IMMUNOGENETIC STUDIES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

Analysis at diagnosis and relapse

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IMMUNOGENETIC STUDIES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

Analysis at diagnosis and relapse

Immunogenetische studies bij acute lymfatische leukemie op de kinderleeftijd

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Voor Janwillem
7 jaar

IMMUNOGENETIC STUDIES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA
ANALYSIS AT DIAGNOSIS AND RELAPSE

CONTENTS

1 GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1.1 EPIDEMIOLOGY OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

Although cancer is not a common disease in children, mortality data demonstrate that after accidents cancer is the second cause of death in children (1,2). Each year approximately 380 children less than 15 years of age are diagnosed with cancer in the Netherlands, with an incidence rate of 13.9 per 100,000 children per year, in boys slightly higher (~14.8) than in girls (~13) (3-5). In general, these incidence rates are comparable within Europe and the rest of the world, but the lowest incidence rates are found in developing countries (2,6-12). The latter can most probably be explained by misdiagnosis, underreporting, or pre-emptive death from infectious diseases (2,7,8). The difference in incidence rates between white and black children is clearly shown in the USA where the annual incidence of cancer is 12.9 per 100,000 white children per year and 9.7 per 100,000 black children per year (6,7,11-13).

Pediatric cancers appear to be a distinct group of diseases (14). They are mainly embryonal in type and evolve from malignant transformation of developing tissues (14), in which genetic influences are probably greater than environmental factors (14). In concordance with this is the fact, that young children (< 5 years) are more frequently stricken by malignant tumors than older children (>5 years) (Table 1) (3,4).

Leukemia is the most common cause of cancer in children, accounting in the Netherlands for 28% of all cases (Figure 1) (3,4). Central nervous system (CNS) tumors are second most common, followed by lymphomas, soft-tissue sarcomas, renal tumors, and tumors of the sympathetic nervous system (SNS) (predominantly neuroblastomas) (Figure 1) (3,4). Each year 100-110 children with leukemia are diagnosed (3-5,16-19). Incidence rates are 3.8 per 100,000 children per year for boys and 3.6 per 100,000 children per year for girls (3,4). These childhood leukemia rates in the Netherlands appear to be more or less similar to those in other countries of similar socio-economic status (7-9,20-23). As in other malignant tumors, leukemia occurs more often in males than in females, a pattern that is consistent across racial and geographic boundaries (20-22,24).

TABLE 1. Incidence rates per 100,000 person-years for invasive tumors among children according to sex and age in 1989/1990 in the Netherlands[a].

Age (years)	Male	Female	Total
<1	17.6	18.3	18.0
1-4	18.6	17.8	18.2
5-9	12.6	9.3	11.0
10-14	13.4	11.8	12.6

a. According to Netherlands Cancer Registry/coordinating Council of Comprehensive Cancer Centres (see references 3 and 4).

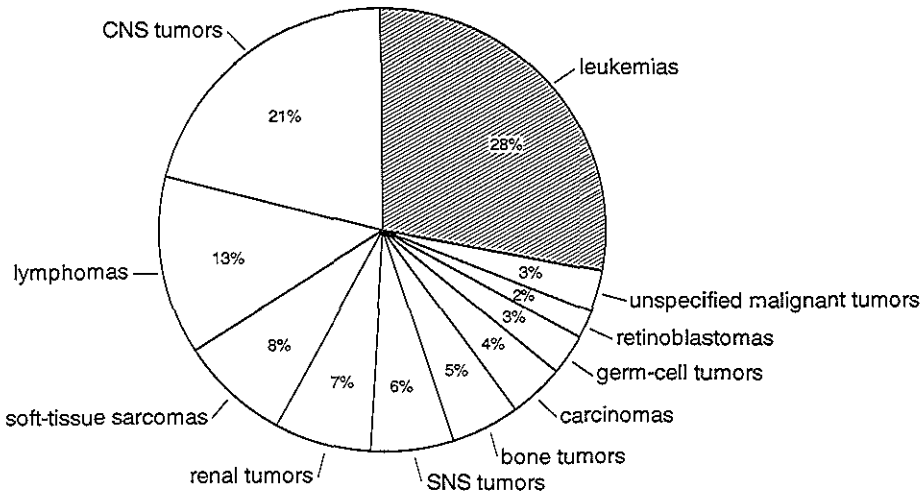

Figure 1. The relative frequency of various malignant tumors among children (age <15 years) in the Netherlands (1989-1990) according to the Netherlands Cancer Registry (3,4). The classification is according to Birch and Marsden (15). Abbreviations used: CNS, central nervous system; SNS, sympathetic nervous system.

About three quarter of all leukemias in children are acute lymphoblastic leukemias (ALL) (Figure 2), with a peak incidence between the ages of two and five years (Figure 3) (19). This peak was first found in Great Britain during the 1920s and 1930s and occurred later on in other industrialized countries (19-22); in the USA in the 1940s and in Japan in the 1960s (20-22). This peak appeared to be an exclusive characteristic of precursor B-ALL (19-22). This has prompted speculation that this peak may reflect environmental exposures associated with modernization and better socio-economic status, all the more since in Africa and many developing countries this peak does not occur (19-22,25).

Childhood ALL subtypes have been identified by immunological marker analysis (Figure 2) (see also section 1.3: Diagnosis of childhood ALL). The geographical distribution and proportional representation of these ALL subtypes was very similar in various developed countries, while precursor B-ALL appeared to occur in a relative lower frequency in developing countries and T-ALL correspondingly in a relative higher frequency (19-22). Although it has been hypothesized that this results from underdiagnosis of precursor B-ALL in developing countries, it has also been suggested that children in industrialized countries are exposed to leukemogens that specifically cause precursor B-ALL (19-22,25).

Over the years, there has been considerable interest in the reported occurrence of so-called leukemic clusters, i.e. the observation of a greater than expected number of leukemic cases within a geographic area or time period (26-28). The documentation of these leukemic clusters would have profound epidemiological implications, potentially permitting identification of common infectious or environmental exposures (26-28). However, in the majority of possible clusters, relations of leukemia with certain risk

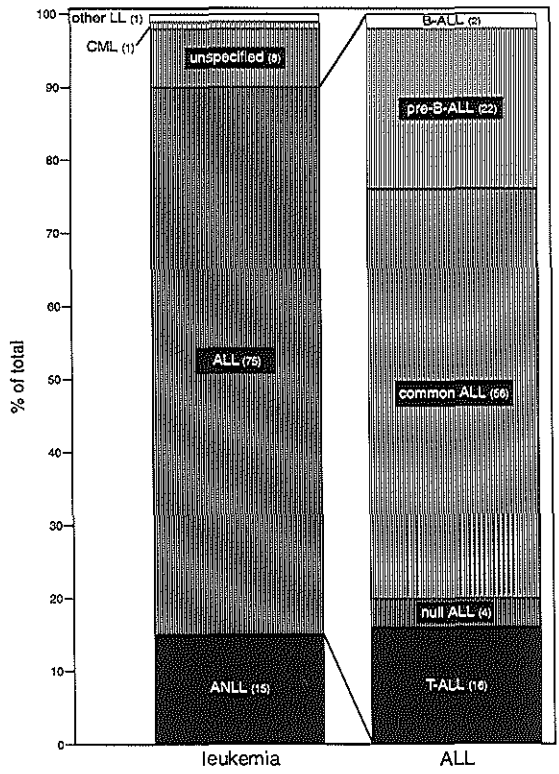

Figure 2. The relative frequency of leukemia and immunological subtypes of ALL in children (age <15 years) in the Netherlands (1987-1992). With permission from the Dutch Childhood Leukemia Study Group (DCLSG) (3,4,19).

factors were difficult to prove (26-28). Recently, new indications were suggested for a transmissible/infectious agent (e.g. virus) as etiologic factor for ALL based on cluster analyses (29).

Survival rates of childhood cancers dramatically improved throughout the last 20 years; this occurred particularly in leukemias, brain tumors, soft tissue sarcomas, and renal tumors (6,9,10,12,30-32). Especially, in ALL the survival rates improved significantly (6,9,10,12,30-33). In the Netherlands, the overall survival rate improved from

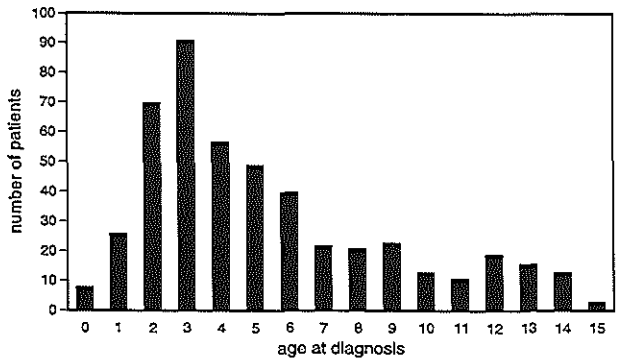

Figure 3. Number of ALL patients and age at diagnosis in the Netherlands (1986-1991). Adapted from Dr. J.W.W. Coebergh et al. (19) with permission.

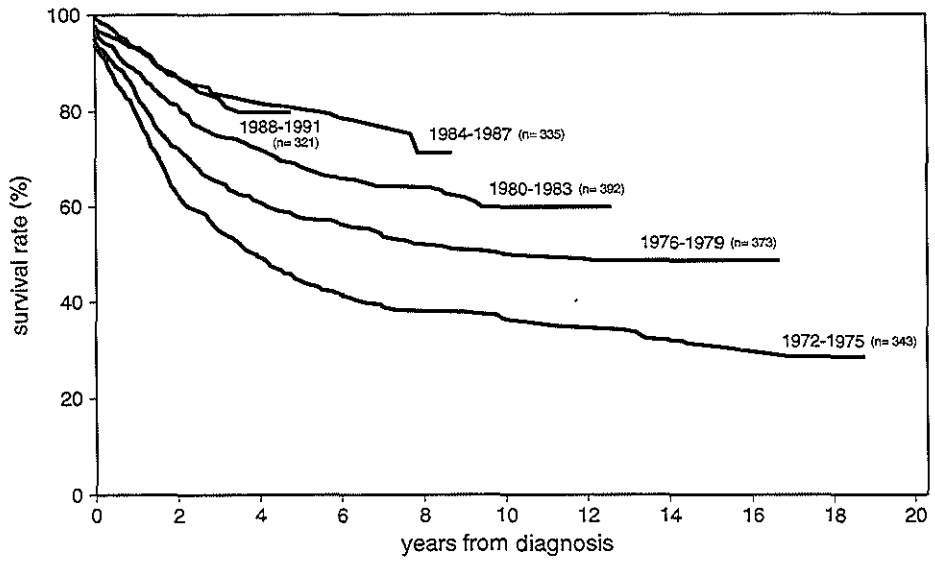

Figure 4. Survival rates of children with ALL in the Netherlands between 1972 and 1991 (33). With permission from the DCLSG (33).

~30% in 1975 till ~70% in 1991 as indicated in Figure 4 (33).

1.2 ETIOLOGY OF CHILDHOOD ALL

Both inherited and environmental factors are assumed to be responsible for the induction of leukemia by various mechanisms (14,20,34,35). The various genetic and environmental factors associated with the etiology of ALL are summarized in Table 2. The relation between genetic abnormalities and leukemia is clearly indicated by the relatively high incidence rates for acute leukemias in Down's syndrome or Klinefelter's syndrome (34-38). In other genetic diseases, characterized by abnormalities of DNA repair processes, also higher incidence rates for ALL are found (Table 2) (34,35,37,39-43). Evidence that inherited factors play a role in the induction of leukemia are weak, although occasional familial clustering has been reported (37,44). However, strong support for an inherited predisposition gives the high concordance rate (up to 25%) of infant ALL in identical twins. This concordance rate appeared to diminish with age (14,37,45). The association between immunodeficiencies and lymphoid malignancies is evident (46,47). The majority of lymphoid malignancies in immunodeficiencies is, however, of mature B-cell type (e.g. B-cell non-Hodgkin lymphomas (NHL)), probably except for T-ALL in ataxia telangiectasia (41,42,46,47).

The relation between environmental factors and ALL was proven by studies on young survivors of the atomic bomb in Japan and possibly a marginally increased rate of ALL in children subjected to diagnostic irradiation in utero (37,48-50). The risk of acute

TABLE 2. Possible factors associated with etiology in childhood ALL[a].

Genetic factors	**Environmental factors**
Down's syndrome	Radiation - atomic bomb explosions
Klinefelter's syndrome	- fallout from nuclear testing or accidents
Bloom syndrome	- therapeutic irradiation
Fanconi's anemia	- diagnostic radiation in utero
Ataxia telangiectasia	Low frequency electromagnetic fields
	Chemicals - benzene
Immunodeficiency	- herbicides and pesticides
Wiskott-Aldrich syndrome	Drugs - alkylating agents
Congenital hypogammaglobulinemia	- Topoisomerase II inhibitors
Ataxia telangiectasia	Viral infections

a. References 14,29,34-68.

leukemia (acute myeloid leukemia (AML) more than ALL) after therapeutic irradiation was proven in several studies, analyzing data of patients treated with radiotherapy for primary cancers or ankylosing spondilitis (51,52). Several studies, suggesting a relation between ALL and radiation from nuclear plants (53), accidents with nuclear plants (54), or fallout from nuclear testing (55,56), could not be confirmed upon re-examining the data (57,58). The role of background or 'natural' radiation as an etiologic factor in the induction of ALL remains unclear so far because it is difficult to investigate (59-61).

Exposure to chemicals, such as benzene, pesticides, and herbicides, is associated with an increased risk of leukemia (62,63). In case of exposure to benzene, the association with the development of AML in adults is clear (62). However, direct evidence linking such exposure to the development of childhood ALL does not exist. A possible association between herbicides and pesticides and leukemia was found in a leukemic cluster in the Netherlands, where a four times higher incidence of hematological neoplasms was found (63).

There is substantial evidence that chemotherapy, particularly alkylating agents has leukemogenic potential (64). Most of these leukemias are AML (64). Other factors studied for possible association with ALL include exposure to electromagnetic fields, maternal use of alcohol, contraceptives, or cigarettes (14,65). Definitive links between these factors and the risk of childhood ALL have not been confirmed.

The role of viral infections in the induction of ALL is, in spite of its clear relation with Burkitt lymphoma (Epstein Barr virus) and adult T-cell leukemia and lymphoma (human T-cell leukemia virus), not proven (66,67). Moreover, the lack of evidence of clustering childhood leukemia argues against a viral factor to the etiology of ALL, despite Greaves' hypothesis (68-71). This hypothesis emphasizes the role of either a non-specific antigenic challenge or infection by a specific agent (e.g. virus) in modulating the late-stage malignant events leading to ALL (68-71). Recently, by re-analyzing several clusters of ALL, new indications were found for a transmissible agent important as an etiologic factor of ALL (29).

Even though in a minority of ALL cases the correlation with one of the above mentioned risk factors is likely, the cause of childhood ALL remains unknown (72). The solution to the 'mystery' of childhood ALL may lie within the developmental biology of the cell type from which this disease arises (73-75). The immature B- and T-cells are,

due to its intrinsic developmental program, at higher risk of '(spontaneous) mutation' than other somatic cells, particularly at early stages of its development (76-80). This mutagenic activity is partly based on rearrangement processes, dependent on several recombination enzymes (e.g. recombination activating genes (RAG1 and RAG2)) (81). Therefore, children are probably more prone to develop ALL than adults, because their immune system goes through a rapid developmental stage and moreover, encounters a tremendous amount of 'new' antigens from their environment (e.g. infectious agents) (76-80).

