The incidence of clonal T-cell receptor rearrangements in B-cell precursor acute lymphoblastic leukemia varies with age and genotype

Caren Brumpt, Eric Delabesse, Kheira Beldjord, Frederic Davi, Jean-Michel Cayuela, Corinne Millien, Patrick Villarese, Pierre Quartier, Agnes Buzyn, Françoise Valensi, and Elizabeth Macintyre

B-cell precursor acute lymphoblastic leukemias (BCP-ALLs) are increasingly treated on risk-adapted protocols based on presenting clinical and biological features. Residual molecular positivity of clonal immunoglobulin (*IG*) and T-cell receptor (*TCR*) rearrangements allows detection of patients at an increased risk of relapse. If these rearrangements are to be used for universal follow-up, it is important to determine the extent to which they are informative in different BCP-ALL subsets. We show that *IGH* V-D-J rearrangements occur in 89% of 163 BCP-ALL, with no significant variation according to age or genotype (*BCR-ABL, TEL-AML1, MLL-AF4*, and *E2A-PBX1*). In contrast, *TCRG* rearrangements, which occur in 60% of patients overall, are frequent in *BCR-ABL* and *TEL-AML1*, are less so in *MLL-AF4*, and are virtually absent in infants aged predominantly from 1 to 2 years and in *E2A-PBX1* ALLs. Incidence of the predominant *TCRD* Vδ2-Dδ3 rearrangement decreases with age but is independent of genotype. These differences are not due to differential recombination activating gene activity, nor can they be explained adequately by stage of maturation arrest. Analysis of *MLL-AF4* BCP-ALL is in keeping with transformation of a precursor at an early stage of ontogenic development, despite the adult onset of the cases analyzed. We postulate that the complete absence of *TCRG* rearrangement in *E2A-PBX1* cases may result from deregulated E2A function. These data also have practical consequences for the use of *TCR* clonality for the molecular follow-up of BCP-ALL. (Blood. 2000;96:2254-2261)

Introduction

B-cell precursor acute lymphoblastic leukemias (BCP-ALLs) represent approximately 85% and 75% of pediatric and adult ALLs, respectively. They include a number of subtypes that can be individualized on the basis of clinical presentation, immunophenotype, and genotype, as assessed by cytogenetic and molecular techniques. This has allowed identification of patients with markedly different prognoses and the increasing use of risk-adapted protocols. BCP-ALL with *MLL-AF4*, for example, is frequent in infants younger than 1 year old who present with marked leukocytosis and a poor response to treatment.[1] It is also common in older adults.[2] *BCR-ABL* identifies a group of poor-prognosis adults.[3] The *TEL-AML1* (*ETV6-CBFA2*) fusion transcript (FT) occurs almost exclusively in childhood BCP-ALL[4] and is of standard or favorable prognostic significance depending on the clinical protocol.[5] *E2A-PBX1* FT predominates in young adults with relatively mature blasts. Its previously poor prognosis has been improved by more intensive treatment (reviewed in Hunger[6]). The approximate incidence of these FTs in pediatric and adult BCP-ALL, respectively, are as follows: *MLL-AF4*, 5% and 5%; *BCR-ABL*, 3% and 30%; *TEL-AML1*, 25% and below 5%; and *E2A-PBX1*, 5% and 5%. Although FTs provide invaluable prognostic markers at diagnosis, their current utility as markers of residual disease at the early stages of remission, when therapeutic decisions are taken, is less clear, at least for qualitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis.

BCP-ALLs also demonstrate clonal rearrangements of the *IGH* and *TCR* genes in the majority of cases (reviewed in Langerak et al[7]). *IGH* rearrangements are reported in more than 90% of cases by Southern blotting and 70% to 90% by PCR.[8] Complete V_H-D_H-J_H rearrangement is preceded by partial D_H-J_H rearrangement, which is detected by Southern hybridization with a J_H probe or by specific D_H-J_H PCR.[9] Illegitimate *TCRG* V-J rearrangements have been described in 40% to 70% of BCP-ALLs,[7,10] and *TCRD* rearrangement in approximately 50%. The commonest rearrangements correspond to partial Vδ2-Dδ3 and Dδ2-Dδ3.[11] Illegitimate rearrangements are thought to result from the fact that ALLs represent cells arrested at a "recombinase-competent" stage of maturation, with *IG* and *TCR* loci in an accessible chromatin configuration.

IG and *TCR* rearrangements provide invaluable markers for patient follow-up, insofar as the vast majority of BCP-ALLs are informative for at least one clonal rearrangement. A variety of molecular strategies allow detection of residual clonal populations with a sensitivity that varies from approximately 5% (5×10^{-2}) to 0.001% (10^{-5}). Two large-scale studies of pediatric ALL have recently demonstrated that residual molecular

From the Department of Biological and Clinical Hematology, and CNRS UMR8603, Hôpital Necker-Enfants Malades and Université Paris V; Biological Hematology, Hôpital de La Pitié-Salpêtrière; and Biological Hematology, Hôpital Saint-Louis; Paris, France.

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C.B. and E.D. contributed equally to this manuscript.

Reprints: Elizabeth A. Macintyre, Laboratoire d'Hématologie, Tour Pasteur, Hôpital Necker, 149-161, rue de Sèvres, 75743 Paris cedex 15, France; e-mail: elizabeth.macintyre@nck.ap-hop-paris.fr.

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positivity during the early stages of morphological complete remission allows identification of approximately 10% to 15% of patients who are at a high risk of relapse.[12,13] It is therefore likely that residual molecular *IGH*/*TCR* positivity will increasingly be used to determine treatment. Both studies were based predominantly on assessment of *TCRG* and *TCRD* clonal markers, but neither compared *IGH*/*TCR* status with genotype.

