor clonal rearrangements were detected in 14/18 (77.8%) patients. V_H-replacement was found in 7/14 patients, V_H to D–J_H joining in 6/14 patients and an unusual type of secondary rearrangement, V_H–D to J_H joining, in one patient. Only a single type of secondary rearrangement was detected in each patient. The type of secondary rearrangement (V_H-replacement or V_H to D–J_H joining) depended on the rearrangement status (VDJ/VDJ or VDJ/DJ, respectively) of the dominant leukemic clone as determined by Southern analysis. We found that in addition to a more 'advanced' IgH rearrangement status patients with V_H-replacements also have a more 'advanced' TCRδ rearrangement status, which possibly reflects exposure of both the IgH locus and the TCRδ locus to recombinase activity in a preleukemic clone. Finally, we investigated a putative relationship between oligoclonality by Southern analysis and S-phase fraction of the leukemic cell population. We found a significantly lower percentage cells in S-phase for oligoclonal patients as compared to monoclonal patients. Our data add to the understanding of ongoing rearrangement of antigen receptor loci in childhood ALL and have implications for the monitoring of minimal residual disease by PCR.

Keywords: MRD; childhood B precursor ALL; PCR; oligoclonality

Introduction

Somatic recombination of the immunoglobulin heavy chain (IgH) variable (V_H), diversity (D) and joining (J_H) gene segments is a key event in early B cell differentiation.[1] Recombination occurs in an ordered sequence, D to J_H joining precedes V_H to D–J_H joining,[2] and is mediated by recombination signal sequences (RSS) which flank the different gene segments.[3] Expression of the recombination activating genes 1 and 2 (*RAG 1/2*) has been shown to be sufficient for the initiation of recombination in fibroblasts.[4] Constitutive expression of *RAG 1/2* has been reported for childhood B precursor acute lymphoblastic leukemia ALL.[5–7]

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B precursor ALL arises from the malignant transformation and clonal expansion of a single B precursor cell which is in the process of IgH recombination. This should lead to monoclonal IgH rearrangement patterns by Southern analysis. However, in 30 to 50% of childhood B precursor ALL multiple rearranged bands (ie more bands than chromosome 14 copies per cell) are observed at diagnosis.[8–10] We and others have shown that IgH oligoclonality by Southern analysis is mechanistically due to ongoing recombination (V_H-replacement, V_H to D–J_H joining, or *de novo* rearrangement of a germline allele) of the IgH loci in the leukemic cell population.[11–14] It is unknown however, what determines the type of secondary rearrangement. This issue is important for the monitoring of minimal residual disease (MRD) by polymerase chain reaction (PCR) as the usefulness of this approach depends largely on the stability of the chosen tumor marker(s). Secondly, it is also unknown what determines the variable degree of IgH oligoclonality in childhood ALL. Possibly, in some patients clones marked by different IgH rearrangements also possess a relative growth advantage. Alternatively, the variable degree of oligoclonality observed between patients may merely reflect differences in kinetic behavior of the leukemic cell populations. A better understanding of the nature of oligoclonality may provide a rational basis for the choice of optimal tumor markers in case of multiple rearrangements at diagnosis.

We previously analyzed IgH rearrangements in four selected patients that all presented with an oligoclonal IgH rearrangement pattern by Southern analysis.[14] The data we obtained suggested that only a single type of secondary rearrangement occurs in each patient and that the type depends on the rearrangement status of the malignant cell. We now confirm and extend these findings by the detailed analysis of the IgH rearrangement status for a random panel of 18 childhood B precursor ALL patients. In addition, to investigate the relationship between kinetic behavior of a leukemic cell population and oligoclonality, rearrangement data were correlated with S-phase fraction.

Methods

Patient materials

Bone marrow samples from 18 childhood B precursor ALL patients were obtained at diagnosis or first relapse for routine diagnostic purposes at the Emma Kinder Ziekenhuis, Amsterdam. Informed consent was obtained according to the rules of the Academic Medical Center, Amsterdam.

All bone marrow samples contained over 90% leukemic cells.

Immunophenotypic analysis was routinely performed using standard techniques. The patient group consisted of one pro-

B ALL ($CD19^+$, $CD10^-$, $cIgM^-$), 13 common ALL ($CD19^+$, $CD10^+$, $cIgM^-$) and four pre-B ALL ($CD19^+$, $CD10^+$, $cIgM^+$).

Cytogenetic analysis was routinely performed at diagnosis, using both the direct method and the 24-h culture method.