It seems logical that several risk factors, which are mentioned above (e.g. genetic diseases, radiation, or viral infections) (Table 2), can influence directly or indirectly the occurrence of mutations (72). Still, leukemias will only develop through mutational events when these mutations affect certain genes (73,82,83). Many of these crucial genes are so-called proto-oncogenes, genes designed to regulate or promote normal growth (84-94). When these genes are made hyperactive by mutations, such as point mutations, translocations, or amplification, they can cause inappropriate growth (84-94). Conversely, tumor suppressor genes normally restrain growth, but inactivation by point mutations, deletions, and chromosomal loss, contributes to oncogenic change (86-95).

The findings of non-random chromosomal abnormalities associated with ALL has focussed on the role of specific genes, which reside nearby chromosomal breakpoints (93,94,96-104). Moreover, the role of proteins encoded by these genes or fusion proteins (also called chimeric oncoproteins) encoded by genes on both sides of the chromosomal breakpoint, increasingly have been the subject of research the last decade (105,106). Many of these proteins are encoded by known proto-oncogenes, some by known tumor suppressor genes (95,99,104). They can be classified by their locations outside the cell, on the cell surface, or in the cytoplasm or nucleus (Figure 5) (91,99,-104,107). Furthermore, in most cases their function can be linked to parts of the signal transduction pathway involving growth and proliferation of the cell (Figure 5) (91,99,-104). It is therefore logical that deregulation of several of the main steps in this signal transduction pathway were found to be associated with human malignancy, in particular ALL (Figure 5) (91,99,104). However, it should be kept in mind that malignant transformation of a cell is a complex phenomenon and generally a multiple-step process. It is likely that the activation of a proto-oncogene, as described here, provides only one of these steps and must be preceded or followed by other genetic or epigenetic alterations to accomplish neoplastic growth *in vivo* (91,94,99,104). Not all chromosomal abnormalities are assumed to be causal factors in the etiology of childhood ALL. Especially numerical chromosomal abnormalities, frequently found in childhood ALL, are probably secondary to the malignant transformation and maturation arrest of an immature lymphoid cell.

ALL, like other lymphoid malignancies, is believed to develop as a consequence of malignant transformation of a single abnormal progenitor cell, which has the capability to expand by unrestrained growth and self-renewal (87,104). The ability to differentiate is decreased or blocked. Therefore, heterogeneity within the ALL cell population is seldomly found. This is in contrast with AML, in which heterogeneity is an important feature. Some AML appeared to have the ability to differentiate (87,104,108).

Evidence for the clonal origin of ALL are derived from studies of glucose-6-

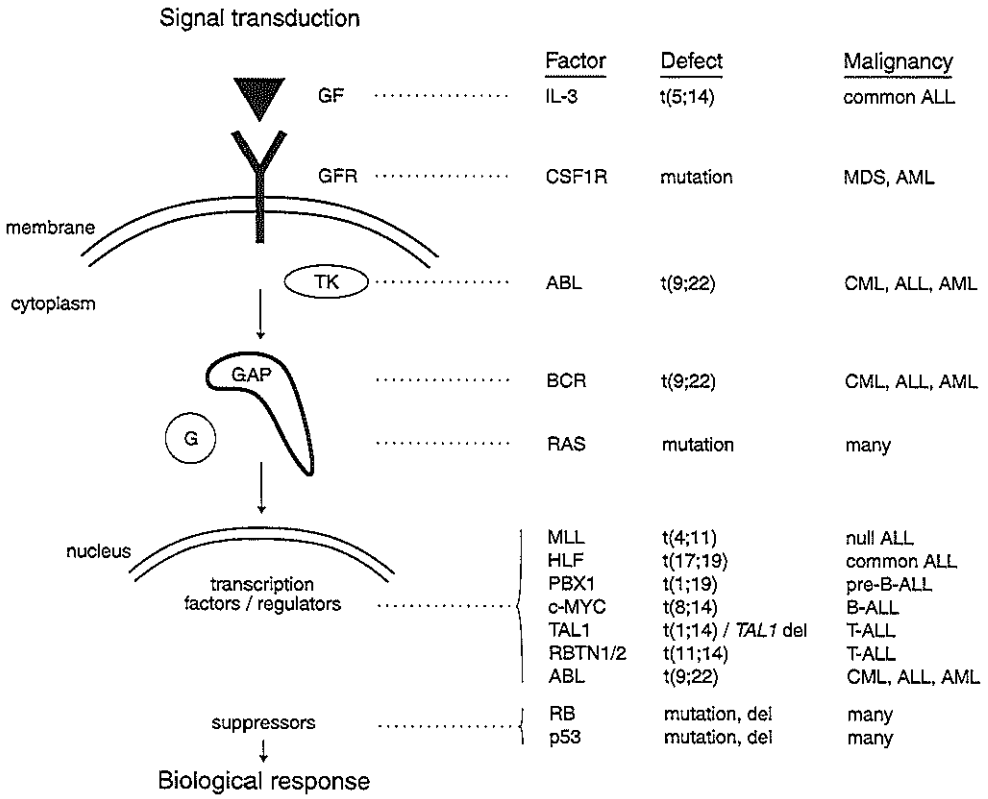

Figure 5. Schematic representation of a signal transduction pathway involving growth factors (GF), growth factors receptors (GFR), tyrosine kinases (TK), G-proteins (G), GTPase activating proteins (GAP), and nuclear transcription factors, transcription regulators, and suppressor genes. Examples of altered signal transduction genes and their relation with malignancy are also shown (Abbreviation used: del, deletion of chromosome or chromosomal region). Adapted from Yunis and Tanzer (99).

phosphate dehydrogenase isoenzyme types (109), immunological marker analysis (110,111), detection of identical immunoglobulin (Ig) idiotypes (112,113), chromosome studies (114-116), and molecular biology studies of leukemic lymphoblasts, such as analysis of Ig and T-cell receptor (TcR) gene rearrangements (117-120), analysis of restriction fragment length polymorphisms (121), determination of X-chromosome inactivation, and determination of X-linked DNA polymorphisms (122-125). Several of these techniques will be discussed in section 1.3: Diagnosis of childhood ALL.

1.3 DIAGNOSIS OF CHILDHOOD ALL

Introduction

ALL is a disorder characterized by the uncontrolled growth and proliferation of immature lymphoid cells, which then represent 25% or more of all bone marrow (BM) cells (14,126). The presenting features of childhood ALL reflect the suppression of normal

hematopoiesis causing anemia, neutropenia, and thrombocytopenia, which can lead to infection and hemorrhage, and/or involvement of lymphatic tissue, causing obstruction of vital organs (e.g. vena cava superior syndrome) (14,127). Symptoms of ALL may be subtle and misdiagnosed for other more common disorders. The majority (85%) of children with ALL develop symptoms more insidiously over weeks or a few months. Common symptoms include pallor, fever, recurrent infections, malaise, abnormal bruising, and bone or joint pain (14,127). Due to these varied presentations, childhood leukemia must be considered in the diagnosis of any unusual illness especially if accompanied by hepatosplenomegaly, lymphadenopathy or bone tenderness (14,127).

The diagnosis of ALL, traditionally based on the cytology of smears of BM and peripheral blood (PB), has become more accurate, since advances in immunophenotyping, cytogenetics, molecular immunology, and other molecular and cellular techniques have improved the specificity of diagnosis and have given the possibility to further subcategorize the disease (126,128,129).

In the Netherlands, ALL in children is diagnosed in the local hospitals and confirmed by the laboratory of the Dutch Childhood Leukemia Study Group (DCLSG). Immunophenotyping and DNA-ploidy measurements are also centrally performed in the DCLSG laboratory. Additional tests, if necessary, are coordinated. Extensive immunophenotyping and immunogenotyping of children with ALL, diagnosed in the Sophia Children's Hospital, Rotterdam, are performed at the Department of Immunology, University Hospital Dijkzigt (immunophenotyping and immunogenotyping). Cytogenetic studies of childhood ALL are performed in several major centers in the Netherlands. In Rotterdam, cytogenetic studies are performed at the Department of Cell Biology and Genetics, Erasmus University, Rotterdam.

Morphological analysis

An useful classification system should be reproducible, should have a high degree of observer concordance, and should provide relevant information for clustering various diseases which share common features of etiology, pathogenesis, biology, clinical presentation and response to therapy. In 1976, the French-American-British (FAB) collaborative group proposed criteria for classifying ALL into three subtypes based on blast cytology, as seen in Romanowsky's or May Grünwald Giemsa stained BM smears (130). Assignment to one of these groups (L1, L2, or L3) is depending on the criteria shown in Table 3 (130). Due to difficulties in distinguishing the L1 and L2 cell types, a modification of this proposal was published in 1981 (131). In the revised form, a scoring system for identifying L1 and L2 was suggested, based on four cytologic features: (1) nuclear cytoplasmic ratio, (2) presence, prominence and frequency of nucleoli, (3) regularity of nuclear membrane outline, and (4) cell size. By this method an overall concordance of 84% between observers was reported (131,132). The occurrence and prognosis of the FAB classification in ALL are shown in Table 3 (128,131-139).

To discriminate between AML and ALL cytochemical characteristics are used (126, 128,140). Cytochemical reactions such as myeloperoxidase (MPO), Sudan black B, and α-naphthyl acetate esterase are positive in the majority of AML cases and negative in the majority of ALL cases (126,128,139-142), while periodic acid-Schiff (PAS) is positive in approximately 90% of ALL cases and negative, or only weakly positive, in

TABLE 3. Morphological FAB classification of ALL and its occurrence and prognosis in children[a].

	L1	L2	L3
Cytological features			
Cell size	small cells predominate (<15μ)	large, heterogeneous in size (>15μ)	large, homogeneous
Nuclear chromatin	homogeneous in any one case	variable in any one case	finely stippled, homogeneous
Nuclear shape	regular, occasional clefting or indentation	irregular, clefting and indentation common	regular, oval to round
Nucleoli	not visible or very small	one or more present, often large	prominent, one or more
Cytoplasm			
- amount	scanty	variable, often moderately abundant	moderately abundant
- basophilia	slight or moderate, rarely intense	variable, deep in some	very deep
- vacuolation	variable	variable	often prominent
Occurrence	~85%	~14%	~1%
Prognosis	good	moderate-good	moderate

a. FAB classification according to Bennett JM et al. (130).

approximately 90% of AML cases (126,128,139). The presence of a strong, localized positivity for acid phosphatase is common in T-ALL, but rare in precursor B-ALL and can be used to discriminate between these two types of ALL (126,128).

Immunological marker analysis

Immunophenotyping of leucocytes began in the mid-1970s with the development of the hybridoma technology by Köhler and Milstein (143). This technology made it possible to produce large amounts of specific monoclonal antibodies (McAb) that recognize distinct epitopes of cellular antigens. During the last two decades many McAb against cellular antigens of leucocytes have become available, in which several McAb recognize identical antigens. To create order within these large panels of McAb an international nomenclature has been designed. Five Leucocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989; Boston, 1993) have been organized and during these conferences a large part of the McAb against leucocyte antigens have been grouped into antibody clusters based on their reactivity with identical antigens (144-148). Each cluster has its own code, the so-called CD ('cluster of differentiation' or 'designation') code. So far a total of 130 CD codes have been assigned (144-148). Clusters, important for immunophenotyping of lymphoid cells, are summarized in Table 4. In this table, the function and gene location of the recognized antigen, and the reactivity pattern of the clustered antibodies are shown.

TABLE 4. Immunological markers which are used for classifying ALL.**

CD no.*	Name(s)/function	Gene location	Reactivity with hematopoietic cells	McAb (mouse isotype)[b]
Precursor marker				
CD34	precursor antigen	1q32 (1q12-qter)	precursors of lymphoid cells, precursors of myeloid cells	HPCA-2/8G12(γ1)[c]
–	TdT/function in Ig and TcR gene rearrangement (insertion of nucleotides at junction sites)	10q23-q24	immature lymphoid cells, small part of precursors of myeloid cells, virtually all ALL and some AML	conventional antisera[d]
B-cell markers				
CD19	pan B-cell antigen/ function in B-cell activation; associates with CD21 antigen (CR2)	?	precursor B-cells and B-cells	Leu-12(γ1)[d], B4(γ1)[e]
CD20	B-cell antigen/function in B-cell activation	11q12-q13.1	subpopulation of precursor B-cells, all B-cells, follicular dendritic reticulum cells	B1(γ2a)[e]
CD21	B-cell antigen/CR2 (C3d receptor); EBV receptor	1q32	subpopulations of B-cells (e.g. follicular mantle cells), follicular dendritic reticulum cells, subset of thymocytes	B2(μ)[e]
CD22	B-cell antigen/function in B-cell adhesion and B-cell activation	?	precursor B-cells and mature B-cells	Leu-14(γ2b)[c]
CD37	B-cell antigen	?	B-cells; weak expression on T-cells, monocytes and granulocytes	Y29/55(γ2a)[f]
CD72	B-cell antigen/ligand for CD5 antigen	9p	precursor B-cells and mature B-cells	J3-109(γ1)[g]
CD79a	mb-1; Igα (disulfide linked to CD79b and associated with SmIg)/signal transduction from SmIg to cytoplasm	(>6 kb)	precursor B-cells (cytoplasmic expression; CyCD79a) and mature SmIg$^+$ B-cells (membrane expression; SmCD79a); plasma cells (?)	HM47(γ1)[h], HM57(γ)[h] detect intracellular epitopes of CD79a (CD79a-Cy antibodies)
CD79b	B29; Igβ (disulfide linked to CD79a and associated with SmIg)/signal transduction from SmIg to cytoplasm	17q23 (30 kb)	precursor B-cells (cytoplasmic expression; CyCD79b) and mature SmIg$^+$ B-cells (membrane expression; SmCD77b)	B29/123[h] detects intracellular epitope of CD79b (CD79b-Cy antibody); SN8(γ1)* detects extracellular epitope
–	weak CyIgμ (weak cytoplasmic expression of Igμ chain)	14q32.3	pre-B-cells; only μ heavy chains are weakly expressed in the cytoplasm (no Ig light-chains)	selected anti-μ antisera
–	SmIg (surface membrane immunoglobulin); IgM	IgH: 14q32 Igκ: 2p12 Igλ: 22q11	SmIg positive cells; each B-cell clone expresses only one type of Ig light-chain (κ or λ), but may express multiple Ig heavy-chains	conventional antisera and McAb
–	IgD, IgA, IgE	IgH: 14q32 Igκ: 2p12 Igλ: 22q11	SmIg positive cells; each B-cell clone expresses only one type of Ig light-chain (κ or λ), but may express multiple Ig heavy-chains	conventional antisera and McAb
–	CyIg (cytoplasmic immunoglobulin)	14q32	CyIg positive cells (immunoblasts, immunocytes and plasma cells)	conventional antisera and McAb