Since patients of different ages and genotype are increasingly treated on different protocols, it is important to determine whether the extent to which *IGH*/*TCR* is informative varies as a function of age and/or genotype. We demonstrate that, while *IGH* rearrangements are common in all categories of adult and pediatric BCP-ALL, *TCRG* and *TCRD* Vδ2-Dδ3 vary significantly with genotype and age, respectively. These data have practical significance for the use of *IGH*/*TCR* molecular markers in clinical management of patients with BCP-ALL.

Patients, materials, and methods

Patient material and immunophenotyping

Ficolled blasts from bone marrow or peripheral blood were collected at diagnosis, with informed consent, and were analyzed fresh or after conservation in 10% dimethyl sulphoxide. BCP-ALL diagnosis was based on morphology, cytochemistry, and immunophenotype. The majority were included in prospective multicenter studies of pediatric (FRALLE93) and adult (LALA94) ALL. Molecular analysis for all patients other than 8 *E2A-PBX1* patients, provided by J.-M. Cayuela and F. Sigaux, was performed at Necker-Enfants Malades (Paris, France). Diagnosis, immunophenotyping, and cytogenetic analysis of several patients were undertaken in referring centers, and material was transferred for molecular analysis. Immunophenotype was performed by flow cytometric analysis of a variety of B- and T-lymphoid and myeloid antigens. Cases expressing the relevant marker on more than 20% of cells were considered positive. We assessed cytoplasmic Igμ (cIgμ) expression by flow cytometric assessment of permeabilized cells.[14] Patients younger than 2 years are classified as infants, although it is recognized that this term generally refers to those younger than 12 months old. Those aged from 2 to 14 years are referred to as children; those from 15 to 55 years as young adults; and those over 55 years as older adults. These cutoffs were chosen to reflect inclusion criteria for the LALA94 (15 to 55 years) protocol, although several adolescents were treated on FRALLE93 protocols.

IGH and TCR analysis

DNA extraction was performed by phenol chloroform and ethanol precipitation from ficolled diagnostic material.

IGH V_H-D_H-J_H PCR was performed from 1 μg DNA in 50 μL using V_H consensus primers FR1 (FR1c) and FR2 (FR2c with 2 V_H5 and V_H6 FR1-family–specific primers) and a mixture of 3 J_H primers.[8] Selected cases were also analyzed with the use of FR1-family–specific (FR1f) and FR3 consensus primers.[8] We analyzed 15% of the PCR reaction by nondenaturing 8% to 12% polyacrylamide gel electrophoresis and ethidium bromide staining (EB PAGE). *IGH* D_H-J_H PCR was performed as previously described.[9]

TCRG analysis was performed in 2 Vγ-Jγ–specific multiplex PCRs.[15] We amplified 1 μg of DNA with a mixture of VγfI, Vγ10, Jγ1/2, JγP1/2, and JγP primers (VγfI/10 PCR) and with a mixture of Vγ9, Vγ11, and the same Jγ primers (Vγ9/11 PCR). Partial identification of Vγ and Jγ utilization was based on PCR product size. Further Vγ and Jγ utilization was done by fluorescent run-off analysis with the use of a mixture of labeled internal Vγ- or Jγ-specific primers, followed by Genescan analysis on an ABI 373 or 310 automated DNA fragment analyzer (PEBiosystems, Foster City, CA).[15]

TCRD

Vδ2-Dδ3 PCR was performed in a total volume of 50 μL as previously described[16] with an initial 94°C step for 2 minutes followed by 35 cycles at 62°C for 20 seconds and 94°C for 20 seconds. The final extension step was at 72°C for 5 minutes. PCR products were analyzed on 8% nondenaturating PAGE.

Detection of FTs and *MLL* rearrangements

RNA was extracted from leukemic cells by a rapid lysis technique (RNAble) (EuroBio, Les Ulis, France), and complementary DNA (cDNA) and RT-PCR reactions were performed as previously described[17] with the use of 2 μg RNA, random hexamers (Pharmacia, Orsay, France), and MMLV reverse transcriptase (Life Technologies, Cergy-Pontoise, France). Porphobilinogen deaminase (*PBGD*) transcripts were amplified in parallel from the same cDNA to control for RNA quality.[17] In keeping with LALA94 and FRALLE93 guidelines, all infants and children were analyzed prospectively for the *BCR-ABL* (e1-a2 and b2/b3-a2), *MLL-AF4*, *TEL-AML1*, and *E2A-PBX1* FTs and adults for all of the above other than *TEL-AML1*, with the use of the following primers: *BCR-ABL* b2/b3-a2 (CMLA: GGAGCTGCAGATGCTGACCAAC; ALLF: GGTCATTTTCACTGGGTCCAGC; annealing 60°C); *BCR-ABL* e1-a2 (BCR1 ExtS TGAGAACCTCACCTCCAG; ABLExtAS: CTCCACTGGCCACAAAAT; annealing 51°C); *TEL-AML1* (B12: CGTGGATTTCAAACAGTCCA; AM3: GCTCGCTCATCTTGCCTGG; annealing 55°C); *E2A-PBX1* (E2AExtS: GGCCTGCAGAGTAAGATAG; PBXExtAS: CACGCCTTCCGCTAACAG; annealing 51°C); *MLL-AF4* (HRX5Ext: GAGGATCCTGCCCCAAAGAAAAG; AF4Ext: TGAGCTGAAGGTCGTCTTCGAGC; annealing 60°C). Certain E2A-PBX1-positive cases were also analyzed for wild-type *E2A* (E2A/1399U19 GCCTCATGCACAACCACG and E2A/2234L20 GAGTGACACGGTGGCTGAGA; annealing 60°C) and *PBX1* (PBX1/243U25 GCAGGACATTGGAGACATTTTACAG and PBX1/732L19 GCTGAACTTGCGGTGGATG; annealing 57°C) and *PBX1-E2A* transcripts (PBX1/243U25 and E2A/2234L20 primers; annealing 57°C) with the use of 6% formamide and 2 U Taq in a final volume of 50 μL. The latter were hybridized with an internal ^{33}P-labeled PBX1 (PBX1294U18 TTTGGATGAGGCGCAGGC) probe.