Southern analysis

The mononuclear cell fraction was isolated by Ficoll–Hypaque (1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density gradient centrifugation prior to cryopreservation. High molecular weight DNA was extracted from cryopreserved BM samples according to standard techniques.[15] Ten micrograms of DNA was digested with the appropriate restriction endonucleases (Promega, Madison, WI, USA) and transferred onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany). Hybridization and washing conditions were as described previously.[16] The H24 probe is a 2.8-kb *Eco*RI–*Bgl*II J_H fragment.[17] The Jδ1 probe is a 1.5-kb *Sac*I–*Sac*I fragment[18] (kindly provided by Dr JJM van Dongen, Erasmus University, Rotterdam, The Netherlands). The 3′-BCR probe is a 1.2-kb *Hin*dIII–*Bgl*II fragment[19] (kindly provided by Dr D van der Plas, Erasmus University, Rotterdam). The VH6 probe is a 0.4-kb PCR-amplified probe spanning the VH6 coding region starting in FR1. The VH6/3′-BCR ratio was determined by densitometry (Quick Scan, Helena Laboratories, Beaumont, TX, USA; Software: Chrompack-Packard, Shimadzu Corporation, Kyoto, Japan) and served as relative measure for the proportion partially rearranged IgH loci.

PCR reactions

Major clonal rearrangements: V_H–D–J_H and D–J_H rearrangements were considered major clonal rearrangements if visible on ethidium bromide stained gels after 30 cycles of PCR amplification with 1 μg template DNA. Eleven different PCR reactions were performed for each patient. V_H–D–J_H rearrangements were amplified with consensus framework region three primer FR3 in combination with consensus joining primer JH21. In addition, V_H–D–J_H rearrangements were amplified with V_H-family-specific framework region one primers VH1–VH6 in combination with consensus joining primer JH26. Primers and PCR conditions for the FR1 PCR and FR3 PCR have been described previously.[14] D–J_H rearrangements were amplified with D-family-specific primers homologous to the 5′-RSS region (DLR 5′-GTGGGGGCTCGTGTCACTG-3′, DXP 5′-TTGGGGTGAGGTCTGTGTC-3′, DN 5′-GAAGGTGTCTGTGTCACAG-3′, DK5′-GTCAGGGGGTGTCAGACTG-3) in combination with consensus joining primer JH21. For patient p81 primer DN-Reverse (5′-CTT- GGAATGGGGTTTCTGGCTGGG-3′) homologous to the 3′-RSS was used in combination with primer FR3 for the amplification of the V_H–DN1 rearrangement. PCR conditions for the D–J_H/V_H–D PCR were as described for the FR3 PCR.

Sequencing reactions: PCR products were directly sequenced with the BRL cycle sequencing kit (BRL, Gaithersburg, MD, USA), using one of the PCR primers end-labeled with ^{32}P (Amersham International, Buckinghamshire, UK) as suggested by the manufacturer.

Amplification of minor clonal rearrangements: Selective amplification of minor clonal rearrangements was achieved applying a two-round semi-nested PCR approach. For each patient the first round of amplification was performed with a mixture of all V_H-family-specific FR1 primers, omitting the primer(s) that amplified a major clonal rearrangement, in combination with consensus joining primer JH26. Thirty cycles were performed with PCR conditions as described for the FR1 PCR. Ten microliters of PCR product was subjected to electrophoresis on a 2% agarose gel in order to separate the PCR product from the template genomic DNA. The appropriate area of the gel (300–450 bp) was excised. PCR product was recovered using the Qiaex gel extraction kit (Diagen, Düsseldorf, Germany) according to the manufacturer's instructions, and dissolved in 25 μl water. One microliter of first-round PCR product served as template for 30 cycles of second-round amplification with primers FR3 and JH21, as described. Second-round PCR product was subcloned into pGEM-T vector using the pGEM-T vector cloning system (Promega) according to the manufacturer's instructions. Ten positive colonies were screened by one lane sequencing analysis (G-tracking) with primer JH21 using the BRL cycle sequencing kit (BRL). Different clones with D–J_H or V_H–D junctional region identity were completely sequenced.

Analysis of third complementarity determining regions (CDR3)

CDR3 sequences were compared to published germline D[20–23] and J_H sequences[24] with the algorithms of the Microgenie DNA software (Intelgenetics, Mountain View, CA, USA). Assignment criteria were as described previously.[25]

Oligonucleotide hybridization

In order to test for the presence of specific D–J_H or V_H–D junctional regions, PCR product was hybridized to D–J_H or V_H–D junctional region oligonucleotide probes using a liquid hybridization technique, as described previously.[26]

Determination of S-phase fraction

The percentage of cells in S-phase was determined by flow cytometry (FACSstar, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA; Software: Modsit 5.0, Ferity) on ethidium bromide stained cells as described previously.[27]

Results

We performed a detailed analysis of the IgH rearrangement status of the leukemic blasts for a panel of 18 childhood B precursor ALL patients (15 at diagnosis and three at relapse). Patients were chosen randomly provided that sufficient cryopreserved cells were available. In addition S-phase fraction of the leukemic cell population was determined.