CD no.[a]	Name(s)/function	Gene location	Reactivity with hematopoietic cells	McAb (mouse isotype)[b]
T-cell markers				
CD1	T6 antigen; common thymocyte antigen/MHC-like protein; can associate with β2-microglobulin	1q22-q23	cortical thymocytes (strong), Langerhans cells, subpopulation of dendritic cells, subpopulation of B-cells	6611C7(γ2a)[l]
CD2	T11 antigen; SRBC receptor (=E rosette receptor); LFA-2/receptor for T cell activation; ligand for CD58 (LFA-3)	1p13	all T-cells, most NK cells; three different antigenic epitopes are known, of which one is the SRBC binding site	T11(γ1)[e]
CD3	T3 antigen (associated with TcR)/signal transduction from TcR to cytoplasm	11q23	immature T-cells (cytoplasmic expression; CyCD3) and mature functional T-(membrane expression); the CD3 antigen consists of at least five protein chains	Leu-4(γ1)[c], UCHT(γ1)[g,k,l,m,n,o,p,q]
CD4	T4 antigen/involved in MHC-class-II-restricted antigen recognition; HIV receptor	12pter-p12	subpopulation of cortical thymocytes, helper/inducer T-cells, subpopulation of monocytes and macrophages; some AML	Leu-3a(γ1)[c]
CD5	T1 antigen/function in T-cell proliferation; ligand for CD72 antigen on B-lymphocytes	11q13	thymocytes and mature T-lymphocytes, subpopulation of B-cells; B-CLL	Leu-1(γ2a)[c]
CD6	T12 antigen; pan T-cell antigen	11	thymocytes and mature T-lymphocytes, subpopulation of B-cells; B-CLL	OKT17(γ2)[r]
CD7	Tp41 antigen/Fc receptor for IgM (FcμR)?	17q25.2-q25.3	almost all T cells, NK cells, subpopulation of immature myeloid cells; some AML	Leu-9(γ2a)[c]
CD8	T8 antigen; the CD8 molecule consists of two disulfide linked chains: α-α homodimer or α-β heterodimer/involved in MHC-class-I-restricted antigen recognition	2p12	subpopulation of cortical thymocytes, cytotoxic/suppressor T-cells, subpopulation of NK cells	most CD8 antibodies detect CD8-α chain: Leu-2a(γ1)[c]
–	TcR-αβ (classical TcR; TCR2)	TcR-α: 14q11 TcR-β: 7q35	TcR-αβ is expressed by a majority of mature $CD3^+$ T-cells	WT31(γ1)[b] and BMA031(γ2b)[s] probably recognize non-polymorphic epitopes of TcR-αβ
–	TcR-γδ (alternative TcR; TCR1)	TcR-γ: 7p15 TcR-δ: 14q11	TcR-γδ is expressed by a minority of mature $CD3^+$ T-cells	anti-TcR-γ/δ-1(γ1)[c] and TCRδ1(γ1)[t] recognize non-polymorphic epitopes of TcR-γδ
NK-cell markers				
CD56	NCAM; PI-linked and transmembrane forms	11q23	NK cells, some T-cells (neuroectodermal cells)	Leu-19(γ1)[c]
CD57	human natural killer cell antigen	11q12-pter	subpopulation of NK cells, subpopulation of T-cells, some B-cells	Leu-7/HNK-1(μ)[c]

CD no[a]	Name(s)/function	Gene location	Reactivity with hematopoietic cells	McAb (mouse isotype)[b]
cross-lineage myeloid markers				
CD13	pan myeloid antigen; aminopeptidase N; differential glycosylation generates different epi-topes, detected by McAb	15q22-q26	almost all myeloid cells, dendritic cells in the skin	My7(γ1)[e], Leu-M7(γ1)[c], CLB-mon-gran/2(γ2a)[u]
CD14	monocytic antigen; PI-linked protein	5q22-q32	monocytic cells, macrophages, follicular dendritic reticulum cells, B-(weak); absent in patients with PNH	My4(γ2b)[e]
CD15	Lewis-X (Le^X); X hapten; 3-FAL (3-fucosyl-N-acetyl-lactosamine)	11q12-qter	cells of the granulocytic lineage, weak expression by monocytes, Reed Sternberg cells	Vim-D5(μ)[v], CLB-gran/2(μ)[u]
CD33	pan myeloid antigen	1q13.3	majority of myeloid and monocytic cells (except for granulocytes)	My9(γ2b)[e], Leu-M9(γ1)[c]
CDw65	myelomonocytic antigen (fucoganglioside; ceramidedodecasaccharide 40)	11q12-qter	majority of myeloid and monocytic cells and a part of their precursors	VIM-2(μ)[m,s,v]
–	MPO (myeloperoxidase); MPO consists of two subunits	17q21.3-q23	majority of cells of the myeloid lineage (granulocytic and monocytic cells)	MPO-7(γ1)[k]
non-lineage restricted markers				
CD9	p24 antigen (tetraspan molecule)/induction of agregation of platelets	12p13	subpopulation of precursor B-cells, subpopulation of B-cells (follicular center cells), monocytes, megakaryocytes, platelets, eosinophils, basophils	BA-2(γ3)[w], CLB-thromb/8(γ2a)[u]
CD10	common ALL antigen (CALLA)/neutral endopeptidase (enkephalinase)	3q21-q27	subpopulation of precursor B-cells, subpopulation of B-cells (follicular center cells), subpopulation of cortical thymocytes, granulocytes	OKBcALLa(γ2a)[o], J5(γ2a)[e,l]
CD23	B-cell antigen/FcεRII (low affinity Fc receptor for IgE); two types of FcεRII exist, which differ in their cytoplasmic domain (FcεRIIa and FcεRIIb)	19p13.3	FcεRIIa is expressed by a subpopulation of B-cells (e.g. follicular mantle cells) and B-CLL cells; FcεRIIb is expressed by subpopulation of B-cells, monocytes, eosinophils, dendritic cells	Leu-20(γ1)[c], TÜ1(γ1)[o,u,x]
CD24	B-cell-granulocytic antigen; PI-linked protein on granulocytes	?	subpopulation of (precursor) B-cells, granulocytes; absent on granulocytes in patients with PNH	BA-1(μ)[w], CLB-gran-B-ly/1(γ1)[u]
CD25	Tac antigen/α chain of the IL-2 receptor (low affinity IL-2R); high affinity IL-2R when associated with β chain (CD122 antigen) and/or γ chain	10p15-p14	activated T-cells, activated B-cells, activated macrophages; HCL	2A3(γ1)[c]

CD no.[a]	Name(s)/function	Gene location	Reactivity with hematopoietic cells	McAb (mouse isotype)[b]
CD71	T9 antigen (homodimer)/transferrin receptor	3q26.2-qter	proliferating cells (e.g. thymocytes), activated cells, macrophages	66IG10(γ2a)[i]
–	HLA-DR, non-polymorphic antigen/MHC-class II molecule	6p21.3	hematopoietic precursor cells, B-cells, activated T-cells, monocytic cells and macrophages	L243(γ2a)[c]
–	nuclear antigen in proliferating cells	10	proliferating cells during late G1, S, G2 and M phases of the cell cycle	Ki-67(γ1)[k]
–	MDR1; P-glycoprotein (P-gp)/transmembrane pump for cytotoxic pro-ducts, including several cytostatics, thereby causing multidrug resistance (MDR)	7q21.1	very low expression (generally not detectable by use of antibodies) in blood and bone marrow cells; high expression in some leukemias, especially a part of AML	JSB-1(γ1)[l] and C219(γ2a)[y] detect intracellular epitope of MDR1; MRK16(γ2a)[g,z] detects extracellular epitopes of MDR1

a. CD = cluster of differentiation, as described during the Leucocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989; Boston, 1993).
b. Only McAb are included, which are routinely used in the Immunodiagnostic laboratory of the Department of Immunology, University Hospital Dijkzigt, Rotterdam, The Netherlands.
c. Becton Dickinson, San Jose, CA.
d. Supertechs, Bethesda, MD.
e. Coulter Clone, Hialeah, FL.
f. Dr. H.K. Forster, Hoffmann La Roche, Basel, Switzerland.
g. Immunotech S.A., Marseille, France.
h. Dr. D.Y. Mason, Oxford, UK.
i. Monosan, Sanbio B.V., Uden, The Netherlands.
k. DAKOPATTS, Glostrup, Denmark.
l. SeraLab, Crawley Down, UK.
m. Serotec, Oxford, UK.
n. PharMingen, ITK Diagnostics B.V., Uithoorn, The Netherlands.
o. Cymbus Bioscience Ltd, Southhampton, UK.
p. Sigma Immunochemicals, Brussels, Belgium.
q. Ansell Corporation, Läufelfingen, Switzerland
r. Ortho Diagnostic Systems, Raritan, NJ.
s. Behring, Marburg, FRG.
t. T-CELL DIAGNOSTICS, Cambridge, MA.
u. Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands.
v. Dr. W. Knapp en Dr. O. Majdić, Vienna, Austria.
w. Boehringer Mannheim, Mannheim, FRG.
x. Biotest, Dreieich, FRG.
y. Centocor Europe, Leiden, The Netherlands.
z. Hoechst Japan LTD, Kawagoe, Japan.
* Recently clustered antibody (Leukocyte Typing Conference V, Boston, 1993), of which the availability is not yet known.

** Adapted from Van Dongen JJM and Comans-Bitter WM. Leukocytentypering en de CD nomenclatuur. In: Van Dongen JJM, Groeneveld K, Adriaansen HJ, and Hooijkaas H, eds: Immunofenotypering in de diagnostiek: Indicatiestellingen, uitvoering en interpretatie. Afdeling Immunologie, EUR Rotterdam, 1994.

Beside morphological characterization, immunological marker analysis can further characterize the cells of the various hematopoietic differentiation stages (110,111,128,-149-153). Although immunological markers represent differentiation antigens, they usually are not specific for one differentiation stage, but are expressed in several stages. However, the expression of a specific set of markers can designate a cell to a particular differentiation stage (110,111,149-153). The combination of the various markers per lymphoid differentiation stage is summarized in Figure 6.

Several markers are not restricted to one differentiation lineage, but are expressed in several lineages (B-, T-, or myeloid lineage) (Figure 6 and Table 4). The enzyme TdT is present in the nucleus of immature lymphoid cells, but is absent in more mature stages (110,111,152). The precursor antigen CD34 is found on most immature cells of both lymphoid and myeloid stages (Figure 6 and Table 4) (110,111,152). The HLA-DR

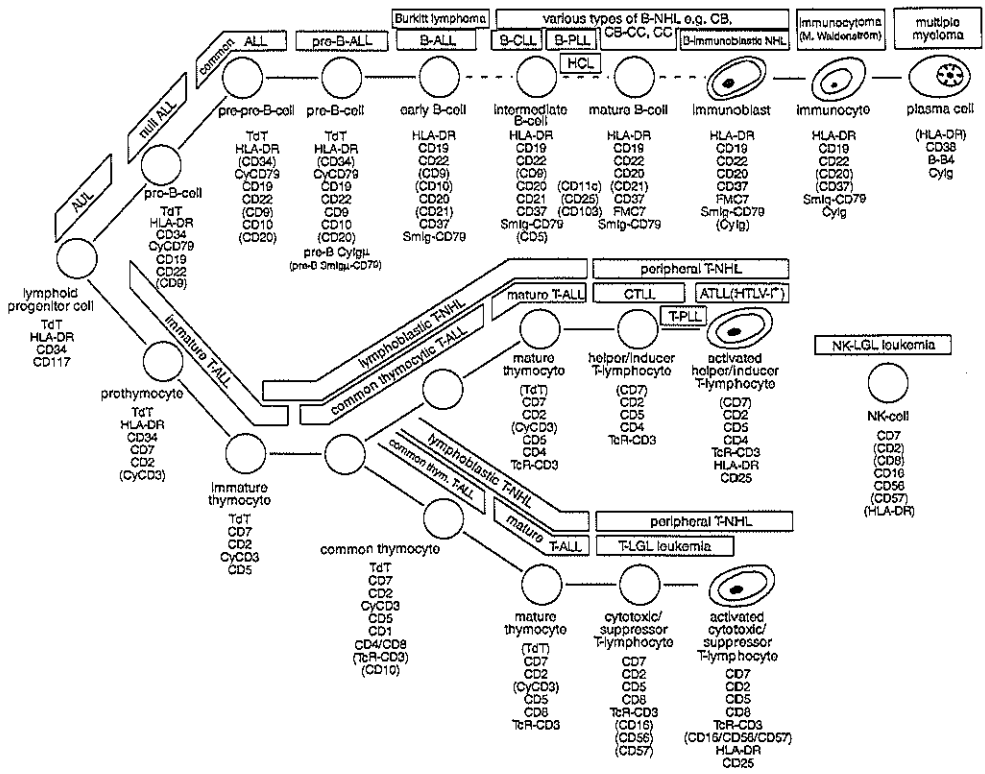

Figure 6. Hypothetical scheme of lymphoid differentiation. The expression of relevant immunological markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias and NHL and indicate where these malignancies can be located according to their maturation arrest: ALL, acute lymphoblastic leukemia; ATLL, adult T-cell leukemia lymphoma; AUL, acute undifferentiated leukemia; CB, centroblastic lymphoma; CB-CC, centroblastic-centrocytic lymphoma; CC, centrocytic lymphoma; CLL, chronic lymphocytic leukemia; CTLL, cutaneous T-cell leukemia lymphoma (mycosis fungoides/Sézary syndrome); HCL, hairy cell leukemia; HTLV, human T-cell leukemia virus; T-LGL, T-cell large granular lymphocytic leukemia; NK cell LGL, NK cell large granular lymphocytic leukemia; NHL, non-Hodgkin lymphoma; PLL, prolymphocytic leukemia. Adapted from ref. 111 and reproduced with permission from the authors.

antigen is expressed by cells in immature hematopoietic differentiation stages, but also by B-cells, monocytic cells, and activated T-lymphocytes (110,111,152). More examples are shown in Table 4.

The reactivity of the McAb can be visualized by staining methods, in which fluorochromes or enzymes are generally used as labels (154,155). These staining techniques can be used for the detection of immunological markers on hematopoietic cells in suspension as well as on cells in tissue sections (154,155). Not only single staining, but double and even triple stainings can be done by use of different fluorochromes or enzymes (156). The evaluation of immunofluorescence (IF) stained cells can be performed with IF-microscopic or flow cytometric techniques (155,157-160).

The maturation arrest of the ALL is localized in the immature differentiation stages

TABLE 5. Immunophenotypes of ALL.

ALL	TdT	HLA-DR and/or CD22	CD19	CD10	Cylgμ	pre-B Smlgμ-CD79	Smlg	pan T-cell marker
null ALL	+	+	+	−	−	−	−	−
common ALL	+	+	+	+	−	−	−	−
pre-B-ALL	+	+	+	+	+	−	−	−
tr-pre-B-ALL	+	+	+	+	+	+	−	−
B-ALL	−	+	+	−/+	−	−	+	−
T-ALL[a]	+	−/+	−	−/+	−	−	−	+

a. Several subtypes of T-ALL can be recognized, based on their positivity for the various T-cell markers.

(110,111,149,150,152,153). Chronic lymphocytic leukemias (CLL) are the malignant counterparts of cells in more mature differentiation stages (110,111,150). Generally, NHL have a mature immunophenotype (Figure 6) (110,111,150). Initially, the ALL were divided in four distinct subgroups according to Nadler et al. (161). Nowadays, we recognize five different types according to their immunophenotype: null ALL, common ALL, pre-B-ALL, B-ALL and T-ALL (110,111,150-153,162). The pre-B-ALL is defined by the cytoplasmic expression of Ig heavy chain μ (Cylgμ). Recently a subgroup of the pre-B-ALL has been defined, which expresses not only Cylgμ but also a pre B-cell complex (pre-B Smlgμ-CD79) on the surface membrane. This subgroup of pre-B-ALL is called transitional pre-B-ALL (tr-pre-B-ALL) (153,162,163). The immunophenotypes of all six ALL are summarized in Table 5. T-ALL can be subdivided into several immature and more mature subtypes, as indicated in Figure 6 (111,164).

The distribution of specific immunophenotypes of ALL is clearly influenced by age (Figure 7) (19). In infants, there is a predominance of null ALL, while in children between the age of two and five years the $CD10^+$ precursor B-ALL dominate (common ALL and pre-B-ALL) (Figure 7). T-ALL are relatively more frequently found in older children starting with the age of six (Figure 7).