Infants were also screened for *MLL* rearrangements by Southern blotting of *Bam*HI and *Hin*dIII digested DNA, with the use of the B859 cDNA probe.[18]

Recombination activating gene transcripts

Detection of recombination activating gene 1 (*RAG1*) and *RAG2* transcripts by RT-PCR were performed in 50 μL with 0.4 μg cDNA, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.4 μmol/L each primer and 2 U *Taq* polymerase. Primers used were as follows: *RAG1* (ACACACTTTGCCTTCTCTTTGGTATT [Ex1]; TCTCACCCGGAACAGCTTAAA [Ex2]); *RAG2* (TTCCCCAAGTGCTGACAATTAA [Ex1a]; TTTGGGCCAGCCTTTTTG [Ex2]). Samples were amplified for 35 cycles (30 seconds at 93°C, 1 minute at 60°C, and 1 minute at 72°C, with a final elongation cycle of 10 minutes at 72°C) and analyzed on 2% agarose gels. *RAG1* PCR products were 209 base pairs (bp) and *RAG2* products were 219 bp. The REH cell line was used as a positive control.

Statistical analysis

Comparison of the incidence of *IGH/TCR* rearrangements in different subgroups was analyzed by the 2-sided χ^2 test, and comparison of the mean ages of *TCR*-rearranged or germline cases was analyzed by the 2-sided Mann-Whitney *U* test.

Results

Diagnostic assessment of *IgH/TCR* configuration and fusion transcripts

In order to determine whether the incidence of *IG* and *TCR* rearrangements varies with patient subgroup, we undertook systematic, prospective assessment of *IGH* and *TCRG* clonality and detection of up to 5 FTs, the latter in accordance with the national

French LALA94 adult (coordinator J. Gabert, Institut Paoli-Calmette, Marseilles, France) and FRALLE93 (coordinator A. Baruchel, Hôpital St Louis, Paris, France) pediatric protocols. Diagnostic samples from 163 B-cell lineage ALLs were analyzed for *IGH* and *TCRG* by PCR and EB PAGE analysis. Detection of *BCR-ABL* (e1-a2 and b2/b3-a2), *E2A-PBX1*, and *MLL-AF4* FTs was undertaken in 140 of 163 patients with sufficient RNA. Detection of *TEL-AML1* was performed systematically only for patients younger than 15 years, since we have found this FT in fewer than 1% of adult cases.[4]

BCR-ABL was detected in 25 cases, (20e1-a2, 2b2-a2, 3b3-a2) *TEL-AML1* in 18, *E2A-PBX1* in 13, and *MLL-AF4* in 14. The incidence of each of these as a function of age is shown in Table 1. Although this corresponds to published incidences for *BCR-ABL* and *TEL-AML1*, the proportion of patients with *E2A-PBX1* and *MLL-AF4* is not representative, because 2 nonprotocol *MLL-AF4* cases were referred for molecular characterization and 8 additional *E2A-PBX1* cases were added to increase this category (see below). The presence of reciprocal *PBX1-E2A* transcripts was looked for in 8 *E2A-PBX1* ALLs, 3 unbalanced and 5 balanced cases, but no specific bands that hybridized to an internal PBX1 probe were seen, despite hybridization to a PBX1 wild-type RT-PCR control. All cases demonstrated wild-type E2A and low-level PBX1 transcripts, and E2A-PBX1 positivity was confirmed in parallel on the same cDNA samples (data not shown).

IGH V_H-D_H-J_H

IGH was assessed with the use of FR1c and FR2 primers and considered to be positive if either or both demonstrated a clonal band. If both were negative, cases were reanalyzed with FR1f and FR3 consensus primers[8] and were classified as positive if either demonstrated a clonal band. Occasional cases were considered to be oligoclonal on the basis of the presence of at least 3 discreet bands. In the majority, at least one PCR system was considered to be clonal. These cases were classified as *IgH*-positive. It should be emphasized that minor clonal PCR products were seen in several cases, particularly following fluorescent analysis (data not shown). Although these are likely to represent minor clones, their presence was not particularly taken into consideration for these consensus PCR strategies. Overall, 145 of 163 (89%) patients demonstrated *IGH* positivity, including 125 of 158 (79%) by FR1c-JH, 121 of 157 (77%) by FR2-JH, 18 of 37 (49%) by FR1f-JH, and 26 of 52 (50%) by FR3-JH.

The incidence of *IGH* rearrangements as a function of age and genotype is shown in Tables 1 and 2 and Figure 1. Rearrangements were frequent in all categories, being highest in *BCR-ABL* and *E2A-PBX1* and lowest in *MLL-AF4* ALL and FT-negative adults, although this was not significant ($P = .3$). *IGH* V_H-D_H-J_H rearrangements therefore occur in approximately 90% of BCP ALLs, with an incidence that varies little with age or genotype.

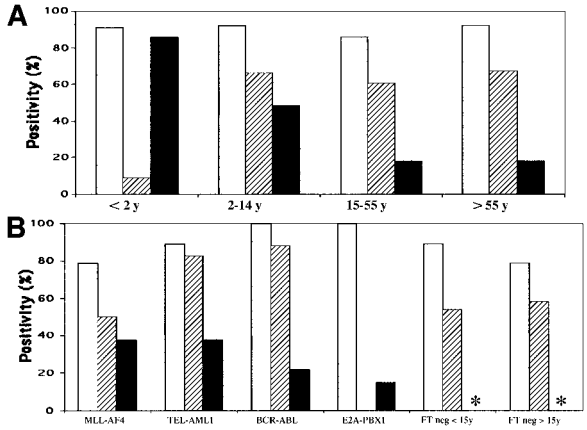

Figure 1. Histograms of *IgH*, *TCRG*, and *TCRD* Vδ2-Dδ3 rearrangements. (A) According to age. (B) According to genotype. □ indicates *IGH* incidence; ▨, *TCRG* incidence; ■, *TCRD* Vδ2-Dδ3 incidence. *Not done.