IgH subclones in the majority of B precursor ALL

Southern analysis of *Bgl*II and *Bam*HI/*Hin*dIII digested DNA was performed with the H24 JH-probe (Table 1). On the basis of Southern analysis a distinction between monoclonal and

a

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33-A*   TGCAAAAGA                                TAGTGCAGCTCGTC atac tACTTT (n4-j4b)
33-A2*  TGCGAGAGAt  ttagga                                 TC atac tACTTT    (j4b)

33-B*   TGCAGGA     cgggg          GGTTCGGGGAGTTATTAT tggcaaat TTTGA (xp'1-j4b)
33-B2*  TGCGAGAGAt  ggaa    acggggGGTTCGGGGAGTTATTAT tggcaaat TTTGA (xp'1-j4b)


36-A*   TGCGAAAGA    tcccgccgg               ATTACGATATTTTGACTGGT cctc gtACTAC (xp1-j4b)
36-A2   TGCGAGAGCgc  gcgccctagt  agatcccgccggATTACGATATTTTGACTGGT cctc gtACTAC (xp1-j4b)

36-B*   TGCGAGAG     tgagtttccc                          TGGGAGG ccaacgtt ACTGG (dir-j5)
36-B2   TGCGAGAGC    ccc                 cgagagtgagtttcccTGGGAGG ccaacgtt ACTGG (dir-j5)
36-B3   TGCGAGATAt   tgg                      gtgagtttcccTGGGAGG ccaacgtt ACTGG (dir-j5)


42-A*                          RSS-GGGTATAGCAGCAGCTGGT ACTGG (n1-j5b)
42-A2*  TACTAGA     cccgg               ATAGCAGCAGCTGGT ACTGG (n1-j5b)
42-A3   TGCGG       tcaggaatt              CAGCAGCTGGT ACTGG (n1-j5b)

42-B*   TGCGAGAGGG  g                        GCAGCTC gtACTAC (n4-j4b)


43-A*   TGCGAGAGAt  ggagttaggt                  TATAGCAGCTCGTCC c GGCcACGA gggc CCTGG (n4-k1-j5b)
43-A2   TGCAAAAGAt  ataggcg     gagagatggagttaggtTATAGCAGCTCGTCC c GGCcACGA gggc CCTGG (n4-k1-j5b)
43-A3   TGCAAAAG    tcgg          gagatggagttaggtTATAGCAGCTCGTCC c GGCcACGA gggc CCTGG (n4-k1-j5b)
43-A4   TA          ggaagg        gagatggagttaggtTATAGCAGCTCGTCC c GGCcACGA gggc CCTGG (n4-k1-j5b)

43-B*   TGCGAGAG    gggtc                                                  TATCGgA AAAAC (xp1-j5b)

43-C*   TGCAC       ttttcttg                                GCcGGTACg gggtgggaagccc GACTA (dn1-j4b)

43-D*   TGCAAGAG    cttcgggggggga                                                    GGGCA (j6c)
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b

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44-A*                    RSS-AGCATATTGTGGTGAcTGCTA ccgaaattcgg ACTGG (lr3-j5b)
44-A2   TGCAAGAC  c                TTGTGGTGGAcTGCTA ccgaaattcgg ACTGG (lr3-j5b)
44-A3   TGCGAGAG  ggggg                GGTGGAcTGCTA ccgaaattcgg ACTGG (lr3-j5b)
44-A4   TGCAAGAG  ggga                   GGAcTGCTA ccgaaattcgg ACTGG (lr3-j5b)

44-B*   TGCGAGAG  aaggcc   TTACGATTTTTGGAGTGGTTA cccaaacgtttac TACTA (xp4-j6c)


45-A*  TGCGAGAGAt                                          ccc        CGAT5GGAGTGGTT ATTAC (xp4-j6c)
45-A2  TGCAAGAGA                                       cccaccc  GTATTACGAT5GGAGTGGTT ATTAC (xp4-j6c)
45-A3  TGCAAG ggggg   ctAGGATATTGTAGTAGTACCAGCTG      ccgattct  GTATTACGAT5GGAGTGGTT ATTAC (xp4-j6c)
45-A4  TGCAAGAGA cca         ATTGTAGTAGTACCAGCTG      ccgattct  GTATTACGAT5GGAGTGGTT ATTAC (xp4-j6c)
45-A5  TGCAAGAG gg             TGTAGTAGTACCAGCTG      ccgattct  GTATTACGAT5GGAGTGGTT ATTAC (xp4-j6c)
45-A6  TGCAAGAG cgg         TATTGTAGTAGTACCAGC    attccctcgtcc   TATTACGAT5GGAGTGGTT ATTAC (xp4-j6c)
45-A7  TGCAAGAG ggg          ATTGTAGTAGTACCAGCTG  ctatgcggcggg    ATTACGAT5GGTGTGGTT ATTAC (xp4-j6c)
45-A8*       (lr4) RSS-AGGATATTGTAGTAGTACCAGCTGTT         ttc  GTATTACGAT5GGTGTGGTT ATTAC (xp4-j6c)
45-A9  TGCAAGAGAtc gg     GATATTGTAGTAGTACCAGCTGTT         ttc  GTATTACGAT5GGTGTGGTT ATTAC (xp4-j6c)
45-A10 TGCGAGAGAtc gcc     ATATTGTAGTAGTACCAGCTGTT         ttc  GTATTACGAT5GGTGTGGTT ATTAC (xp4-j6c)
45-A11 TGCAAGAG t            ATTGTAGTAGTACCAGCTGTT         ttc  GTATTACGAT5GGTGTGGTT ATTAC (xp4-j6c)
45-A12 TGCAAGAG ggaggg         TGTAGTAGTACCAGCTGTT         ttc  GTATTACGAT5GGTGTGGTT ATTAC (xp4-j6c)
45-A13*TGCAAGAAA ggc          TTGTAGTAGTACCA    cctcctattcggcg  GTATTACGAT5GGTGTGGTT ATTAC (xp4-j6c)