The prognostic significance of subtyping childhood ALL is based on the fact that the different subtypes of ALL have different outcomes when compared to each other (153,165-167). Common ALL have the best prognosis, while null ALL and T-ALL have the worst (153,165,167). Upon looking at single markers or combination of markers as individual prognostic features, such as CD10 or CD34 expression or activation status (CD38 or CD71 positivity) of the malignant cells (168-171), the results are uncertain because in the majority of studies no adjustments were made for other risk factors, such as age, high white blood cell (WBC) count, or therapy (168-171). Recently, several articles have been published about cross-lineage marker expression in ALL (e.g. myeloid marker positive precursor B-ALL) (167,172-178). Although large groups of childhood precursor B-ALL patients have been analyzed, the conclusions of these results are not concurrent. Still a tendency towards a worse prognosis for myeloid marker positive precursor B-ALL is observed (153,167,173-178). The clinical outcome of the various subtypes of ALL seems to be also dependent on the presence of specific karyotypic abnormalities (153,179). The prognosis of these ALL can, therefore, not only be

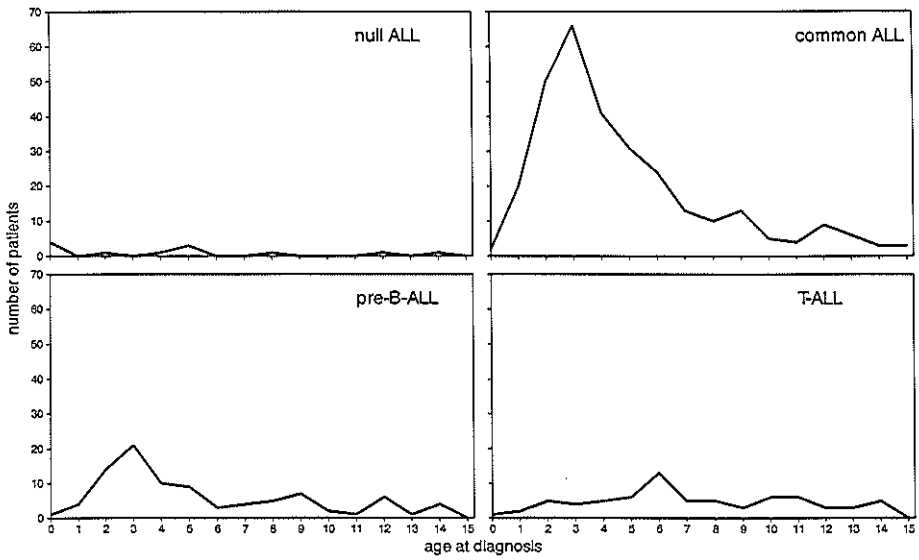

Figure 7. Number of ALL patients divided in four subgroups (null ALL, common ALL, pre-B-ALL, and T-ALL) according to age at diagnosis in the Netherlands (1986-1991). Adapted from Coebergh JWW et al. (19) with permission.

determined by immunological marker analysis.

Cytogenetic analysis

Cytogenetic analysis in ALL is more difficult than in AML. In ALL, despite high cellularity and high percentages of leukemic cells in BM and PB, only a low yield of leukemic metaphases is obtained, with chromosomes which are very fuzzy and of poor morphology (180,181). The success rate of chromosome studies in ALL is dependent on the time between harvesting and analyzing the leukemic cells and on the experience of cytogenetic laboratories (180,181). In multicenter trials an average of ~70% successful cytogenetic analyses is reached (182), whereas in several single-center trials, cytogenetic analysis is successful in 90-95% (180,181,183).

In childhood ALL the abnormal karyotypes are distributed in five categories, based on changes in ploidy: high hyperdiploid with more than 50 chromosomes, hyperdiploid with 47-50 chromosomes, pseudodiploid, normal (diploid), and hypodiploid (116,183-187). The frequencies of these different groups in childhood ALL are summarized in Table 6 (116,181,183-188). The DNA content of leukemic cells can easily be estimated by flow cytometry in nearly all ALL cases (189-191). With this method, a DNA index (ratio of the DNA content of leukemic G_0/G_1 cells versus that of normal diploid cells) can be derived, which serves to distinguish two prognostic categories of patients based on the findings: < 1.16 or ≥ 1.16 (approximately equal to < 53 or ≥ 53 chromosomes) (191). These hyperdiploid groups show a specific pattern of gain of chromosomes with mainly trisomy of chromosomes X, 4, 6, 10, 14, 17, 18 and often tetrasomy 21 (116,181,183,187,192). Several studies about large groups of ALL patients have con-

TABLE 6. Distribution of karyotypes in childhood ALL[a].

Ploidy-group	Frequency (%)
Hypodiploid	5
near haploid	<1
hypodiploid (30-40 chromosomes)	<1
hypodiploid (41-45 chromosomes)	5
Pseudodiploid	25-35
Normal (diploid)	10-20
Hyperdiploid	40
hyperdiploid (47-50 chromosomes)	10-15
hyperdiploid (>50 chromosomes)	25-30
near triploid	<1
near tetraploid	1

a. References 116,181,183-188,212.

sistently indicated that a DNA index ≥ 1.16 equates with a favorable outcome, especially in precursor B-ALL (192-202).

The largest ploidy group in ALL is the pseudodiploidy group, characterized by a chromosome number of 46 per cell with structural abnormalities, mainly translocations (Table 6) (116,181,183,187). As recently indicated in the literature, it is likely that the occurrence of these chromosomal abnormalities is underestimated when analysis is performed with standard cytogenetic techniques (203). However, molecular screening for such abnormalities by the polymerase chain reaction (PCR) or with the fluorescence in situ hybridization (FISH) technique will identify more cases that are missed by cytogenetic studies, either because of technical difficulties or because these abnormalities were not visible by light microscopy (203-208). Two other advantages of the FISH technique is the detection of both numerical and structural aberrations not only in metaphase spreads but also in interphase nuclei, and the possibility to combine the FISH technique with morphological or immunological analysis (207-211). The incidence of ALL cases that lack apparent cytogenetic abnormalities will probably diminish when these new techniques are used routinely.

Hypodiploidy is a relatively uncommon finding in ALL, affecting only 5-7% of patients (116,181,187). Most cases (> 80%) have 45 chromosomes with loss of chromosome 20 (187). Recently, three subdivisions of the hypodiploid category were recognized (Table 6) (212). Prognostic implications can not be determined for each of these categories due to the limited number of cases reported (187,212).

In the majority of ALL cases at least one non-random chromosomal abnormality could be detected, often in association with a specific immunophenotype (179,187,-213,214). Describing ALL by the types of structural abnormalities found in chromosomes of the leukemic cells has led to impressive advances in the understanding of the origin and development of leukemia (94,105). Molecular analysis of genes adjacent to the breakpoints of these structural abnormalities and studies on the functions of their protein products have helped to gain understanding in the complex interactions that support leukemogenesis and perpetuate the leukemic cell phenotype (see also section

1.2: Etiology of childhood ALL) (94,105). Table 7 summarizes the cytogenetic abnormalities associated with childhood ALL, their frequency of occurrence, the genes involved, and their association with specific leukemic immunophenotypes.

Within each ALL subgroup specific structural chromosomal abnormalities are found. In null ALL, 11q23 abnormalities, especially t(4;11)(q21;q23) have the highest incidence (187). These 11q23 abnormalities are associated with a high WBC count, CNS involvement, young age at diagnosis, often absence of hyperdiploidy, and a poor prognosis (116,187,215-217,219,220). The Philadelphia chromosome or t(9;22)(q34;q11) is the most frequent translocation in adult ALL and is the hallmark of chronic myeloid leukemia (315). It is found less frequently in childhood precursor B-ALL and rarely in T-ALL (116,251-253,255); it is associated with a FAB-L2 morphology and has a poor prognosis (254,255,316). The t(1;19)(q23;p13) is clearly correlated with the pre-B-cell immunophenotype and is one of the most common translocations in childhood ALL (116,187). This translocation occurs in either a balanced, t(1;19)(q23;p13) (25%), or an unbalanced, -19, +der(19), t(1;19)(q23;p13) (75%) form (261). They are associated with several adverse prognostic features: such as high WBC count and the lack of hyperdiploidy and they have an intermediate outcome (265,266). Recently, it was found that patients with the balanced t(1;19) have a significantly worser outcome when compared with the unbalanced der(19) form (317). B-ALL contains one of three specific chromosomal translocations: the t(8;14)(q24;q32) (90%), or less commonly, either the t(2;8)(p12;q24) (4-5%), or the t(8;22)(q24;q11) (6-10%). This translocation is associated with FAB-L3 morphology and extramedullary disease and has an intermediate prognosis (271).

A large part of chromosomal abnormalities in T-ALL involve one of the TcR genes, located on either 14q11 (TcR-α/δ), 7q35 (TcR-β), or 7p15 (TcR-γ) (187,287,318). Apparently, these chromosomal abnormalities originate from TcR gene rearrangements during T-cell differentiation. Alteration of the *TAL1* gene (located on 1p32) occurs either by translocation t(1;14)(p32;q11) or by a submicroscopic deletion of ~90 kb (298-300,303). The *TAL1* deletion is the most frequent genetic lesion associated with T-ALL (10-30%) (299). Also in these cases the recombinase system, used in Ig and TcR gene rearrangements, is active via recombination signal sequences found in these gene regions (299).

Lineage independent chromosomal aberrations occur rather frequently in ALL and encompass abnormalities of 6q, 9p, and 12p (187). In the majority of cases these abnormalities are deletions (187). No correlation with a specific immunophenotype is found although most cases with a 12p aberration have a precursor B-ALL immunophenotype (248). The critical region involved in 9p deletions is p21-p22, which encloses the interferon α and β gene clusters (313,314). These deletions possibly lead to loss of tumor suppressor genes located in this 9p region (313,314).

The prognostic value of specific karyotypic abnormalities in ALL is high as indicated before. Table 8 summarizes the outcome of the various cytogenetic abnormalities found in the different childhood ALL. The group of patients with high hyperdiploidy shows the highest response rate and a probability of cure of about 85% with current therapy. Favorable response rate and survival time is found for the group of patients with normal karyotype and with slight aneuploidy (45-50 chromosomes), provided that the poor-risk

TABLE 7. Cytogenetic abnormalities associated with childhood ALL[a].

Chromosome abnormality	Frequency (%) (within immunophenotypic subgroup)[b]	Immuno-phenotype	Genes involved[c]	References
precursor B-lineage				
t(1;11)(p32;q23)	<1	null ALL	–[d];*MLL*	114,215-217
t(4;11)(q21;q23)	2 (80)	null ALL	*AF4;MLL*	114,215-228
t(9;11)(p22;q23)	<1	null ALL	*AF9;MLL*	114,229-233
t(11;14)(q23;q11)	<1	null ALL	*MLL*;TcR-α/δ	234
t(11;17)(q23;q21)	<1	null ALL	*MLL*; –	235,236
t(11;19)(q23;p13)	<1	null ALL	*MLL;ENL*	217,237-242
t(5;14)(q31;q32)	<1	common ALL	*IL3;IGH*	243-245
dic(7;9)(p13;p11)	<1	common ALL	– ; –	116,246,247
dic(9;12)(p11-p12;p12)	1-2 (15)	common ALL	– ; –	248-250
t(9;22)(q34;q11)	2-5 (20-30)	CD10$^+$ precursor B-ALL	*ABL;BCR*	251-255
t(10;11)(p14-p15;q23)	<1	common ALL	– ; *MLL*	215,217
t(12;21)(p11-p12;q22)	<1	common ALL	– ; –	256
t(17;19)(q22;p13)	<1	common ALL	*HLF;E2A*	247,257,258
t(1;19)(q23;p13)	5-6 (90)	pre-B-ALL	*PBX1;E2A*	114,259-267
B-lineage				
t(2;8)(p11;q24)	<1 (4-5)	B-ALL	*IGK;MYC*	268-271
t(8;14)(q24;q32)	1-2 (90)	B-ALL	*MYC;IGH*	268,271-274
t(8;22)(q24;q11)	<1 (6-10)	B-ALL	*MYC;IGL*	268,271,275,276
T-lineage				
t(9;22)(q34;q11)	<1	T-ALL	*ABL;BCR*	116
t(10,11)(p13-14;q14-21)	<1	T-ALL	– / –	277
TcR-α/δ locus, located on 14q11				
t(8;14)(q24;q11)	<1 (2)	T-ALL	*MYC*;TcR-α/δ	278,279
t(10;14)(q24;q11)	1 (5-10)	T-ALL	*HOX11*;TcR-α/δ	280-286
t(11;14)(p13;q11)	1-2 (7)	T-ALL	*RBTN2*;TcR-α/δ	114,287-294
t(11;14)(p15;q11) 287,290,291,293,294	<1 (1)	T-ALL	*RBTN1*;TcR-α/δ	
inv(14)(q11;q32)	<1	T-ALL	TcR-α/δ;*IGH*	287,295
TAL1 locus, located on 1p32				
tal-1 deletion (1p32)	3 (10-30)	T-ALL	*TAL1*	296-301
t(1;7)(p32;q35)	<1	T-ALL	*TAL1*;TcR-β	302
t(1;14)(p32;q11)	<1 (3)	T-ALL	*TAL1*;TcR-α/δ	303
TcR-β locus, located on 7q35				
t(1;7)(p34;q35)	<1	T-ALL	*LCK*;TcR-β	304,305
t(7;9)(q35;q34)	<1	T-ALL	TcR-β;*TAN1*	302,304,306
t(7;9)(q35;q32)	<1	T-ALL	TcR-β;*TAL2*	302,304,306
t(7;10)(q35;q24)	<1	T-ALL	TcR-β;*HOX4*	187,307
t(7;11)(q35;p13)	<1	T-ALL	TcR-β;*RBTN2*	116,187,292
t(7;19)(q35;p13)	<1	T-ALL	TcR-β;*LYL1*	187,308
inv(7)(p15;q35)	<1	T-ALL	TcR-γ;TcR-β	187,309
Non-lineage specific				
del(6q)	4-13			187,188,310
t/del(9p)	7-13			187,230,311-314
t/del(12p)	~10			187,197,246

a. Adapted from Pui C-H et al. (116), Crist WM et al. (185), and Raimondi SC (187).

b. If known, the frequency of the chromosomal abnormality within a specific ALL immunophenotypic subgroup is given between brackets.

c. Genes involved: *MLL* is also named *ALL1* and *HRX*; *AF4* is also named *FEL*; *AF9* is also named *MLLT3*; *ENL* is also named *MLLT1*; *TAL1* is also named *SCL* and *TCL5*; *HOX11* is also named *TCL3*; *RBTN2* is also named *TTG2*; *RBTN1* is also named *TTG1*.

d. –, the involved gene or gene locus is not known.

TABLE 8. Immunophenotype and prognostic value of childhood ALL with various karyotypes[a].

Karyotype	Immunophenotype	Outcome
Hyperdiploid (>50 chromosomes)	common ALL	Good
dic(9;12)(p11-p12;p12)	common ALL	
Hyperdiploid (47-50 chromosomes)	no specific	Favorable
Normal	immunophenotype	
Hypodiploid (45 chromosomes)		
Translocations (pseudodiploid)		Intermediate
t(1;19)(q23;p13)	pre-B-ALL	
t(8;14)(q24;p13)	T-ALL	
t(11;14)(p13;q11)	T-ALL	
inv(14)(q11;q32)	T-ALL	
t(8;14)(q24;q32)	B-ALL	
Hypodiploid		
near haploid	common ALL	Poor
t(9;22)(q34;q11)	common ALL	Very poor
t(4;11)(q21;q23)	null ALL	
t(11;19)(q23;p13)	null ALL	
$6q^-$	no specific	Unclear
$9p^-$	immunophenotype	
$12p^-$		
near tetraploid/triploid		

a. References 116,181,183,185,187,192,197-202,212,220,241,271,291,316,317.

translocations are absent. Poor outcome is found in patients with a near haploid karyotype. Translocations, particularly t(9;22), t(4;11) and t(11;19) have been shown to identify patients with the shortest survival (Table 8).