TCRG

Cases were considered to be *TCRG*-positive if either multiplex reaction (VγfI/10-Jγ or Vγ9/11-Jγ, Figure 2) demonstrated at least one discrete band. Overall, rearrangements were observed in 98 of 163 (60%) cases. The incidence varied with age (Table 1, Figure 1) and was significantly less frequent in infants younger than 2 years (9%) compared with all other groups ($P = .0011$). Mean age for the 11 infants (5 boys, 6 girls) was 13.5 months (range, 3-21) and 9 were older than 12 months. The group included 2 boys aged 3 and 5 months with *MLL-AF4*, a 14-month-old girl with *TEL-AML1*, a 21-month-old boy with *E2A-PBX1*, a 16-month-old girl with an unidentified *MLL* rearrangement, and 8 *MLL* germline cases. The only *TCRG* rearrangement was seen in the uncharacterized *MLL* rearrangement. *TCRG* rearrangements were seen in 16 of 20 (80%) children aged from 2 to 3 years, 13 of 23 (56%) from 3 to 4 years, 10 of 16 (63%) from 6 to 8 years, and 10 of 15 (67%) from 9 to 14 years. The absence of *TCRG* rearrangements was therefore relatively specific to children aged predominantly from 1 to 2 years.

With regard to genotype (Table 2), *TCRG* rearrangements were frequent in *BCR-ABL* and *TEL-AML1*, were less so in *MLL-AF4*, and were not seen in *E2A-PBX1* cases. The incidence in FT-negative ALLs varied with age, as above (Table 1). These data demonstrate that illegitimate *TCRG* rearrangements are absent in *E2A-PBX1* ALLs, infrequent in *MLL-AF4*, and rare in infants from

Table 1. Incidence of fusion transcripts and IGH/TCR rearrangements as a function of age

	Genotypes					Rearrangements		
Age (mean)	MLL-AF4 (%)	TEL-AML1 (%)	BCR-ABL (%)	E2A-PBX1 (%)	FT-negative (%)	IGH (%)	TCRG (%)	Vδ2-Dδ3 (%)
<2 y (1.1 yr)	2/10* (20)	1/10 (10)	0/10 (0)	1/10 (10)	6/10* (60)	10/11 (91)	1/11 (9)	6/7 (86)†
2-14 y (5.9 y)	0/52 (0)	17/52 (33)	1/52 (2)	3/52 (6)	31/52 (60)	68/74 (92)	49/74 (66)	10/23 (43)
15-55 y (32.3 y)	10/66 (15)	nd‡	17/66 (26)	8/66 (12)	31/66 (46)	57/66 (86)	40/66 (61)	6/33 (18)
>55 y (65.3 y)	2/12 (17)	nd	7/12 (58)	1/12 (8)	2/12 (17)	11/12 (92)	8/12 (66)	2/11 (18)
Total	14/140 (10)	18/62 (29)§	25/140 (18)	13/140 (9)	70/140 (50)	145/163 (89)	98/163 (60)	24/74 (32)

FT indicates fusion transcript; nd, not determined.
*The infant with an uncharacterized MLL rearrangement is not included.
†Three-fourths in FT-negative infants.
‡Not systematically tested, but 0 of 23 were positive.
§29% of children aged younger than 15 years.

	BCP-ALL						T-ALL
		Children		Adults			
	Total Mean age	*TEL-AML1* 6.1	FT neg 7.4	*MLL-AF4* 45.3	*BCR-ABL* 49.9	FT neg 29.7	
Patients	*62*	*15*	*10*	*7*	*17*	*13*	*45*
biallelic	37	8	8	3	10	8	37
monoallelic	23	6	2	4	6	5	8
oligoclonal*	2	1 (3 all*)	0	0	1 (5 all*)		
Alleles							
Vfl-J1/2	*54*	*20*	*9*	*1*	*11*	*13*	*58*
V9-J1/2	*20*	*4*	*1*	*3*	*7*	*5*	*4*
Vψ10-J1/2	*1*			*1*			*3*
Vψ11-J1/2	*4*	*1*		*3*			*3*
Total J1/2	79	25 100%	10 55%	8 80%	18 69%	18 86%	68 83%
Vfl-JP1/2	*16*		*7*		*6*	*3*	*9*
V9-JP1/2	*2*		*1*		*1*		*1*
Vψ10-JP1/2	*1*			*1*			*1*
Vψ11-JP1/2	*1*			*1*			*3*
Total JP1/2	20	0 0%	8 44%	2 20%	7 27%	3 14%	13 16%
Vfl-JP#	1				1		1 1%
Total Vfl	71 71%	20 80%	16 89%	1 10%	18 69%	16 76%	68 83%
Total alleles typed	100	25	18	10	26/31*	21	82
Mean/patient	1.61	1.66	1.8	1.43	1.82*	1.62	1.82

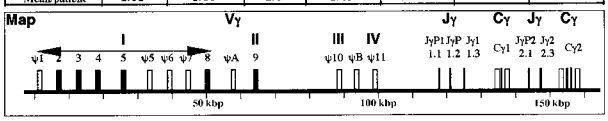

**Figure 2. TCR Vγ and Jγ utilization in BCP-ALL. ***Several patients demonstrated minor fluorescent peaks that were not included. Only those patients in whom oligoclonality was evident by ethidium bromide analysis or in whom more than 2 equal intensity peaks were observed were classified as oligoclonal. The large number of peaks observed in the BCR-ABL case precluded their inclusion for relative Vγ and Jγ usage, but they were included in the total number of alleles. #One patient demonstrated VγfI-JγP1/2 and VγfI-JγP rearrangements. FT indicates fusion transcript.

1 to 2 years old, and that in the last-named, this is not due to *MLL* rearrangement.