54-A*                             RSS-GTATTACGATATTTTGACTGGTTATTATA cgtt TACTA (xp1-j6a)
54-A2*  TGCGAGAGA  anncc              GTATTACGATATTTTGACTGGTTATTATA cgtt TACTA (xp1-j6a)
54-A3   TGCGAAAAG  gtcgaga              ATTACGATATTTTGACTGGTTATTATA cgtt TACTA (xp1-j6a)


57-A*                      RSS-AGCATATTGTGGTGGTGA ccttattctgccacccaca ATTAC (lr3-j6b)

57-A2*  TGC         ctgaaggtcttt                                                  (j6b)
57-A3   TGCAAAAGAt attgc            ATTGTGGTGGTGA ccttattctgccacccaca ATTAC (lr3-j6b)
57-A4   TGC         tcctga                GTGGTGA ccttattctgccacccaca ATTAC (lr3-j6b)


74-A*   TGCG  ggggt            TTACT (j6)
74-A2   TAC   ccagg    cgggggtTTACT (j6)
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oligoclonal was made. Restriction fragment length patterns were considered oligoclonal if either the number of bands exceeded the number of chromosome 14 copies per cell or if there were extreme differences in intensity of the bands, indicating the existence of subpopulations. For patient p45 the assignment of oligoclonal/monoclonal could not be made on the basis of the above criteria alone as data on the karyotype was not available. However, this leukemia was considered oligoclonal as sequencing data showed that all three predominant clonal rearrangements (45-A, -A8, -A13) were related by ongoing rearrangement (presented later). On the basis of Southern analysis 10/18 patients were considered oligoclonal

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C  75-A*   TTACTG     gaa          AGCAGCTCG ATTAC (n4-j6b)
   75-A2   TGCGAGAG           ggaaAGCAGCTCG ATTAC (n4-j6b)
   75-A3   TGC           ttactggaaAGCAGCTCG ATTAC (n4-j6b)
   75-A4   TGC       actaag   ggaaAGCAGCTCG ATTAC (n4-j6b)
   75-A5   TGCGAGAGAt ag      ggaaAGCAGCTCG ATTAC (n4-j6b)

   76-A*   TGCGAGAA    ccgccttatat      ATATTGTACTAATGGTG gtatgctat ACTGG (lr1-j2)

   76-B*                          RSS-GTGGATATAGTGGCTACGATT ggttcga ATGGA (k1-j6b)
   76-B2   TGCGAGAGAt  ttc                     GTGGCTACGATT ggttcga ATGGA (k1-j6b)
   76-B3   TACTGTT     cc                    AGTGGCTACGATT ggttcga ATGGA (k1-j6b)
   76-B4   TGC         tccg                   GTGGCTACGATT ggttcga ATGGA (k1-j6b)

   78-A*   TGCAAGAGAt    gggcggatggg                        TTGTAGTAGTACCAGCTGCTAT tggg TACTG (lr4-j5)
   78-A2   TGCGAGACA    gcacggccaccccgaa  aagagatgggcggatgggTTGTAGTAGTACCAGCTGCTAT tggg TACTG (lr4-j5)
   78-A3   TGCGAGAC     gccggggcgac             ggcggatgggTTGTAGTAGTACCAGCTGCTAT tggg TACTG (lr4-j5)

   79-A*   TGCAAGAGGc  gag       TACTATGGTggGGGGAGTTATG gggggggc TACTA (dxp'1-j6)

   79-B*                              RSS-GTATTACGATTTTTGGA taggg TACTT (xp4-j4)
   79-B2   TGCAAG    cggggtaactgg                                    AACCG   (j4/5)

   81-A*   TGCGAGACA    gac    (dxp'1b) CTATGGTTCGGGGAGT   gatatac       GGTTC  (j5)

   81-B    TGCAAAGA     ag     (n1) AGCAGCTG               tcccccct      TTCGA  (j5)
   81-B2   TGCAAAGA     ag     (n1) AGCAGCTG               cccagggtc     CTGGT  (j5)
   81-B3   TGCAAAGA     ag     (n1) AGCAGCTGGTAC           atgg          TGGTT  (j5)
   81-B4   TGCAAAGA     ag     (n1) AGCAGCTGGTA                          ACAAC  (j5)
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Figure 1 Nucleotide sequence analysis of predominant clonal rearrangements and related minor clonal rearrangements. Shown are the nucleotide sequences of V_H–D–J_H junctional regions. Only the first five nucleotides of the J_H region are shown. Related sequences are shown in groups and labeled A, B, C and D. Sequences are fractionated into V_H-, D- and J_H-region (upper case) and first and second N-region (lower case). Nucleotides that differ from the published V_H, D or J_H germline sequence are shown in lower case. At the V_H–D junction, N-nucleotides derived from pre-existing rearrangements by V_H-replacement are shown directly adjacent to the D-region, separate from the 'true' N-nucleotides. Palindromic (P) nucleotides[35] at the V_H–N or N–J_H junction are shown directly adjacent to the V_H- or J_H-region (lower case). Sequence homologies at the D–J_H junction are underscored. Major clonal rearrangements are indicated with *. RSS, recombination signal sequence; n, unreadable base pair at that position.