Molecular genetic analysis

ALL is a clonal disease, in which each cell has quite similar characteristics. Based on this fact, recombinant DNA techniques, which enable analysis of genes at the DNA or mRNA level, can be used to determine clonality of suspected cell populations (120,319). The techniques used are Southern blotting and PCR (319-321). With both techniques gene rearrangements, deletions, or mutations can be detected. In the case of Southern blotting this is done with specific probes and in the case of the PCR technique by amplification of a specific piece of DNA (319-321). Nowadays, both techniques have earned their place in research of leukemias and lymphomas and both are frequently used in the diagnosis of hematopoietic malignancies, especially in those cases in which other techniques are not conclusive (120,323). In the ensuing chapters of this thesis the usage of both techniques in the analysis of childhood ALL will be discussed in more detail.

Characterization of ALL with other techniques

In addition to the above described techniques, characterization of ALL can further be

done by ultrastructural morphology and cell culture analysis (323-328). Ultrastructural morphology and cytochemistry are not essential for the diagnosis of ALL. However in case of undifferentiated or minimally differentiated acute leukemias, electron microscopy and cytochemistry (e.g. MPO) provide conclusive information for diagnosis and classification of these acute leukemia cases (325-328).

Cell culture analysis has a low diagnostic value in ALL. Still, for the detection of residual ALL cells in cultures of BM obtained during remission, this technique has shown its merit (323,326,329-331). Especially for research purposes, such as specific growth patterns or determination of cell death after addition of different growth factors, cytokines or cytostatic drugs, the *in vitro* culture experiments of ALL cells at diagnosis appear to be important (330-336). Furthermore, these *in vitro* findings may have prognostic significance for obtaining remission or developing relapse and for stratification of treatment protocols (330-332,334,336).

1.4 PROGNOSIS OF CHILDHOOD ALL

The prognosis of children with ALL has improved remarkably during the last two decades (337-339). Presently, 70-80% of newly diagnosed children may be expected to survive with current treatment protocols (339). However, still 20-30% of children will eventually die as a cause of this disease. The prediction which children with ALL have a high probability of treatment failure has become possible through the definition of prognostic factors that may be determined at the time of diagnosis (339-341). A variety of factors that have been associated with prognosis in ALL is summarized in Table 9.

The initial leucocyte count is perhaps the most significant prognostic factor identified (342). Children with a high WBC count (more than 50,000 cells/mm^3) tend to have a poorer prognosis (342). There is also a clear relationship between age at diagnosis and outcome. Patients who are young when diagnosed (<2 years of age) and older patients (>10 years of age) have a relatively poor prognosis in contrast to children in the intermediate age group (343,344). Studies analyzing the prognostic importance of sex revealed that boys have a poorer prognosis than girls (345,346). Immunophenotype and FAB morphology also correlate with prognosis, in which patients with a B-ALL/L3 phenotype have an intermediate prognosis (153,347). Cytogenetic abnormalities both in chromosomal number (ploidy) and structure appeared to have prognostic significance (see section 1.3: Cytogenetic analysis) (116,185,348). Except for race, all other factors

TABLE 9. Factors that have been associated with poor prognosis in ALL[a].

Initial WBC count (>50.10^9 cells/l)	Organomegaly and lymphadenopathy
Age at diagnosis (<2y and >10y)	Low hemoglobulin level
Cytogenetics/ploidy	Race (black > white children)
Sex (M>F)	Low platelet count
Immunological subtype	Low serum immunoglobulins
FAB morphology	Slow leukemic cytoreduction (>4-6 weeks)
Mediastinal mass	CNS leukemia at diagnosis

a. References 116,153,185,342-350.

summarized in Table 9 are more or less associated with the tumor burden at diagnosis and its effect on normal hematopoiesis (349,350). The prognostic importance of the factors shown in Table 9 is dependent on the applied therapy protocol. They may lose prognostic significance with more intensive and specific chemotherapy (351-353). So in some categories, therapy appeared to be the most important prognostic feature.

1.5 TREATMENT OF CHILDHOOD ALL

Introduction

Nowadays, the majority of children with ALL in developed countries achieve long-term survival and cure, caused by improved treatment protocols during the last decades (338,339,353-360). The current principles of curative treatment of childhood leukemia are based on eradication of the malignant cells with aggressive strategies involving the earlier use of intensive therapy, prophylactic treatment for relapse of leukemia in the meninges, optimal supportive therapy and reduction of side effects of treatment. The combination of these principles in treatment is one of the reasons for this success.

The therapy of first choice in childhood ALL is chemotherapy. Bone marrow transplantation (BMT) (allogeneic, syngeneic, or autologous) will be done after first relapse during second remission.

Chemotherapy

In the Netherlands, children with ALL are treated according to DCLSG protocols since 1972. The current protocols (ALL VII 1988-1991 and ALL VIII 1992-not closed) are based on treatment principles of the International Berlin-Frankfurt-Münster (BFM) Study Group. Children with ALL are stratified into three treatment groups according to the BFM risk factor (based on WBC count, liver and spleen size), the presence or absence of mediastinal mass, CNS leukemia, chromosomal aberrations, and a specific immunophenotype. The stratification of the ALL patients over the three treatment groups is: 34% in the standard risk group (SRG), 59% in the medium risk group (MRG), and 7% in the high risk group (HRG) in case of ALL VII. Each risk group starts with an identical induction treatment of five weeks. After this, the treatment is divided into separate protocols. Each of these protocols consist of an intensive chemotherapy (consolidation treatment) until 28 to 32 weeks, starting from diagnosis, after which maintenance treatment is applied until two years of therapy.

The initial aim of ALL treatment is induction of remission. Complete remission (CR) is achieved when BM has a normal cellularity with less than 5% lymphoblasts and absence of leukemic cells in PB, CNS, or elsewhere in the body (129). The induction treatment includes a combination of prednisone, vincristine, daunorubicin, and L-asparaginase. Presently a CR is achieved in ~97% of ALL cases. During this induction phase intrathecal CNS prophylaxis is given containing methotrexate, cytosine arabinoside (ARA-C), and prednisolone. After induction treatment intensive consolidation chemotherapy is continued with several other cytostatic drug combinations. During maintenance treatment all ALL patients receive 6-mercaptopurine daily and methotrexate weekly.

Unfortunately, treatment failure because of drug resistance occurs. The resistance to cytostatic drugs may represent 'apparent' drug resistance or 'true' drug resistance. Apparent drug resistance may be caused by hiding of leukemic cells in locations which are less accessible for the cytostatic drugs such as CNS, or by pharmalogical factors such as drug scheduling or dosage (361,362). True drug resistance is considered to be a resistance at the cellular and/or molecular level, such as an increased production of the enzyme which is the target for the drug. An example is the resistance to methotrexate due to amplification of the dihydrofolate reductase gene (363,364). Another type of drug resistance is the multidrug resistance (MDR), which implies resistance against a variety of drugs including anthracyclines, vinca-alkaloids, and actinomycin D (365-367). The significance of the latter form of drug resistance appeared to be low in ALL (368-370).

Bone marrow transplantation

BMT involves the administration of intensive cytoreductive therapy (high-dose chemotherapy in doses lethal to normal BM), in combination with total body irradiation and subsequently intravenous infusion of BM obtained from an appropriate compatible donor (371).

As mentioned above, BMT as therapy for childhood ALL is only applied after first relapse, because the results of standard intensive chemotherapy regimens in children are sufficiently good (339,372). In some treatment protocols, BMT is performed as part of the primary therapy in ALL with very poor prognosis such as in cases with special translocations (e.g. t(9;22) and t(4;11)) (339,373,374).

Allografting in second remission with a HLA-matched sibling (allogeneic BMT) has an equal overall survival as with autologous BMT (ABMT). This was shown in a study comparing both types of BMT in relapsed childhood ALL (375). A higher rate of subsequent relapses was found in ABMT, due to the presence of residual leukemic cells in the graft, while a higher rate of deaths was found in the allogeneic BMT group, caused by graft versus host disease (375). Survival rates of ~30% are obtained after allogeneic BMT in relapsed childhood ALL patients, as shown in a recent study which analyzed 123 patients during 12 years follow-up (376). A second course of chemotherapy for childhood ALL after relapse, instead of BMT, results in lower survival rates (5-30% for chemotherapy, and 45% for BMT or ABMT), but long-term follow-up is needed to be conclusive (377).

The role of growth factors (such as GM-CSF, G-CSF, and IL-3) in the treatment of ALL has to be investigated. They are able to shorten the duration and severity of chemotherapy-induced myelosuppression and can therefore provide a potential means for intensifying treatment in the future. Furthermore, they are able to stimulate the hematopoietic stem cells after BMT to a more rapid development of sufficient hematopoiesis and thereby reducing the isolation period and prophylactic and supportive treatment of ALL patients.

Late effects of ALL treatment

The improved survival of children with ALL has focussed attention on the late effects of anti-leukemic therapy (339,378-380). Most important are the effects of therapy,

especially radiotherapy, on the CNS, resulting in cortical atrophy, mineralizing microangiopathy, impaired intellectual and psychomotor function, and neuro-endocrine abnormalities resulting in growth retardation and, fortunately, rarely in reproductive problems (339,378-382). In addition to these adverse sequelae affecting the CNS, other late effects may occur, such as organ failure (e.g. cardiomyopathy, hemorrhagic cystitis, or hepatotoxicity), occurrence of second malignancies (383-385), sterility, and impaired psychosocial status (e.g. behavorial problems) (339,378-380,386).

To reduce these late effects of ALL treatment optimal supportive care during and after therapy must be provided not only for the child but also for his or her environment. Application of methods for minimal residual disease (MRD) detection in ALL patients during follow-up can be used to monitor the effectiveness of the applied chemotherapy and subsequently for adaptation of treatment protocols in patients with or without MRD.

1.6 RELAPSE OF ALL

Relapse is defined as a reappearance of morphologically distinct leukemic blasts (>25% in the BM) following a complete remission, or leukemic blasts in PB or elsewhere in the body (129). CNS leukemia (relapse) is defined as the presence of > 5 cells/mm^3 and cytomorphologically proven leukemic blasts in the liquor cerebrospinalis (387,388). The most common site of leukemia relapse is the BM. BM relapse is often accompanied by the same hematologic features and clinical signs and symptoms present at the time of diagnosis. These include anemia, bruising, and bone pain (14,127).

Extramedullary spread is not an unusual feature in ALL (14,389). Although not always detectable without invasive diagnostic procedures, extramedullary relapse is significant, because it may cause morbidity at a localized site and because such relapses frequently precede a BM relapse, by "seeding" from this extramedullary site (390). The CNS and testes are the most common sites of extramedullary relapse in childhood ALL. Currently, the incidence of CNS relapse is reduced to 5-10%, due to CNS prophylaxis. Leukemia in the CNS and other sanctuaries are believed to develop as a result of leukemic metastases (391). Recently, clonal evolution at the Ig gene level of the leukemic clone in CNS was found when compared with the BM clone (392), but in the majority of CNS diseases the leukemic clone has an identical (immuno)phenotype and karyotype. The clinical signs and symptoms of CNS leukemia are among others headache, vomiting, lethargy, and papilledema caused by increased intracranial pressure (14).

The testes are a common site of extramedullary relapse in boys (393). At present, clinically overt testicular relapse manifested by painless enlargement of one or both testes, occurs in less than 5% of boys. A number of factors are associated with an increased likelihood of developing testicular relapse, including a high initial WBC, T-ALL, prominent lymphadenopathy, splenomegaly, and thrombocytopenia. The time to development of overt testicular relapse ranges from 2 months to several years (14,393).

On rare occasions ALL may recur in other isolated sites. These sites are lymph nodes, pleural space (especially T-ALL), the ovary, the anterior chamber of the eye, the kidney, the skin, spleen, muscles, bones (femur or rib) and the gastrointestinal tract

(394-401). Extramedullary relapses are generally followed by BM relapse.

Relatively little is known about the nature of relapse, although in most cases regrowth of leukemic cells usually represents the emergence of resistance to cytostatic drugs applied to the patient (see chapter 1.5). That this is not always the case is shown by a prompt leukemic response when identical drugs are used again at relapse. Other factors that cause relapse are not associated with characteristics of the leukemic cell but with factors causing individual variability among patients that result in decreased exposure of the malignant cells to the administered drugs (e.g. poor compliance in taking medication, intestinal malabsorption, or variations in the pharmacokinetics of the antileukemic drugs) (402). An extramedullary relapse in a pharmacologic sanctuary (e.g. the eye) is an example in which the leukemic cells may still be drug sensitive.

In the majority of relapse cases a relation with the initial leukemia can be proven by identical morphology, immunophenotype, or genotype. However in a large percentage of cases, changes are found (see Chapter 4). When major shifts occur and no relation is found between the initial and relapse leukemia a second malignancy, either or not induced by the applied treatment (especially in case of Topoisomerase II inhibitors), is diagnosed (383-385,403). It has been suggested that the occurrence of secondary leukemia and the 11q23 translocation are related (404).

1.7 INTRODUCTION TO THE EXPERIMENTAL WORK

ALL patients

According to age, ALL can be divided into three groups: First, adult ALL (age >15 years); second, childhood ALL (age 1-15 years); and third, infant ALL (age <1 year). Approximately 7% of adult leukemias represent ALL with an incidence of 1-2 per 100,000 adults (4,5). Adult ALL form a distinct group with several adverse prognostic factors (e.g. age, t(9;22), and T-ALL phenotype) (405). Results of treatment have not been improved considerably during the last decades. Although remission rates are high (68-91%), overall survival and disease free survival did not change significantly (25-41%), regardless the intensity of the applied chemotherapy (405). Still for some subgroups (T-ALL and B-ALL) better outcome is achieved (405). These findings contrast sharply with the experiences in childhood ALL as described in the previous sections.

Although infants with ALL have their own characteristics (e.g. null ALL phenotype and t(4;11)) and a poor prognosis (339,406-409). This thesis does not distinguish between infant and childhood ALL. This was due to the fact that the number of infant ALL was too small in the studied series of childhood ALL cases.

The majority of childhood ALL patients described in this study were treated at the Sophia Children's Hospital, Rotterdam. Their diagnosis and relapse cell samples were harvested by pediatricians of the Division of Hematology/Oncology, Department of Pediatrics, Sophia's Children Hopital, Rotterdam and send to the Department of Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam. The other childhood ALL patients were treated at different hospitals in the Netherlands and their diagnosis and relapse cell samples were collected by the DCLSG, The Hague. In case of a relapse, both diagnosis and relapse cell samples were send to the Department of

Immunology, University Hospital Dijkzigt/Erasmus University, Rotterdam for immunogenetic analysis.

Aim of the study

The objective of this study was to get more insight into the factors and mechanisms which influence the development of a relapse in childhood ALL. For this purpose, extensive analyses have been performed on the leukemic cells at diagnosis and relapse in both a nationwide retrospective study (in collaboration with the DCLSG, The Hague) and a prospective study (in collaboration with the Sophia Children's Hospital, Rotterdam). The retrospective study was expected to provide information about differences between the cells at diagnosis and at relapse, whereas the prospective study could provide information about differences between ALL which relapse and those which remain in remission.