TCRD Vδ2-Dδ3

TCRD Vδ2-Dδ3 PCR was assessed in all subjects with known FTs, aged younger than 2 years and/or *IGH* V_H-D_H-J_H–negative, with available DNA. Overall, clonal rearrangements were detected in 32%. The incidence correlated more closely with age than genotype, since rearrangements were present in a progressively lower proportion with increasing age (Table 1, Figure 1). Mean age of Vδ2-Dδ3–positive subjects was 15 years compared with 33 years for negative subjects ($P = .0004$). In contrast, the mean age of patients with *TCRG*-rearranged cases was 23 years compared with 18 years for those with negative cases ($P = .13$). *TCRG* rearrangements were seen in 13 of 24 (54%) TCR Vδ2-Dδ3–rearranged and 30 of 50 (60%) Vδ2-Dδ3–unrearranged cases. Vδ2-Dδ3 rearrangements were present in all genotype categories (Table 2, Figure 3), with no significant differences. It is noteworthy that *TCRD* rearrangements occurred in a proportion of *E2A-PBX1* cases, when they were seen in the 2 youngest subjects.

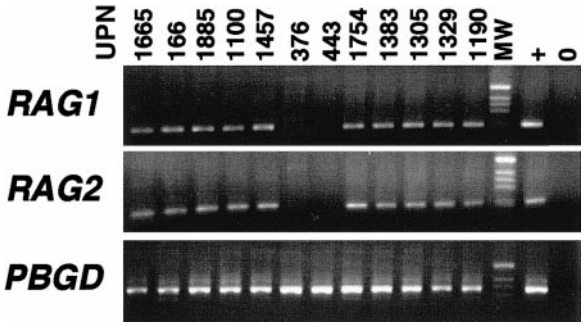

Figure 3. *RAG1* and *RAG2* expression in BCP-ALL. The 39 BCP-ALLs included 9 *MLL-AF4*, 11 *E2A-PBX1* (1 sIg^+ and 10 $cIg\mu^+$), 7 *BCR-ABL* (5 $cIg\mu^+$), 1 $cIg\mu^+$ *TEL-AML*, 3 sIg^+ (not from the present series), 10 $cIg\mu^+$, and 6 $cIg\mu^-$, FT-negative cases, including 6 infant ALLs. *TCRG* rearrangement was seen in 12 of 39 cases.

IGH and *TCRG* rearrangements according to immunophenotype

It was possible that the lower incidence of *TCRG* rearrangement in *MLL-AF4* and *E2A-PBX1* ALL resulted from relative immaturity and maturity of the recombinase complex, respectively. *MLL-AF4* ALLs classically present with a $CD34^+$, $CD19^+$, $CD10^-$, $CD15^+$ profile.[19] *BCR-ABL* and *TEL-AML1* are usually $CD34^+$, $CD19^+$, and $CD10^+$, and frequently express the CD13 and/or CD33 myeloid markers.[20,21] *E2A-PBX1* ALLs demonstrate a more mature phenotype, insofar as they often express cIgμ heavy chains and are $CD34^-$ but $CD10^+$ and $CD22^+$.[22] Although surface CD20 (sCD20) appears before sCD22 during B-lymphoid development,[23,24] the latter has been considered to represent an earlier marker of differentiation arrest.[25]

Immunophenotypic data for selected groups are shown in Table 2. All cases expressed CD19. *MLL-AF4* cases were indeed relatively immature, as assessed by the absence of CD20 and cIgμ expression. It is, however, noteworthy that CD34 expression was less frequent than in *TEL-AML1* and *BCR-ABL* cases, and sCD22 was seen in 50% of cases. CD15 was expressed in 7 of 9 (78%)

Table 2. Incidence of IGH/TCR rearrangements and immunophenotype as a function of genotype

	MLL-AF4 (%)	TEL-AML1 (%)	BCR-ABL (%)	E2A-PBX1 (%)	FT-negative < 2 yrs (%)	FT-negative 2-15 yrs (%)	FT-negative* > 15 yrs (%)	Total
IGH	11/14 (79)	16/18 (89)	25/25 (100)	13/13 (100)	5/6 (83)	28/31 (90)	26/33 (79)	124/140 (89)
TCRG	7/14 (50)	15/18 (83)	22/25† (88)	0/13 (0)	0/6 (00)	20/31 (65)	19/33 (58)	83/140 (59)
Vδ2-Dδ3	5/13 (38)	6/16 (38)	5/23‡ (22)	2/13 (15)	3/4 (75)	—	—	—
CD34	8/13 (62)	14/18 (78)	23/25 (92)	1/13 (8)	4/5 (80)	—	—	—
CD10	0/14 (0)	18/18 (100)	25/25 (100)	13/13 (100)	5/6 (83)	—	—	—
CD22	7/14 (50)	13/15 (87)	18/21 (86)	12/12 (100)	5/5 (100)	—	—	—
CD20	0/14 (0)	6/18 (33)	11/24 (46)	4/13 (31)	4/6 (67)	—	—	—
cIgμ	0/6 (0)	1/11 (9)	5/20 (25)	10/12 (83)	0/5 (0)	—	—	—
CD13	0/13 (0)	7/18 (39)	8/24 (33)	1/12 (8)	0/5 (0)	—	—	—
CD33	3/13 (23)	5/16 (31)	8/24 (33)	1/13 (8)	0/6 (0)	—	—	—

FT indicates fusion transcript.

*Includes 2 cases > 55 years, neither of which demonstrated TCRG rearrangement. All cases expressed CD19. Only surface expression is considered positive, except for cIgμ. FT-positive cases are represented with their genotypic group.

†18/20 (90%) in e1-a2 cases.

‡5/18 (28%) in e1-a2 cases.

MLL-AF4 but was not tested in the other categories. *TEL-AML1* and *BCR-ABL* cases demonstrated a similar immunophenotypic profile, apart from more frequent cIgμ and CD20 expression in the latter. *E2A-PBX1* cases showed relative maturity, with rare CD34 positivity, universal CD22 expression, and frequent cIgμ positivity. CD20 expression was not, however, more frequent than in *TEL-AML1* and *BCR-ABL* cases.