and 8/18 were considered monoclonal (Table 1). In order to identify minor clonal rearrangements below the Southern analysis detection level, and also to determine the type of secondary IgH rearrangement, we systematically PCR amplified and sequenced V_H–D–J_H and D–J_H rearrangements. A panel of six V_H-family specific FR1 primers (VH1–6), four D-family specific primers (DLR, DXP, DN, DK) and a consensus V_H–FR3 primer, was used in combination with consensus J_H primers (see Methods). By PCR minor clonal rearrangements generated by ongoing IgH rearrangement were identified for 14/18 (77.8%) patients (Figure 1). Four of these patients (p44, p57, p74, p76) were considered monoclonal by Southern analysis.

Type of secondary IgH rearrangement

Minor clonal rearrangements were mostly identified on the basis of identical D–J_H junctional regions. In these cases, sequence analysis of the V_H–D junctional regions allowed distinction between V_H to D–J_H joining and V_H-replacement. V_H to D–J_H joining, characterized by cessation of sequence identity in related rearrangements in a region with D-homology, was observed in 6/14 patients (p42, p44, p45, p54, p57, p76). In all six patients the presence of the expected pre-existing D–J_H rearrangement was confirmed by PCR amplification with a D-family-specific primer homologous to the 5′ RSS (all visible after 30 PCR cycles) followed by sequence analysis. Also in patient p79, V to D–J_H joining was considered to account for subclone formation. In this patient a D–J_H rearrangement (79-B) was identified by PCR and search for minor clonal rearrangements consistently identified 79-B2, which probably is derived from 79-B by V to D–J_H joining and exonucleolytic loss of the D–J_H junctional region. In order to obtain a relative quantification of the proportion partially (D–J_H) or non-rearranged IgH loci, we performed Southern analysis with a VH6 probe (Figure 2). VH6 is the most 3′-located V_H-gene and is deleted or rearranged upon V_H to D–J_H joining. The VH6 signal was compared to the signal of a reference probe (3′-BCR) by densitometry and the VH6/3′-BCR ratio served as relative measure for the proportion D–J_H rearranged IgH loci. It could be shown that for all but two patients with V_H to D–J_H joining (p42, p76), a significant proportion of the IgH loci was partially rearranged (VH6/3′-BCR ratio: 0.8–2.1) (Table 1). In patients p42 and p76, the absence of significant germline VH6 signal suggests that the D–J_H rearrangement is present in a small percentage of leukemic cells only. Alternatively, in these patients the VH6 gene may have been deleted in the process of an additional rearrangement (possibly a V_H to D joining) upstream of the D–J_H joining. In patient p45 the high VH6/3′-BCR ratio indicates that a large proportion of the IgH loci is D–J_H rearranged and that most identified V_H–D–J_H rearrangements represent small clones, not detected by Southern analysis.

V_H-replacement, characterized by sequence identity in related rearrangements that extends into the V_H–D junctional

Table 1 Patient characteristics

Patient/Age (years)	*Ch 14*[a]	*SB H24 BglII/B + H*[b]	*IgH clonality*	*Type*[c]	*DJ*[d]	*V/B ratio*[e]	*SB Jδ1*[f]	*S-phase %*	*WBC 10^9/l*
9 cALL(D)/6.5	2	RD/RD	mono	VDJ	ND	0.4	G	9.6	185
22 cALL(R)/15.1	2	RR/RR	mono	VDJ VDJ	ND ND	1.5	ND	4.7	15.4
32 cALL(D)/2.2	3	R^{2+}/R^{2+}	mono	VDJ VDJ	ND ND	0.5	GR	7.6	4.9
33 cALL(D)/6.2	ND	RRRR/RRRR	oligo	V→VDJ V→VDJ	– –	0.3	DD	5.4	470
35 Pre-B(D)/20.1	ND	RR/RR	mono	VDJ	ND	0.2	GR	3.3	ND
36 Pre-B(D)/5.4	ND	RR/RRr	oligo	V→VDJ V→VDJ	– ND	0.3	DD	3.8	ND
42 cALL(D)/3.2	2	RRR/RR^{2+}	oligo	V→DJ VDJ	+ ND	BG	DD	4.0	24.9
43 cALL(R)/7.2	3	RRRr/RRRr	oligo	V→VDJ VDJ VDJ	– – ND	0.4	DDD	8.1	ND
44 Pre-B(D)/3.0	2	RR/RG	mono	V→DJ VDJ	+ ND	1.2	GG	8.1	248
45 Pro-B(D)/0.5	ND	RRR/RRR	oligo	V→D→DJ	+	2.1	GR	3.8	1.5
54 cALL(D)/8.0	ND	Gr/Rr	oligo	V→DJ	+	1.2	GR	0	ND
57 cALL(R)/3.4	ND	RR/RR	mono	V→DJ	+	ND	RR	7.5	12.2
74 cALL(D)/8.3	2	RR/RR	mono	V→VDJ	–	<0.7	DD	4.8	127
75 pre-B(D)/3.5	2	Rrr/R	oligo	V→VDJ	–	ND	ND	2.2	21.6
76 cALL(D)/4.9	3	RR/RR	mono	V→DJ VDJ	+ ND	0.02	RRD	ND	ND
78 cALL(D)/3.5	ND	RR/RRr	oligo	V→VDJ	–	BG	RR	4.6	510
79 cALL(D)/13.5	ND	RRrrr/RRrrr	oligo	VDJ V→DJ	ND +	0.8	RR	2.8	140
81 cALL(D)/5.5	ND	Rr/Rr	oligo	VDJ VD→J	ND VD+	0.4	RR	2.7	2.0