The analyses of the cell samples aimed at determination of phenotypic characteristics (e.g. morphological and immunological phenotype) as well as genotypic characteristics (e.g. cytogenetic aberrations and configuration of Ig and TcR genes). If necessary, the results were correlated with other features of these patients, especially clinical characteristics.

We studied the antigen specific receptor genes (Ig and TcR) and gene rearrangements of B- and T-lymphocytes, (especially the IgH and Igκ gene rearrangements) by Southern blot analysis. The latter is important for clonality studies in patients with lymphoproliferative disorders (e.g. ALL) (Chapter 2).

In addition, the occurrence of Ig and cross-lineage TcR gene rearrangements in precursor B-ALL at diagnosis were analyzed, with accent on the prognostic implications of these gene rearrangements as well as the significance for the junctional region minimal residual disease (MRD)-PCR technique (Chapter 3).

Furthermore, we investigated the phenotypic (morphological and immunological) and immunogenotypic changes in ALL at relapse. The possibilities of development of ALL relapse and the consequence of the changes in phenotype or genotype at relapse for the detection of MRD by immunological marker analysis and PCR, respectively are discussed (Chapter 4).

The molecular biology (cytogenetics and immunogenotype) of ALL and the applications for detection of MRD with the PCR technique are summarized. Furthermore, we analyzed a new PCR target for the detection of MRD (Chapter 5).

Finally, the significance of the presented experimental data for the origin of precursor B-ALL are discussed. In addition, the relevance of the usage of leukemias as experimental model for B- and T-cell differentiation is addressed.

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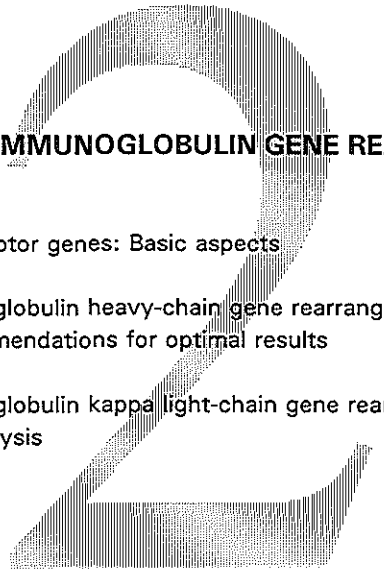

DETECTION OF IMMUNOGLOBULIN GENE REARRANGEMENTS

CHAPTER 2.1

ANTIGEN SPECIFIC RECEPTOR GENES: BASIC ASPECTS

Introduction

The daily production of lymphopoiesis results in 10^6 to 10^7 mature B- and T-lymphocytes (1,2). These B- and T-lymphocytes form the antigen specific part of the immune system (2-4). The ability of the immune system to recognize specifically a large variety of antigens is based on the presence of an enormous diversity of antigen receptors, which are expressed on the cell membrane of lymphocytes (2-4). Surface membrane bound immunoglobulin (SmIg) molecules represent the antigen specific receptors of B-lymphocytes, while the T-cell receptor (TcR) is the antigen specific receptor of T-lymphocytes (5-7). The huge diversity of these receptors is based on rearrangement processes in the Ig and TcR genes, which occur early during lymphoid differentiation (5-8).

Antigen specific receptors

Ig molecules are composed of two Ig heavy (IgH) chains and two Ig light (IgL) chains, which are held together by disulfide bonds (Figure 1). The IgL chains of each B-lymphocyte are of the same isotype: either Igκ or Igλ (7,8).

The TcR is composed of two chains, which are generally held together by a disulfide bond. Four TcR chains are known: TcR-α, TcR-β, TcR-γ, and TcR-δ. The majority of T-lymphocytes expresses a classical TcR composed of a TcR-α and TcR-β chain, while a minority (2-15%) expresses the alternative TcR composed of a TcR-γ and TcR-δ chain (Figure 1) (5,6,8).

Both surface membrane bound antigen specific receptors are closely associated with specific transmembrane molecules, which are probably involved after antigen recognition in signal transduction of the Ig or TcR molecule to the cytoplasm (9-12). These transmembrane molecules are in case of the Ig molecule two protein chains (CD79 complex) and in case of the TcR molecule a CD3 complex which contains five

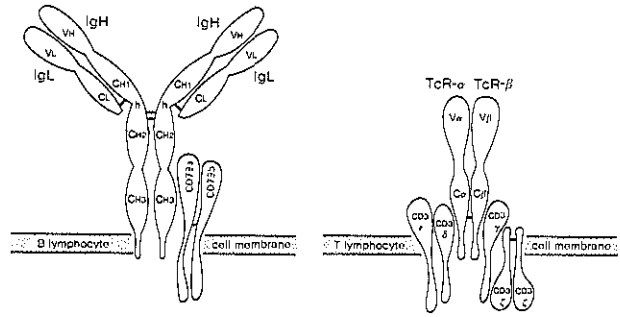

Figure 1. Schematic diagram of an Ig molecule, closely associated with CD79 chains, forming the SmIg-CD79 complex, on the cell membrane of a B-lymphocyte (left) and the TcR-CD3 complex on the membrane of a T-lymphocyte (right).

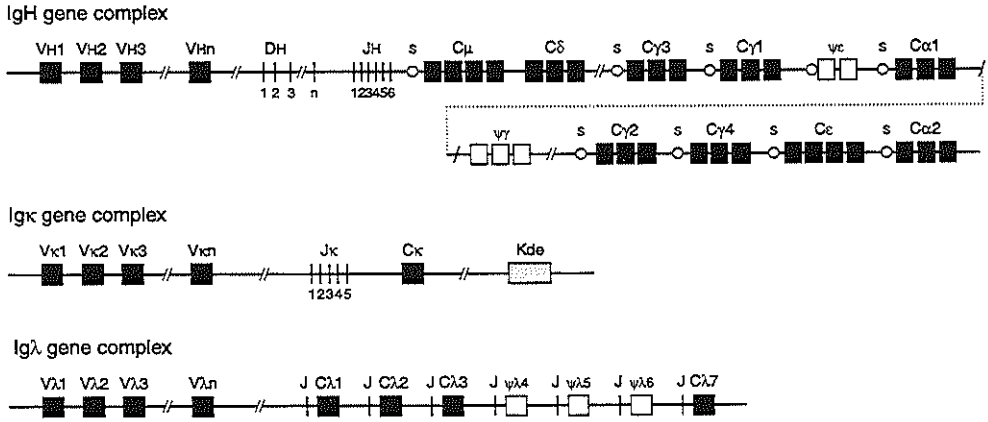

Figure 2. Schematic diagram of human Ig genes. The IgH gene complex consists of many (>100) V gene segments, at least 30 D gene segments, six functional J gene segments, and C gene segments for the constant domains of the various IgH classes and subclasses. Most C gene segments are preceded by a switch gene (s), which plays a role in IgH (sub)class switch. The Igκ gene complex consists of >50 V gene segments, five J gene segments, and one C gene segment. The Kde (κ deleting element) plays a role in the deletion of the Cκ gene in B-cells, which rearrange their Igλ genes. The Igλ gene complex consists of >40 V gene segments and four functional C genes, all of which are preceded by a J gene segment. Pseudo genes (ψ) are indicated with open symbols.

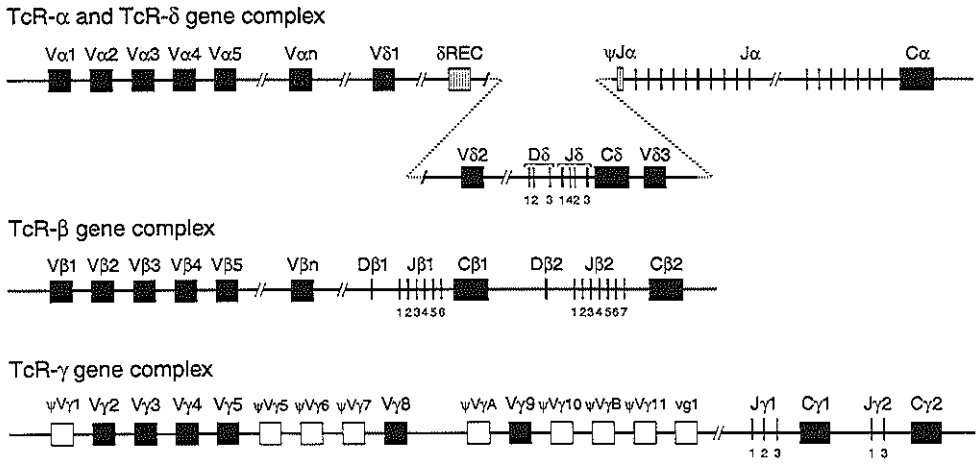

Figure 3. Schematic diagram of human TcR genes. The TcR-α gene complex consists of >50 V gene segments, a remarkably long stretch of ~55 functional J gene segments, and one C gene segment. The TcR-β gene complex consists of >70 V gene segments and two C gene segments, both of which are preceded by one D and six or seven J gene segments. The TcR-γ gene complex consists of a restricted number of V gene segments (six functional V gene segments and nine pseudo genes) and two C gene segments, each preceded by two or three J gene segments. The major part of the TcR-δ gene complex is located between the Vα and Jα gene segments and consists of six V, three D, and four J gene segments and one C gene segment. The δREC and ψJα gene segments play a role in TcR-δ gene deletions, which precede TcR-α gene rearrangements. Pseudo genes (ψ) are indicated with open symbols. Recently, a new functional Jδ gene segment (Jδ4) is found and located between the Jδ1 and Jδ2 gene segments (73,74).

protein chains (Figure 1) (9-13).

Each of the Ig chains and TcR chains consists of a variable and one or more constant domains (8). The variable domains are encoded by combinations of gene segments: a variable (V) gene segment, a diversity (D) gene segment in case of IgH, TcR-β, and TcR-δ chains, and a joining (J) gene segment (Figure 2 and 3) (8,14-36). The constant domains of the different Ig and TcR chains are encoded by constant (C) gene segments (Figure 2 and 3) (8). The location of the three Ig gene loci (IgH, Igκ, and Igλ) is on chromosomes 14q32, 2p12, and 22q11, respectively (37-40). The location of the four TcR gene loci (TcR-α, TcR-β, TcR-γ, and TcR-δ) is on chromosomes 14q11, 7q35, 7p15, and 14q11, respectively (41-45).

Antigen specific receptor diversity and gene rearrangement

As indicated before, the large diversity in specificity of the two types of antigen specific receptors of lymphocytes is based on gene rearrangements (8,46-50). Early during B- and T-cell differentiation these gene rearrangements result in specific combinations of V, (D), and J gene segments, thereby deleting all intervening sequences (Figure 4) (8,46-50). These gene rearrangements are performed by a recombinase enzyme complex, including the protein products of recombining activating genes (RAG1 and RAG2) and are precisely regulated via recombination signal sequences (RSS) (49-52). These RSS generally consist of heptamer-nonamer sequences, separated by a spacer of 12 or 23 basepairs, which flank the V, D, and J gene segments (8,50). After this recombination process the gene complex can be transcribed into precursor messenger(m)RNA, which

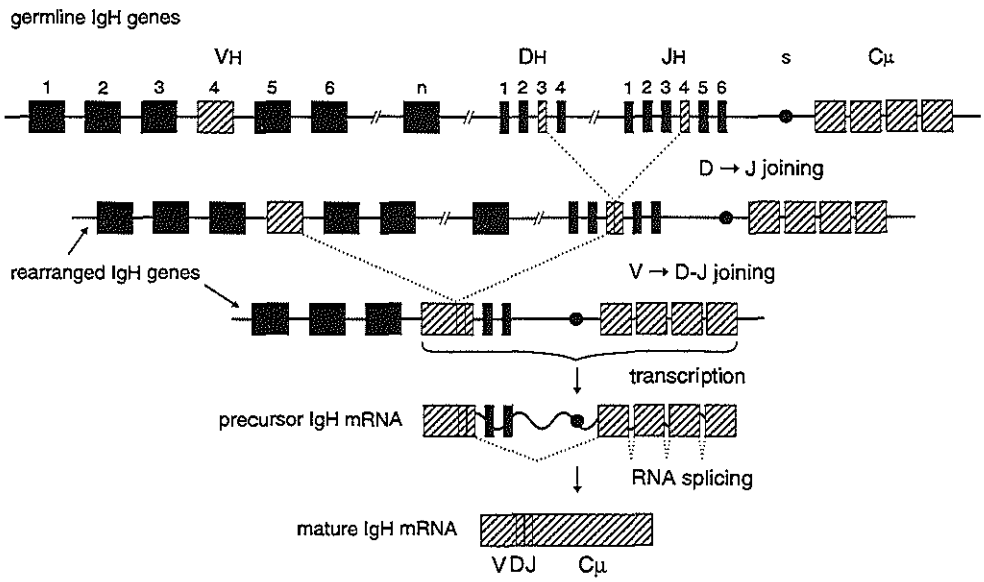

Figure 4. Schematic diagram of human IgH gene rearrangement. In this example first DH3 is joined to JH4, followed by VH4 to DH3-JH4 joining, thereby deleting all intervening sequences. The rearranged gene complex can be transcribed into precursor mRNA, which will be transformed into mature mRNA by splicing out all non-coding intervening sequences.

will be transformed into mature mRNA by splicing out all non-coding intervening sequences (Figure 4). Protein chains can now be produced via translation of the mRNA.

The various combinations of V, (D,) and J gene segments of each Ig or TcR molecule and the combination of two different chains (IgH and IgL in case of B-cells and either TcR-α and TcR-β, or TcR-γ and TcR-δ in case of T-cells) results in a large combinatorial diversity (Table 1) (8,46-50). This diversity is increased by random deletion and insertion of nucleotides at the junctions of the rearranging gene segments, which together form the junctional diversity. The insertion of N-region nucleotides is mediated by terminal deoxynucleotidyl transferase (TdT) (53-55). TdT is able to add nucleotides, DNA template independable, to 3´ ends of DNA breakpoints. Insertion of so-called P-region nucleotides increases the junctional diversity (56). P-region nucleotides represent junctional region nucleotides that are derived from an untrimmed rearranging gene segment (56). Based on these data, the theoretical repertoire (combinatorial and junctional diversity) of Ig and TcR molecules can be estimated (Table 1) (8). The actual repertoire is probably lower due to preferential usage of particular V, (D), or J gene segments.

TABLE 1. Estimation of repertoire of human Ig and TcR molecules.

	Ig molecules			TcR-$\alpha\beta$ molecules		TcR-$\gamma\delta$ molecules	
	IgH	Igκ	Igλ	TcR-α	TcR-β	TcR-γ	TcR-δ
Number of functional gene segments:							
- V gene segments	>100	>50	>40	>50	>70	8	6
- D gene segments	30*	–	–	–	2*	–	3*
- J gene segments	6	5	4	55	13	5	3
Combinatorial diversity	$>5 \times 10^6$			$>5 \times 10^6$		>5000	
Junctional diversity	++	±	±	+	++	++	++++
ESTIMATION OF TOTAL REPERTOIRE	$>10^{12}$			$>10^{12}$		$>10^{15}$	

* In TcR-δ gene rearrangements multiple D gene segments might be used; this implies that the number of junctions can vary from one to four. In IgH and TcR-β gene rearrangements generally only one D gene segment is used.

Lymphoid differentiation and gene rearrangement

Ig and TcR genes are rearranged early during lymphoid differentiation. These Ig and TcR gene rearrangements seem to occur in a hierarchical order (57-59). During B-cell differentiation IgH gene rearrangements precede Igκ gene rearrangements, which in turn precede Igλ gene rearrangements. In general, B-cells start to rearrange their Igλ genes after both Igκ alleles are non-functionally rearranged and subsequently deleted via kappa deleting element rearrangements (60-63). Eventually, this will result in Igκ or Igλ bearing B-lymphocytes (Figure 5).