The incidence of *TCRG* rearrangements in cIgμ-expressing, *E2A-PBX1*–negative patients was 7 of 14 (50%), compared with 0 of 10 in cIgμ$^+$, *E2A-PBX1*–positive cases. The latter showed less frequent CD34 (1 of 10 vs 8 of 14, respectively) and CD20 (4 of 10 vs 9 of 14) expression but similar CD22 (10 of 10 vs 13 of 14) expression when compared with *E2A-PBX1*–negative cIgμ$^+$ ALLs, thus not clearly demonstrating a distinct stage of maturation arrest. These data suggest that a relatively late stage of maturation arrest is unlikely to explain the complete absence in *E2A-PBX1* cases.

Identification of an immunophenotypic category equivalent to *MLL-AF4* cases was more difficult, insofar as CD10 negativity was the most characteristic abnormality in this group but was identified in only 3 *MLL-AF4*–negative (*MLL* germline) cases. We therefore compared the incidence of *TCRG* rearrangement among *MLL-AF4* cases using CD34 positivity as an indicator of relative immaturity, or CD22 positivity as an indicator of relative maturity. No differences were observed (data not shown), suggesting that the lower incidence of *TCRG* rearrangement in *MLL-AF4* cases is not due purely to arrest at a particularly early stage of B-lymphoid development.

The absence of *TCRG* rearrangements in the 6 FT-negative infants was not due to a particular immunophenotypic stage of maturation arrest (Table 2).

RAG1 and *RAG2* expression

To determine whether the absence of *TCRG* rearrangement was secondary to loss of recombinase activity, *RAG1* and *RAG2* expression was assessed by RT-PCR in selected patients. Analysis of 2 patients with essential thrombocythemia showed *RAG1* and *RAG2* transcripts in bone marrow but not in peripheral blood, consistent with their detection in B-lymphoid precursors (data not shown). We analyzed 17 blood and 24 bone marrows from 2 mature B-cell leukemias and 39 B-lineage ALLs (Figure 2). All cases other than the 4 surface Ig$^+$ (sIg$^+$) ALLs (1 of which was *E2A-PBX1*–positive) and both mature B-cell leukemias were *RAG1*/*2*-positive, although 1 cIgμ$^+$ *E2A-PBX1* bone marrow was only *RAG2*-positive. Positive cases included 10 cIgμ *E2A-PBX1*, 9 *MLL-AF4*, and 6 infant cases (Figure 3). Failure to rearrange *TCRG* is not therefore due to absence of RAG activity.

TCRG Vγ and Jγ utilization

TCRG Vγ and Jγ utilization was assessed by multifluorescent run-off (FluRO) analysis[15] for 62 patients demonstrating a clonal *TCRG* rearrangement by EB PAGE. Three *TCRG*-negative patients all demonstrated only residual polyclonal rearrangements on FluRO analysis (data not shown). Data from 45 T-ALLs[15] are shown for comparative purposes (Figure 2). As expected,[10] BCP-ALLs demonstrated a higher proportion of monoallelic rearrangements and a lower mean number of rearranged alleles per patient than T-ALLs. Vγ9 rearrangements were commoner in B-lineage ALL ($P = .006$) and VγfI-Jγ1/2 less so ($P = .03$), but no other major differences were noted.

The types of rearrangement differed in the subgroups (Figure 2). *E2A-PBX1* and infant cases are obviously absent. *TEL-AML1* cases demonstrated only Jγ1/2 rearrangements, predominantly VγfI. Among *TCRG*-positive *MLL-AF4* ALLs (all adults), 90% of rearrangements involved Vγ9-Vγ11, predominantly with Jγ1/2. VγΨ10 and VγΨ11 pseudogene utilization was virtually restricted to this category. Among FT-positive cases, VγfI-JγP1/2 rearrangements were restricted to *BCR-ABL*, and the only JγP rearrangement occurred in this category. These differences could not be explained by age differences, insofar as the ages of *BCR-ABL* and *MLL-AF4* cases were similar (Figure 2), as were those of *TEL-AML1* and FT-negative children. Certain Vγ-Jγ rearrangements did, however, differ with age. VγfI composed the vast majority of rearrangements in pediatric cases, whereas Vγ9-Jγ1/2 rearrangements were commoner in adult cases. VγfI-JγP1/2 rearrangements were common in FT-negative children.

Subtyping of VγfI members[15] was undertaken for 65 alleles (48 Jγ1/2, 16 JγP1/2, and 1 Vγ2/4-JγP), but no particular differences were noted between different subgroups (data not shown). VγΨ7 rearrangements were restricted to the *BCR-ABL* and *TEL-AML1* categories (2 cases each).

IGH V_H-D_H-J_H–negative patients by PCRs

Fifteen patients (9%), including 3 *MLL-AF4* and 2 *TEL-AML1*, were negative for all 4 *IGH* according to PCRs. To determine whether these patients had undergone J_H rearrangement, Southern hybridization with a J_H6 probe and/or PCR detection of partial D_H-J_H rearrangements were performed in 14 cases. Results of both analyses will be presented in greater detail elsewhere (unpublished data, F.D. et al, 1999). Nine of 13 (69%) evaluable patients had undergone partial D_H-J_H rearrangements, which were oligoclonal in 4. All 3 *MLL-AF4* belonged to this category, in keeping with an early stage of maturation arrest. Four ALLs, including both *TEL-AML1* cases, had undergone J_H deletion.

From a practical point of view, 7 of 163 (4%) patients demonstrated neither *IGH* V_H-D_H-J_H nor *TCRG* clonal rearrangements by PCR. One was *MLL-AF4*–positive; 2 were *TCRD* Vδ2-Dδ3–positive, and 2 demonstrated clonal D_H-J_H rearrangements, thus allowing PCR follow-up. One demonstrated oligoclonal D_H-J_H, and as such, would be difficult to follow. Only 1 case demonstrated no PCR marker.