Pro-B, progenitor-B ALL; cALL, common ALL; Pre-B, precursor-B ALL; D, diagnosis sample; R, relapse sample; r, minor band; G, germline; D, deleted; WBC, white blood cell count.
[a]Chromosome 14 copy number per cell.
[b]Southern analysis with the H24 J_H probe; B + H = *Bam*HI/*Hin*dIII double digest, R = major band, $^{2+}$ = double band intensity.
[c]Rearrangement type as determined by PCR/sequence analysis; V→VDJ = V_H-replacement, V→DJ = V_H to D–J_H joining, VD→J = V_H–D to J_H joining.
[d]Pre-existent D–J_H or V_H–D rearrangement; +, detectable by PCR/oligonucleotide hybridization; –, not detectable by PCR/oligonucleotide hybridization; ND, not determined.
[e]VH6/3′BCR ratio (see Methods); BG, VH6 signal equal or below background level.
[f]Southern analysis with the Jδ1 probe; two chromosome 14 copies per cell were assumed unless trisomy 14 was proven.

N-region,[28–30] was observed in 6/14 patients (p33, p36, p43, p74, p75, p78). In these patients we could neither detect pre-existing D–J_H rearrangements by PCR/oligonucleotide hybridization, nor a significant fraction partially rearranged IgH loci by Southern analysis with the VH6 probe (VH6/3′-BCR ratio: below background level, <0.7). Detectable low VH6 signal in some of these patients is due to contaminating nonleukemic cells in the bone marrow sample. In all six patients the V_H–D junctional N-region shared between related rearrangements was always also completely present in the predominant rearrangement(s), which is compatible with all minor clonal rearrangements being derived from the predominant clonal rearrangement(s). It should be noted that in patient p75 the V_H-gene in the major clonal rearrangement (p75-A) lacks the last two nucleotides of the internal heptamer RSS (TACTGTG), which therefore unlikely mediated V_H-replacement. Possibly, in this patient a more upstream located RSS mediated V_H-replacement. However, this could not be confirmed as sequencing data were not available.

In addition to V_H-replacement and V_H to D–J_H joining, a variant type of secondary rearrangement was observed in patient p81. In this patient, related rearrangements shared identical V_H–D junctional regions in the absence of D–J_H homology, which is compatible with V_H–D to J_H joining. This type of rearrangement was confirmed by amplification of the expected preexisting V_H–D rearrangement followed by hybridization with a V_H–D junctional region oligonucleotide probe. In patient p45, a variation on the V_H to D–J_H type of rearrangement was seen. Apart from related V_H–D–J_H (dxp4-J6) rearrangements (45-A, 45-A2), also related V_H–D–D–J_H (dlr4-dxp4-J6) rearrangements and a D–D–J_H intermediate (45-A8) were present.

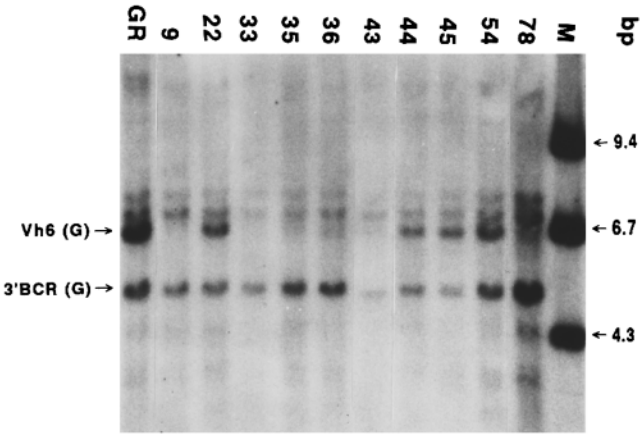

Figure 2 VH6 Southern analysis. For a panel of patients Southern analysis with the VH6 probe is shown. Southern blots (*Bgl*II digests) were hybridized with probes VH6 and 3′-BCR. The VH6/3′-BCR ratio as determined by densitometry served as a relative measure for the proportion partially rearranged IgH loci in the sample. Patient number is indicated above the lanes. Gr, granulocytes; M, lambda *Hin*dIII marker. The 6.6 kb VH6 and 5.0 kb 3′-BCR germline fragments are indicated (G).