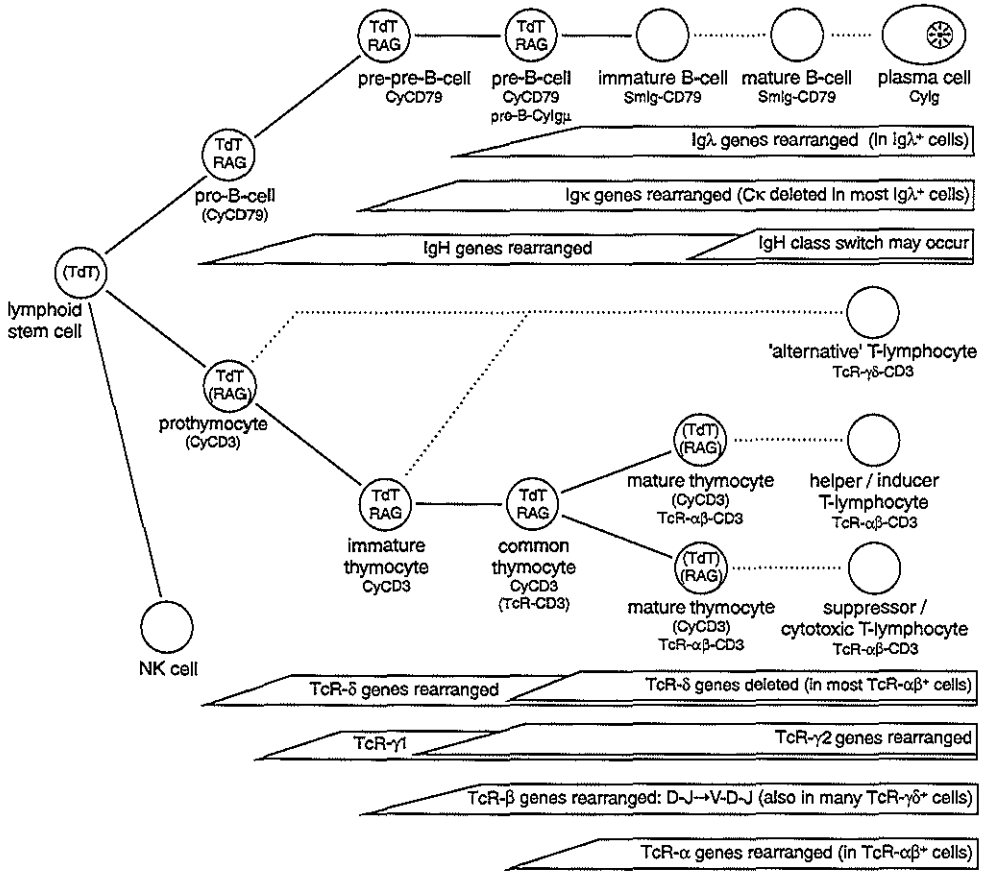

Figure 5. Hypothetical scheme of lymphoid differentiation, summarizing the available data about the ordered rearrangement of Ig and TcR genes, as well as the expression of TdT, Ig molecules and TcR molecules. The horizontal bars represent the rearrangement of the Ig genes (IgH, Igκ, and Igλ) and TcR genes (TcR-α, TcR-β, TcR-γ, and TcR-δ). The different forms of Ig and TcR expression (pre-B Igμ-CD79, SmIg-CD79, CyIg, and TcR-CD3 (TcR-αβ or TcR-γδ) is indicated, as well as the CyCD3, CyCD79, and TdT expression per differentiation stage.

During T-cell differentiation probably the TcR-δ and TcR-γ genes will rearrange first, resulting in TcR-γδ bearing T-lymphocytes when both TcR genes are functionally rearranged. It is not yet clear whether the TcR-γδ differentiation pathway is separated from the TcR-αβ differentiation pathway, in which TcR-β genes rearrange first, followed by TcR-α genes. Before TcR-α genes can rearrange, the TcR-δ gene locus, which is located between the Vα and Jα gene segments, will be deleted (Figure 3). Therefore, the rearrangement and deletion processes in the TcR-α/δ locus are probably important for the separation of the γδ and αβ differentiation pathways. It is still not known where in T-cell differentiation this bifurcation occurs (Figure 5) (64).

The SmIg-CD79 complex is then expressed when a functional rearrangement of both the IgH and IgL molecules has occurred (Figure 5) (65). The first signs of functional

Ig gene rearrangements can be found in the pre-B-cell stage: weak cytoplasmic expression of Igμ chain (pre-B CyIgμ) occurs as a result of a functional IgH gene rearrangement (Figure 5). In addition, expression of the so-called pre-B-cell complex (pre-B Igμ-CD79) on the cell membrane can occur. In that case the pre-B-cell complex consists of CD79 chains, Igμ chains, and a pseudo IgL chain, encoded by non-rearranging Igλ like genes (Figure 5) (66).

Analogous to the SmIg-CD79 complex, a TcR-CD3 complex will be expressed when the involved TcR genes are functionally rearranged. Recently, also a so-called pre-T-cell complex is found on the surface membrane of thymocytes of the $\alpha\beta$ differentiation pathway. This pre-T-cell complex consists of CD3 molecules and a complex of a TcR-β chain and a second unidentified TcR chain (67,68).

Strikingly, early during B- and T-cell differentiation cytoplasmic expression of CD79 protein chains (CyCD79) and the CD3 protein chains (CyCD3) is found (65,69). Apparently, these signal transduction molecules are prepared early and ready to be joined with functionally rearranged antigen specific molecules. Finally, TdT is expressed in all immature lymphoid cells and it disappears when the antigen specific receptors are expressed (53-55). Activation of the RAG genes, shown by RNA expression, is also found during these stages (70-72).

Conclusion

B- and T-lymphocytes form the cells of the specific immune system and therefore play a crucial role in the host defense processes via their antigen specific receptors. The structure of the Ig and TcR genes, the rearrangement processes, and the random deletion and insertion of nucleotides in the junctional regions form the basis of the diversity of these antigen receptors of B- and T-lymphocytes.

Lymphoid malignancies consist of clonal cells which are arrested at certain stages of differentiation. Since Ig and TcR gene rearrangements are different in each lymphocyte or lymphocyte clone, they can be used as markers for clonality. The Southern blot technique allows a detailed analysis of clonally-rearranged Ig or TcR genes. For this purpose detailed information about the gene configuration, restriction maps, and probes should be available as well as information about the occurrence of polymorphisms and their frequency in the population. This will be discussed in the next chapters of this thesis.

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CHAPTER 2.2

DETECTION OF IMMUNOGLOBULIN HEAVY-CHAIN GENE REARRANGEMENTS BY SOUTHERN BLOT ANALYSIS: RECOMMENDATIONS FOR OPTIMAL RESULTS*

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SUMMARY

Southern blot analysis of immunoglobulin (Ig) and T-cell receptor (TcR) genes has proven to be important for detection of clonal rearrangements in patients with lymphoproliferative diseases. To improve the detection of clonal Ig heavy chain (IgH) gene rearrangements, we carefully determined the precise restriction map of the joining (J)H and constant (C)μ region of the IgH locus, and evaluated relevant combinations of restriction enzymes with JH and Cμ probes.

Our extensive Southern blot analyses revealed that rearrangements in the JH region are optimally detectable by use of a JH probe in combination with at least two restriction enzyme digests which are not affected by polymorphisms, and which produce small germline bands (e.g. *Bgl*II and *Bam*HI/*Hin*dIII), thereby reducing the chance of comigration of germline and/or rearranged bands. Application of a JH or a Cμ probe in combination with *Bam*HI or *Eco*RI digests should be avoided, because of the large size of the restriction fragments and the occurrence of polymorphisms. Comparison of different types of JH probes demonstrated that optimally reproducible signals, independent of the rearranged JH gene segment, are only obtained if the JH probe is complementary to the 3´ flanking sequences of the JH gene region, such as our IGHJ6 probe.

INTRODUCTION

B-cell malignancies can be regarded as malignant counterparts of B-cells in the various stages of lymphoid differentiation, because malignant B-cells have comparable phenotypic and genotypic characteristics, as normal B-lymphocytes (1-3). Since rearrangements of Ig variable, (diversity,) and J gene segments occur early during lymphoid differentiation, virtually all B-cell malignancies have rearranged Ig genes (4-8), which can be used as unique

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clonal markers for diagnostic purposes. Southern blot analysis of Ig genes has proven to be important for (a) distinguishing monoclonal from polyclonal B-cell proliferations, (b) detection of subclones within a malignancy, (c) determination of the cell lineage when analysis is performed in association with immunophenotyping, and (d) assessment of residual disease and early relapse (8-11).

Since J gene segments are assumed to be involved in all rearrangements of IgH genes, these rearrangements can be detected by use of Southern blotting with appropriate restriction enzymes and a JH region probe or a Cμ region probe (12). The choice of restriction enzymes is dependent on the precise position of the restriction sites in the IgH gene locus, and the size of the resulting restriction fragments (12), as well as on the occurrence of polymorphisms, such as polymorphic restriction sites, resulting in restriction fragment length polymorphisms (RFLP) or variable numbers of tandem repeats (VNTR) resulting in hypervariable polymorphic (HVP) regions (12-15).

During the last decade Cμ probes and several types of JH probes hybridizing to different parts of the JH gene region have been used in rearrangement studies (6,12,16-21). The position of the different JH probes varies remarkably from the 5′ side, down to the 3′ side of the JH gene region. Theoretically, JH probes which only recognize sequences at the 3′ side of the JH gene region will result in optimally reproducible signals, independent of the rearranged JH gene segment. Therefore, we developed a JH probe, which recognizes 1.0 kb of the JH gene region 3′ of the JH6 gene segment.

Based on sequence data from the literature and our extensive Southern blot analysis of cell samples with germline IgH genes, the precise position of restriction sites, and the occurrence of polymorphisms in the JH-Cμ region was determined. Furthermore, our JH probe (IGHJ6) was compared with Cμ probes, and two examples of frequently used JH probes (*Sau*3A-JH and H24-JH; Figure 1), in a detailed study of B-cell leukemias. The latter comparison demonstrated the importance of the position of the JH probe in routine Southern blot analysis of IgH genes for diagnostic purposes.

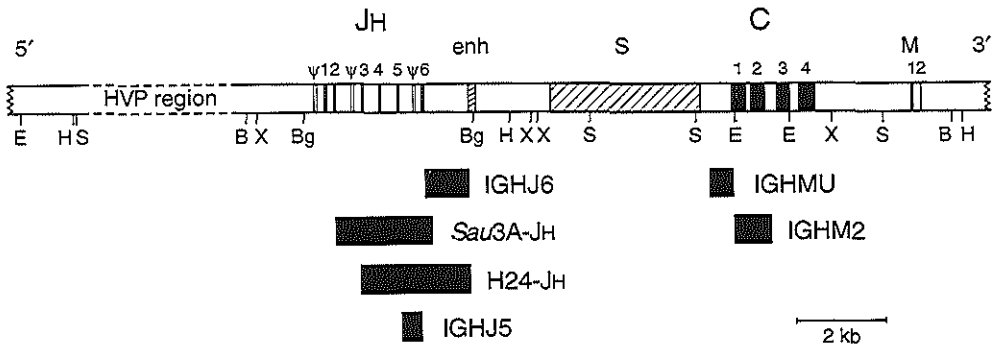

Figure 1. Position of the DNA probes in the JH and Cμ gene region of the human IgH genes. The location of relevant *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S), and *Xba*I (X) restriction sites are indicated. Also the location of the HVP region at the 5′ side of the JH region as well as the IgH enhancer (enh), the μ switch region (Sμ) and membrane μ (Mμ) gene segments are depicted (12-17,24-26). The solid bars represent the four JH probes and two Cμ probes: the IGHJ6, the *Sau*3A-JH, the H24-JH, the IGHJ5, the IGHMU, and the IGHM2 probe.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) or bone marrow (BM) samples were obtained from 20 precursor B-cell acute lymphoblastic leukemia (ALL) patients, and 45 chronic B-cell leukemias. Mononuclear cells (MNC) were isolated by Ficoll-Paque centrifugation (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). These MNC samples were frozen and stored in liquid nitrogen. For polymorphism studies, we used PB-MNC from 75 patients with germline IgH genes, as well as granulocytes from 75 healthy individuals. The granulocytes were obtained from the cell pellet after Ficoll-Paque centrifugation. Granulocytes and non-hematopoietic human cell lines were used as the source for germline control DNA.

Southern blot analysis

DNA was isolated from frozen MNC, freshly isolated granulocytes, and cell lines as previously described (12,22). Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia) as previously described (12). The restriction fragments were size-separated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) (12). The membranes were hybridized with the appropriate ^{32}P-random oligonucleotide labeled probes (12).

Restriction map and polymorphisms

DNA from granulocytes and non-hematopoietic cell lines was used for digestion with *Bgl*II, *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, *Xba*I and several combinations of these restriction enzymes (Pharmacia) to locate the precise position of the restriction sites in the JH and Cμ regions. In addition, granulocyte DNA from 75 healthy individuals, and/or DNA from PB-MNC of 75 patients with germline IgH genes was used to determine the occurrence of RFLPs and to evaluate which germline restriction fragments are affected by the VNTR-HVP region ~1.8 kb upstream of the JH gene segments.

*Sau*3A-JH probe and H24-JH probe

The *Sau*3A-JH probe is a 2.2 kb *Sau*3A fragment containing the JH2-JH6 region and only 230 bp of the 3′ flanking sequences. This probe was isolated from the 6.0 kb *Bam*HI/*Hin*dIII JH clone (pLJH), kindly provided by Dr. M.H. Siegelman (23). The H24-JH probe is a 2.5 kb *Eco*RI/*Bgl*II fragment containing the JH3-JH6 region, as well as 1.1 kb of the 3′ side of the JH gene region, and was isolated from the H24 clone, kindly provided by Dr. T. Honjo (16). The precise position of the *Sau*3A-JH and H24-JH probes is indicated in Figure 1.

Construction of IGHJ5, IGHJ6, IGHMU and IGHM2 DNA probes

The IGHJ5 probe (462 bp), IGHJ6 (1020 bp), IGHM (1365 bp) probes were obtained by cloning the purified polymerase chain reaction (PCR) amplification products of granulocyte DNA from a healthy individual, using specific oligonucleotide primer sets and pUC19 as cloning vector (22). The oligonucleotide primers with aspecific tails containing *Eco*RI or *Hin*dIII restriction sites for cloning are indicated in Table 1. All oligonucleotide primers were synthesized according to published sequences of the JH and Cμ gene region (17,24-26) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA, USA) with the solid-phase phosphotriester method and used without further purification.

PCR was essentially performed as previously described (22,27). A 1.0 μg sample of granulocyte DNA, 12.5 pmol of the upstream and downstream oligonucleotide primers and one unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used in each PCR of 100 μl. The PCR products were sized fractioned by 0.7% agarose gel electrophoresis. After electrophoretical recovery from the agarose gel using a separation chamber (Biotrap; Schleicher and Schuell), the PCR products were digested with *Eco*RI and/or *Hin*dIII and cloned, using pUC19 as cloning vector (22). All probes were partly sequenced (at least 150 bp) at both sides to confirm their position in the IgH gene region.

The IGHJ5 probe recognizes sequences between the JH5 and JH6 gene segment (Figure 1). The IGHJ6 probe recognizes ~1.0 kb of the 3′ flanking sequences of the JH locus (Figure 1). The IGHM probe can be divided into the IGHMU probe (552 bp) and IGHM2 probe (813 bp) by use of the restriction enzyme *Eco*RI

TABLE 1. Oligonucleotide primers used in construction of IgH gene DNA probes.