Discussion

In this manuscript, we demonstrate that, whereas *IGH* V-D-J rearrangement varies little with BCP-ALL subtype, this is not the case for "illegitimate" *TCR* rearrangements. We confirm that the incidence of the *TCRD* Vδ2-Dδ3 rearrangements decreases with age[11] and show that the incidence and type of *TCRG* rearrangements vary with genotypic subgroup and is virtually absent in infants aged from 1 to 2 years. The overall incidence and type of *TCRG* rearrangements are similar to those reported in 202 childhood BCP-ALLs, based predominantly on Southern blot analysis.[26] Minor differences include a higher incidence of *TCRG* JγP½ rearrangements in our series (21% vs 13%), which probably reflects the fact that these rearrangements are more easily detected by PCR than by Southern blot,[11] and the presence of Vγ8-Jγ½ rearrangements in the present series (8% of rearrangements) but not in Szczepanski et al.[11] Rearrangement incidence in Szczepanski et al[26] was correlated with immunophenotype but not genotype. It is likely that the CD10$^-$ pro-B category (fewer than 5% of patients) corresponded to our *MLL-AF4* cases and part of the cIgμ$^+$ pre-B

category (30% of patients) to our *E2A-PBX1* cases. Absence of *TCRG* rearrangement was seen in 25% of pro-B and 15% of pre-B cases, compared with only 2% of common ALL, in keeping with our observations.

Several explanations for the variable incidence of *TCRG* rearrangements are possible.

RAG activity

Differential expression of *RAG1* and 2 could not explain the aforementioned differences, since the only *RAG*-negative ALLs were those expressing sIg, as previously described.[27] This was, in any case, an unlikely explanation, since it would imply coordinate occurrence of illegitimate *TCRG* and *TCRD* rearrangement, which is not the case.

Maturity

The low incidence of *TCRG* rearrangement in *MLL-AF4*, infant cases, and *E2A-PBX1* cases may reflect oncogenic transformation of a particularly immature or mature lymphoid precursor. *MLL-AF4* cases were, as expected, immunophenotypically immature. Interestingly, CD34 negativity was more common in *MLL-AF4* ALL than in all categories other than *E2A-PBX1*, including the potentially immature *IGH* V_H-D_H-J_H–unrearranged group. The recent demonstration[28] that the earliest hematopoietic progenitors are $CD34^-$ suggests that $CD34^-$ *MLL-AF4* cases may be more immature than their $CD34^+$ *MLL-AF4* counterparts. In keeping with this, CD33 expression, which is found on immature hematopoietic progenitors demonstrating multilineage potential,[29] was seen in 3 of 5 $CD34^-$ *MLL-AF4* cases but in 0 of 8 $CD34^+$ cases. Complete *IGH* V_H-D_H-J_H rearrangement was, however, seen in 4 of 5 $CD34^-$ cases.

The arguments for relative maturity as an explanation for the total absence of *TCRG* rearrangement in *E2A-PBX1* ALL are weaker. *TCRG* rearrangements were seen in 50% of cIgμ-expressing *E2A-PBX1*–negative patients. It is not therefore the presence of cIgμ that renders the *TCRG* locus inaccessible. Although we cannot exclude the possibility that *E2A-PBX1* cases are more mature than their *E2A-PBX1*–negative cIgμ-expressing counterparts, comparison of CD34, CD20, and CD22 expression was not in favor of this, and the relatively subtle differences observed would be expected to lead to a diminution, rather than a complete absence, of *TCRG* rearrangement. Similarly, the virtual absence of *TCRG* rearrangement in infants cannot be explained on the basis of maturity, since they are immunophenotypically similar to *TEL-AML1* and *BCR-ABL* cases.

Fetal vs adult-type precursors

It is possible that different types of BCP-ALL undergo oncogenic transformation at distinct stages of ontogenic development with respect to *TCR* accessibility. Accessibility of the *TCRG* and *TCRD* loci must be independently controlled, since we show that rearrangement at these loci does not occur in the same subgroups. Physiological *TCRD* rearrangement precedes *TCRG* in human thymus,[30] with Dδ2-Dδ3 occurring prior to Vδ-Dδ. Vδ2-Dδ3 rearrangements were not seen; these are virtually restricted to BCP-ALLs. During lymphoid development, early fetal and immature precursor *IGH*,[31-33] *TCRG*,[34,35] and *TCRD*[36-38] gene rearrangements preferentially involve 3′, J proximal V segments, whereas a wider range, including 5′ V segments, are found in mature lymphoid cells. Identification of Jγ proximal Vγ utilization may also reflect preliminary attempts to rearrange the locus that have not been superseded by subsequent attempts.

Several arguments from our series of, predominantly adult (12 of 14), *MLL-AF4* ALLs favor relative genotypic immaturity: the low level of *TCRG* rearrangements predominantly involving Vγ9-11, overrepresentation of partial *IgH* D_H-J_H rearrangement, and the relatively high incidence of partial *TCRD* Vδ2-Dδ3 rearrangements (38% overall, 27% of adults). The partial *TCRD* Vδ2-Dδ3 rearrangements occur more frequently in children than adults, respectively representing 70% and 40% of *TCRD* rearrangements.[11] TCR Vγ utilization was strikingly different from all other BCP-ALLs, insofar as Jγ proximal Vγ9-11 were involved in 90% of rearrangements, compared with 18% of non–*MLL-AF4* cases. This may reflect absence of "secondary rearrangement" by upstream Vγ segments. These data are in favor of oncogenic conversion at an early, immature stage of ontogenic development, at the transition of D_H-J_H to V_H-D_H-J_H rearrangement, after the *TCRD* locus becomes accessible to at least Vδ2-Dδ3 rearrangement and at the onset of illegitimate *TCRG* rearrangement. This is in keeping with observations that *MLL-AF4* rearrangements in children occur during fetal development.[39,40] It is, however, striking that the present series represents predominantly adult cases (mean age, 47 years).