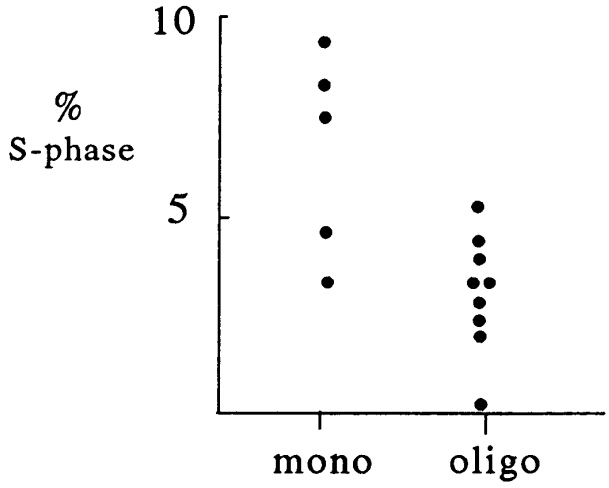

Figure 3 Correlation between S-phase fraction and clonality. Percentage of leukemic cells in S-phase is shown for patients with a monoclonal (mono) and with an oligoclonal (oligo) IgH rearrangement pattern by Southern analysis with the H24 joining probe.

In each patient only a single type of secondary rearrangement (V_H to D–J_H joining, V_H-replacement or V_H–D to J_H joining) was detected and mostly only one of the IgH loci appeared to be subject to ongoing rearrangement. In two patients with V_H-replacement (p33 and p36) related rearrangements were identified for two IgH alleles. It should be noted that for patient p33 the type of secondary rearrangement that generated rearrangement 33-A2 cannot be determined with certainty as additional related rearrangements were not identified. However, the nucleotide sequence of 33-A2 is compatible with V_H-replacement and exonucleolytic loss of most of the original V_H–N–D region and a D–J_H rearrangement was not identified in this patient.

In order to investigate a correlation between IgH and TCRδ rearrangement we compared the TCRδ rearrangement status for five patients with V_H-gene replacement and seven patients with V_H to D–J_H joining by Southern analysis with the Jδ1 probe (Table 1). The first group showed a clearly more 'advanced' TCRδ rearrangement status (two rearranged and nine deleted alleles) than did patients with V_H to D–J_H joining (four germline, eight rearranged and three deleted alleles).

Clonality and S-phase fraction

At diagnosis, 6/15 patients were considered monoclonal by Southern analysis (2/15 also by PCR) and 9/15 patients oligoclonal by Southern analysis. We compared the percentage of cells in S-phase for monoclonal (for only five patients S-phase fraction was determined) and oligoclonal patients (Table 1, Figure 3). The S-phase fraction in the oligoclonal patient group was significantly lower than in the monoclonal patient group ($P = 0.004$ two-sided Student's *t*-test). Notably, the two patients that were also monoclonal by PCR analysis (p9, p32) showed the highest percentage of cells in S-phase. A correlation with white blood cell count (WBC count) or age at diagnosis was not apparent ($P \geqslant 0.05$ two-sided Student's *t*-test).

Discussion

IgH oligoclonality in childhood B precursor ALL as determined by Southern analysis is the result of ongoing rearrangement of the IgH locus.[11–14] We previously reported a detailed analysis of ongoing IgH rearrangement in four selected oligoclonal childhood ALL patients, which suggested that only a single type of secondary rearrangement occurs in each patient.[14] We now substantiate and extend these observations for a random panel of 18 patients.

In our patient group 10/18 (55.6%) patients were classified as oligoclonal on the basis of Southern analysis. This percentage is relatively high as compared to previous reports (30–50%).[8–10] Possibly, this is due to the fact that not only Southern patterns with an excess of bands over chromosome 14 copies were classified oligoclonal, but that also differences in band intensity were considered indicative for the presence of minor clonal rearrangements. Alternatively, the patient group we analyzed may be biased for patients with a relatively high initial WBC count as sufficient cryopreserved cells had to be available for the analyses. An association between high WBC count and IgH oligoclonality has been reported.[31] In contrast to the Southern analysis data, by PCR/sequence analysis we demonstrated the presence of subclones that resulted from ongoing IgH rearrangement for 14 of 18 (77.8%) patients. Apparently, ongoing IgH rearrangement occurs in the large majority of patients. In agreement herewith, we previously demonstrated ongoing cross lineage TCRδ rearrangement in over 80% of B precursor ALL.[32]

By nucleotide sequence analysis of the V_H–D junctional region of multiple related rearrangements, the type of secondary rearrangement, V_H-replacement or V_H to D–J_H joining can be objectively determined.[14] Moreover, for all patients with V_H to D–J_H joining the expected pre-existing D–J_H rearrangement could be demonstrated, whereas D–J_H joinings were never present in patients with V_H-replacement. Taken together these data show that the type of secondary rearrangement that mainly occurs depends on the rearrangement status of the predominant malignant clone. One explanation is that in case of a DJ/VDJ-rearranged cell, V_H to D–J_H joining will preferably occur as this type of rearrangement is mediated by a complete RSS. In contrast, V_H-replacement depends on a heptamer sig-

nal within the original V_H-gene[30] and is likely to be a relatively inefficient process that only becomes apparent if no partial rearrangements are present. Alternatively, partially rearranged alleles may be transcriptionally more active.