Applying the same model to infant BCP-ALLs would suggest that they have undergone transformation of a more immature lymphoid precursor in which the *TCRG* locus is not yet accessible, yet in which *TCRD* accessibility is much more pronounced. *E2A-PBX1* cases would have to be explained as transformation at a much later ontogenic stage, after *IGH* rearrangement is complete and some residual *TCRD* accessibility remains, but after *TCRG* has become inaccessible.

E2A

The most interesting potential explanation is that *E2A-PBX1* expression is directly or indirectly responsible for rendering the *TCRG* locus inaccessible to illegitimate recombination. Wild-type E2A plays a fundamental role in regulating lymphoid development. $E2A^{-/-}$ mice demonstrate a total block in B-lymphoid development with absence of D_H-J_H and V_H-D_H-J_H rearrangement.[41,42] Loss of *E2A* also leads to a block in T-lymphoid development, with development of thymic lymphomas.[43] Cell lines derived from the latter undergo programmed cell death in the presence of enforced *E2A* expression,[44] suggesting that *E2A* products can act as tumor suppressors. $E2A^{-/-}$ mice demonstrate a switch from adult to fetal-type TCRγδ lymphocytes and *TCRG* and *TCRD* rearrangements.[45] E2A does not appear to modulate germline transcription of *TCRG* but may target the recombinase to specific recognition signal sequences (RSSs) since consensus E2A-binding motifs were identified in the linker sequences of almost all murine Vγ and Vδ RSSs.[45]

Cytogenetically, the majority (approximately 75%)[46] of t(1;19) are unbalanced, being associated with loss of the der(1) and potential *PBX1-E2A* reciprocal transcripts and duplication of the normal chromosome 1.[47] Consequently, only *E2A-PBX1* has been considered to be oncogenic, strengthened by the fact that *PBX1-E2A* transcripts have not been seen in t(1;19) cell lines or patient material,[48,49] as confirmed here. Against this, however, is the observation that patients with the unbalanced form have a relatively good prognosis,[47] suggesting that something on the der(1) may add oncogenic potential. This difference was, however, only seen as a trend by Pui et al.[50]

E2A-PBX1 leads in all cases to haploinsufficiency. $E2A^{+/-}$ mice demonstrate a partial reduction in B lymphocytes[41] and deregulated

TCRD gene segment usage,[45] suggesting that *E2A* is rate limiting. It is therefore possible that *E2A* haploinsufficiency in *E2A-PBX1* BCP-ALL may directly interfere with B-lymphoid development and/or *IG/TCR* rearrangement. The similar phenotype observed in *E2A-HLF* and *E2A-PBX1* transgenic mice has been suggested to represent an oncogenic effect mediated by interference with wild-type *E2A*.[51] Loss of wild-type *E2A* activity would be expected to lead to impairment of *IGH* V_H-D_H-J_H rearrangement, which we show is not the case. It might also be expected to inhibit adult in favor of fetal-type *TCRG* rearrangements. The absence of both adult and fetal *TCRG* rearrangements in *E2A-PBX1* ALLs suggests either that *E2A* loss is not relevant to this process or that, in a B-lymphoid background, *E2A* dosage may be even more limiting than in a T-lymphoid background and may be necessary even for fetal-type rearrangements.

Infant ALLs

The basis for the virtual absence of *TCRG* rearrangements in infants aged 1 to 2 years is unclear but is not due to *MLL-AF4* rearrangement or immunophenotypic immaturity. The change from an infant *TCRG* profile to a pediatric one occurs abruptly at the onset of the peak of childhood ALL. It suggests that infant ALLs without *MLL* rearrangement represent a distinct subtype of ALL rather than the lower end of the spectrum of pediatric-type BCP-ALLs.

TEL-AML1 and *BCR-ABL* BCP-ALLs are similar

TEL-AML1 and *BCR-ABL* ALLs showed similar phenotypic and genotypic features. The only immunophenotypic difference observed was more frequent cIgμ expression in *BCR-ABL* cases, suggesting relative maturity. In contrast, *TEL-AML1* cases essentially demonstrated end-stage VγfI-Jγ1/2 rearrangements, whereas immature Vγ9 and JγP1/2 rearrangements were relatively common in *BCR-ABL* cases. These data suggest that, despite the striking differences in demographic and prognostic features, *BCR-ABL* and *TEL-AML1* ALLs are arrested at a similar stage of development with pronounced phenotypic and genotypic lineage infidelity or promiscuity. The recent demonstration that loss of *PAX5* expression uncovers multilineage potential in pro–B-lymphoid cells, including myeloid and T-lymphoid development under appropriate condition,[52,53] suggests that assessment of *PAX5* status may be interesting in these BCP-ALLs.

Practical significance

From a practical point of view, the variable incidences of *TCRG* and *TCRD* rearrangements observed in BCP-ALLs of different ages and genotypes suggest that the relative representation of the different subgroups identified here should be taken into account when these rearrangements are used as universal markers for molecular follow-up. As an example, use of *TCRG* will lead to underrepresentation of *E2A-PBX1*, *MLL-AF4*, and infant cases, and use of *TCRD* Vδ2-Dδ3 to underrepresentation of older children. The use of certain Vγ-Jγ combinations will exacerbate this. Obviously this tendency will be minimized by the use of several *IG/TCR* targets per patient, as is widely recommended in order to reduce the risk of false negative results. Based on this series, only 3 of 160 (fewer than 2%) patients failed to demonstrate at least 1 PCR-amplifiable *IGH*, *TCRG*, or *TCRD* rearrangement. It is likely that the wide applicability of these markers, along with recent encouraging data with regard to their predictive value in childhood ALL,[12,13] will lead to their increasing use in the management of BCP-ALL. Our data suggest that results using these markers should be interpreted in the light of accurate genotyping at diagnosis.

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