Apart from a more 'advanced' IgH rearrangement status in the patient group with V_H-replacement (VDJ/VDJ) as compared to patients with V_H to D–J_H joining (VDJ/DJ), we also found that the TCRδ locus had clearly undergone more recombination steps in the former group. Apparently, there is a correlation between rearrangement of the IgH and TCRδ locus in B precursor ALL cells. Possibly, this is due to prolonged (as compared to normal B cells) exposure of the IgH and TCRδ loci to recombinase activity, for variable periods of time. We previously postulated that prior to leukemic transformation recombinase activity in a preleukemic clone leads to the more 'advanced' TCRδ rearrangement status observed for B precursor ALL (70% rearranged) as compared to normal B cells and B cell malignancies that arise from mature B cells (mostly germline TCRδ alleles).[32]

In patient p81 the V_H–D to J_H type of secondary rearrangement violates the normal sequence of recombination (D to J_H before V_H to D–J_H).[2] The presence of a V_H–D rearrangement together with a D–J_H rearrangement on the same allele was previously reported for a cell line of human origin obtained by Epstein–Barr virus transformation. This rearrangement status was postulated to be an intermediate stage in the formation of V_H–D–D–J_H joints, which are frequently found in human CDR3 sequences.[25,33,34] In patient p81, the V_H–D segment was joined directly to a J_H gene segment, suggesting that for this allele V_H to D joining was the first recombination step. Alternatively, a pre-existing D–J_H joint, not suitable for further recombination due to loss of the 5′-RSS, may have been present as all the identified subclones used the J_H5 gene segment. The data on patient p45 show that in this patient V_H–D–D–J_H rearrangements are the result of V_H joining to a pre-existing D–D–J_H segment as demonstrated by the presence of a pre-existing D–D–J_H rearrangement (45-A8) and groups of related minor clonal rearrangements with identical D–D–J_H junctional regions (A3-A5 and A9-A12). Apparently multiple mechanisms can be involved in the formation of D–D fusions.

Our data show that ongoing IgH rearrangement is not restricted to patients with oligoclonal Southern analysis patterns, but occur in the large majority of B precursor ALL. On the basis of the V_H–D junctional sequences found in minor clonal rearrangements generated by V_H-replacement, it appears that all minor clonal rearrangements are derived from the same predominant clonal rearrangement which argues against selectional processes. Also the finding of others that in some patients oligoclonality is observed for both the IgH and Ig kappa locus,[10] supports nonselection. Therefore, ongoing IgH recombination most likely continuously occurs in most ALL blasts and factors other than selection determine whether or not this results in oligoclonality at the Southern analysis level. We found a significantly lower S-phase fraction for the leukemic cell population in oligoclonal patients as compared to monoclonal patients. Possibly, for oligoclonality to become apparent by Southern analysis the number of secondary rearrangements generated in a given time interval must be relatively high as compared to the number of cell divisions in the same time interval. Alternatively, some leukemic cells may still have a tendency to differentiate and leave the proliferative cell pool. If 'differentiation' is frequently accompanied by a secondary rearrangement event this could also explain the lower number of cells in S-phase in oligoclonal patients. For childhood ALL, a correlation between low S-phase fraction and treatment failure has been reported.[35] Some authors have reported a worse prognosis for patients with an oligoclonal IgH rearrangement pattern as compared to 'monoclonal' patients,[8,10,36] whereas others did not confirm this finding.[37] Although this issue is still not totally resolved, it is possible that a low S-phase fraction in 'oligoclonal' patients results in worse prognosis.

A good understanding of ongoing IgH rearrangement processes in childhood ALL is important for the monitoring of minimal residual disease (MRD) by PCR as a pitfall of this approach is loss of tumor marker in the course of the disease. Although we may have missed unrelated minor clonal rearrangements due to the technical approach we took, our data suggest that for a random patient group V_H-replacement and V_H to D–J_H joining occur approximately equally frequently and that variant types of secondary rearrangements are rare. If D–J_H junctional region oligonucleotide probes are used for the follow-up of MRD, only variant types of recombination such as V_H–D to J_H joining are expected to result in loss of hybridization in the case of clonal changes at relapse. In addition, mostly only a single allele appears to recombine actively. These data provide a rational basis for the empirical finding that the simultaneous use of D–J_H junctional region probes for all identified IgH rearrangements reduces false negativity at relapse to approximately 10% of patients.[38,39]

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