Probe name	Size	Cloning sites	Code	Relative position[a]	Sequence[b] 5′ 3′	Reference[c]
IGHJ5	462 bp	*Eco*RI–*Hin*dIII	JH5-p5′	+21 bp	tctgaattCACTTAGGGAGACTCAGC	17
			JH5-p3′	+471 bp	cgaaagCTTCTGTCTCCGGGCGTC	17
IGHJ6	1020 bp	*Eco*RI–*Hin*dIII	JH6-p5′	−1bp	GGTAAGAATtcCCACTCTAGGGCCTT	17
			JH6-p3′	+1002bp	acacaaGCTTCCTAAAATAGACTCTCGCGGTG	26
IGHM[d]	1365 bp	*Hin*dIII–*Hin*dIII	Sμ-p5′	−516 bp	TGGCTCCAaGCTTCACATTCAGGTA	26
			Cμ-p3′	−100 bp	cacaagCTTGGCCGCTCTGGGAAGCCA	25

a. The position of the oligonucleotide primers is indicated upstream (−) or downstream (+) relative to an exon splice site. The positions of the DNA probes are indicated in Figure 1.
b. The sequences in lower case characters represent the aspecific nucleotides, which generate restriction sites (underlined).
c. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references.
d. The IGHM probe can be divided into the IGHMU probe (552 bp) and IGHM2 (813 bp) probe by digestion with *Eco*RI.

(Table 1 and Figure 1). The IGHMU probe recognizes 509 bp of the 5′ flanking sequences of the Cμ locus and 43 bp of the Cμ exon 1. The IGHM2 probe recognizes sequences of the Cμ exon 1 (296 bp), the complete Cμ exon 2 and a part of the intron sequences (Figure 1).

Comparison of three JH probes

Each Southern blot filter with *Bgl*II or *Bam*HI/*Hin*dIII digested MNC-DNA from the 65 leukemia patients was successively hybridized with the ^{32}P-labeled *Sau*3A-JH, H24-JH, and IGHJ6 probes. Rearrangements to the JH6 gene segment were proven, by demonstrating deletion of the JH5 gene segment, via hybridization with the ^{32}P-labeled IGHJ5 probe. After each hybridization the membranes were washed according to the following protocol: one washing in buffer I (40 mmol/l $NaHPO_4$, pH 7.2; 2% SDS; 1 mmol/l EDTA; 0.5% BSA) for 5 min at 65°C; four washes in buffer II (40 mmol/l $NaHPO_4$, pH 7.2; 1% SDS; 1 mmol/l EDTA) for 3-5 min at 65°C; and four washes in buffer III (10 mmol/l $NaHPO_4$, pH 7.2; 1% SDS) for 3-5 min at 65°C. Finally, the membranes were rinsed with 100 mmol/l $NaHPO_4$ (pH 7.2) and autoradiographed. Before rehybridization, filters were stripped with 50% formamide and 6x SSC for 30 min at 65°C (12).

Southern blot dilution experiment

The detection limit of the Southern blot technique with the *Sau*3A-JH probe and the IGHJ6 probe was determined by diluting B-chronic lymphocytic leukemia (CLL) cells with two rearranged IgH genes in PB-MNC from a healthy individual, resulting in mixtures with the following leukemic cell content: 100%, 85%, 70%, 60%, 50%, 40%, 30%, 20%, 15%, 10%, 7.5%, 5%, 3%, 2%, 1%, and 0% of B-CLL cells. DNA was isolated from all mixtures, digested with *Bgl*II or *Bam*HI/*Hin*dIII and subjected to Southern blotting and successive hybridization with the ^{32}P-labeled *Sau*3A-JH probe and ^{32}P-labeled IGHJ6 probe.

RESULTS

Restriction map of the JH-Cμ region

Sequence data from the literature (17,24-26), and extensive Southern blot analysis of DNA from granulocytes of healthy individuals and cell lines with germline IgH genes, were used to determine the precise position of the restriction sites. The obtained detailed restriction map of the germline JH and Cμ region is presented in Figure 1, whereas the sizes of the

TABLE 2. DNA probes and restriction enzymes for detection of IgH gene rearrangements.

DNA probe[a]	Size of insert in bp (restriction enzymes)	Restriction enzymes used for digestion of genomic DNA	Size of germline restriction fragments in kb[b]	Allelic frequency of polymorphisms[c]
IGHJ6	1020 (*Eco*RI-*Hin*dIII)	*Bgl*II	3.8	0.3% (1/300)
		*Bam*HI/*Hin*dIII	6.0	0% (0/300)
		*Xba*I	6.2	0% (0/150)
		*Bam*HI/*Sac*I	7.8	0% (0/150)
		*Hin*dIII	9.9	VNTR
		*Sac*I	11.6	VNTR
		*Bam*HI	16.0	0% (0/150)
		*Eco*RI	16.2	VNTR
IGHMU	552 (*Eco*RI-*Hin*dIII)	*Bgl*II	**16.7**	0% (0/150)
		*Bam*HI/*Hin*dIII	**10.0**	0% (0/150)
		*Xba*I	**6.6**	0% (0/150)
		*Hin*dIII	**10.2**	0% (0/150)
		*Sac*I	**4.2**	0% (0/150)
		*Bam*HI	16.0	0% (0/150)
		*Eco*RI	16.2	VNTR
IGHM2[d]	813 (*Eco*RI-*Hin*dIII)	*Eco*RI	**1.2**	0% (0/150)

a. The precise position of the probes is presented in Figure 1.
b. The sizes of the germline restriction fragments, based on published sequence data, are presented in bold (17,24-26). The sizes of all other germline fragments were determined by extensive Southern blot analysis of granulocyte DNA from 75 healthy individuals (all restriction enzymes) as well as DNA from 75 patients with germline IgH genes (in case of *Bgl*II and *Bam*HI/*Hin*dIII).
c. The *Hin*dIII, *Sac*I, and *Eco*RI germline restriction fragments of the JH region vary in size due to the presence of the VNTR-HVP region at the 5′ side of the JH region (12-15).
d. The germline restriction fragments obtained with the IGHM2 probe are similar to those obtained with the IGHMU probe except for the *Eco*RI restriction fragment (Figure 1).

germline restriction fragments are summarized in Table 2.

Optimal choice of restriction enzymes and probes

Based on our extensive Southern blot analyses and published sequence data, we conclude that rearrangements in the JH region are optimally detectable by use of *Bgl*II, *Bam*HI/*Hin*dIII, *Xba*I, and *Bam*HI/*Sac*I digests (resulting in small germline bands of 3.8 kb, 6.0 kb, 6.2 kb, and 7.8 kb, respectively) in combination with a JH probe (Figures 1, 2 and Table 2). However, digestion with *Bam*HI and *Eco*RI (resulting in large germline bands of 16.0 kb and 16.2 kb, respectively) in combination with a Cμ probe or a JH probe frequently leads to comigration of germline bands and/or rearranged bands (Figures 1, 2 and Table 2).

Polymorphisms

The main polymorphism in the JH-Cμ region is a HVP caused by the presence of a VNTR, located ~1.8 kb upstream of the JH region (Figure 1) (12-15). In 80% of individuals this HVP causes differences between the restriction fragments of the two IgH alleles, which can vary up to 2.2 kb (13). This implies that interpretation of JH region Southern blot analysis with the restriction enzymes *Eco*RI, *Hin*dIII and *Sac*I might be difficult, because their germline restriction fragments include the VNTR region (Figure 1).

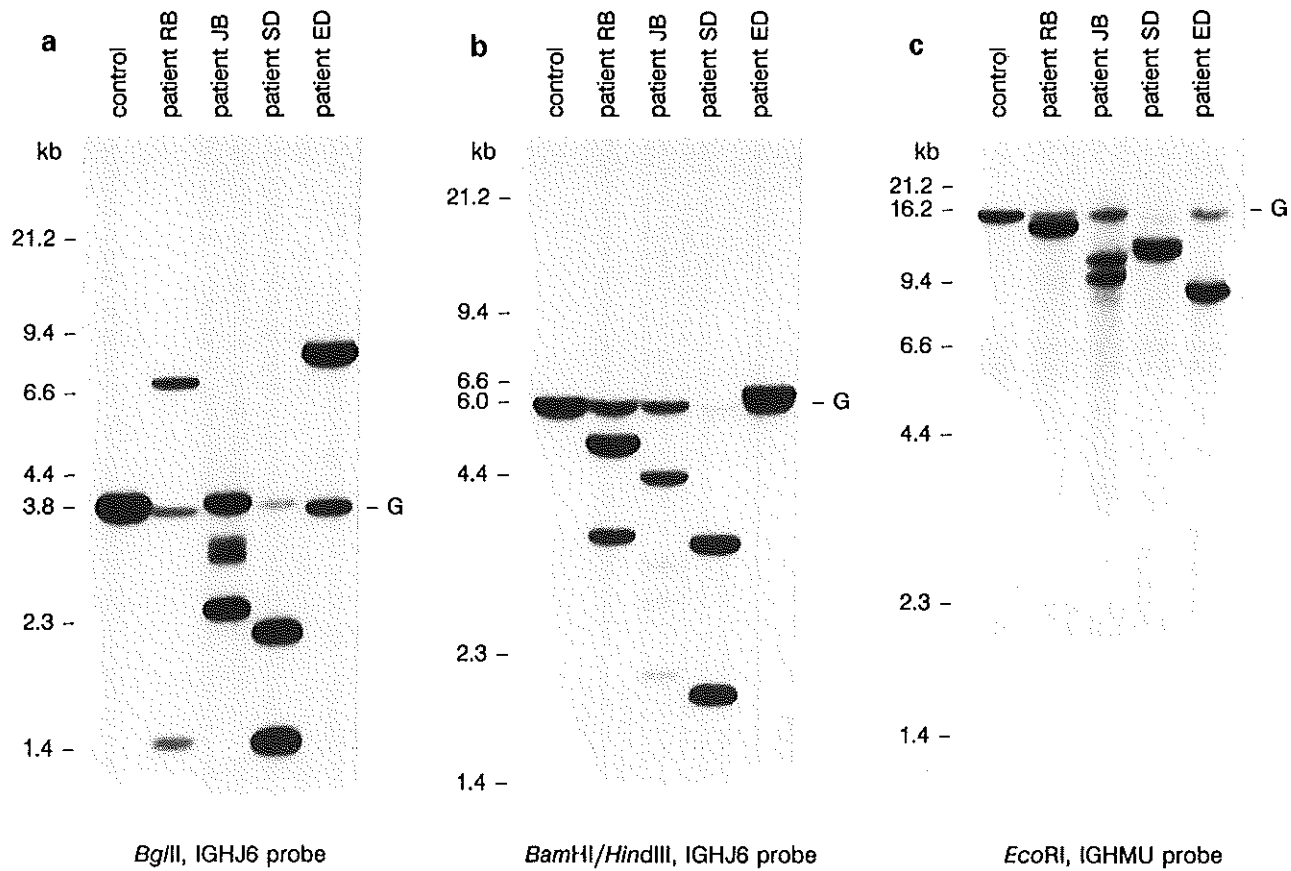

Figure 2. Southern blot analysis of IgH genes in four representative precursor B-ALL at diagnosis. Control DNA and DNA from precursor B-ALL samples were digested with (A) *Bgl*II, (B) *Bam*HI/*Hin*dIII, and (C) *Eco*RI. The *Bgl*II and *Bam*HI/*Hin*dIII filters were hybridized with the IGHJ6 probe and the *Eco*RI filter with the IGHMU probe. In the *Eco*RI filter the rearranged IgH gene bands varied from 9.1 to 16.2 kb, while in the *Bgl*II filter the rearranged bands varied from 1.4 to 8.9 kb. Comigration of IgH gene bands occurred in several lanes of the *Eco*RI filter, but optimal separation of most rearranged bands was obtained in the *Bgl*II and *Bam*HI/*Hin*dIII filters.

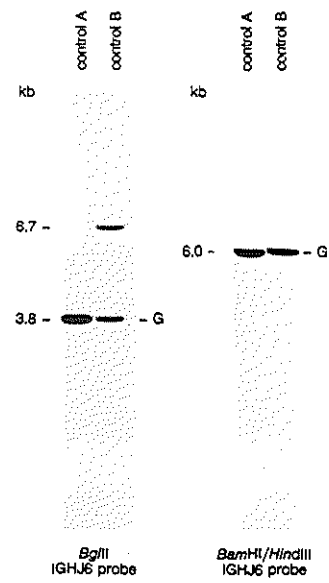

Figure 3. Rare RFLP caused by absence of the *Bgl*II site located 172 bp upstream of the JH locus. In control B two germline bands were found in *Bgl*II digests after hybridization with the IGHJ6 probe. All other digests (including *Bam*HI/*Hin*dIII) resulted in single germline bands, indicating that the IgH genes were in germline configuration.

Our additional studies on polymorphic restriction sites in the JH and Cμ region revealed only one polymorphic *Bgl*II site (1/300 IgH alleles; 0.3%), i.e. the *Bgl*II site upstream of the JH gene region (Figures 1 and 3). The other relevant restriction sites downstream of the VNTR region did not show any polymorphism (Table 2).

Comparison of JH probes

In a large series of 20 precursor B-ALL and 45 chronic B-cell leukemias, IgH gene rearrangement patterns were determined by successive hybridization of Southern blot filters with the *Sau*3A-JH, H24-JH, and IGHJ6 probes (6,16,17). The *Sau*3A-JH probe and H24-JH probe recognize JH gene segments, including ~0.2 kb and ~1.1 kb of the 3′ flanking sequences, respectively, whereas the IGHJ6 probe only recognizes ~1.0 kb of the 3′ flanking sequences (Figure 1). The IGHJ6 probe resulted in distinct rearranged IgH gene bands of comparable density in all chronic B-cell leukemias, and in a large part of the precursor B-ALL. In 32% (14/44) of rearranged IgH alleles in precursor B-ALL and in 30% (26/87) of rearranged IgH alleles in chronic B-cell leukemias the rearranged bands were weaker upon hybridization with the H24-JH probe, and faint or absent upon hybridization with the *Sau*3A-JH probe (Figure 4). These weak, or faint rearranged bands, represented rearrangements to the JH6 gene segment, as proven by hybridization with the IGHJ5 probe, which shows deletion of the JH5 gene segment if rearrangement to the JH6 gene segment has occurred (Figures 1 and 4).

Detectability of subclones

In many precursor B-ALL more than two rearranged IgH gene bands, generally differing in density, were found by use of the IGHJ6 probe, proving subclone formation (biclonality or oligoclonality) within the leukemias. In ten cases (eight biclonal and two oligoclonal), only a part of the multiple rearranged bands were detected by use of the *Sau*3A-JH probe

Figure 4. Comparison of JH probes for Southern blot analysis of IgH genes in three precursor B-ALL patients at diagnosis. The *Bgl*I filter was successively hybridized with the *Sau*3A-JH probe, the H24-JH probe, the IGHJ6 probe, and the IGHJ5 probe. In all three precursor B-ALL hybridization with the IGHJ6 probe resulted in rearranged bands of comparable density, whereas in two patients one band (3.2 kb in patient EY and 1.7 kb in patient GA) was weaker upon hybridization with the H24-JH probe or faint upon hybridization with the *Sau*3A-JH probe. Upon hybridization with the IGHJ5 probe the weak or faint bands were absent (arrows), thereby proving that they represented rearrangements to the JH6 gene segment. Densitometry of the rearranged bands in these two patients disclosed that the hybridization signals of the JH6 rearrangements were only ~5% with the *Sau*3A-JH probe and ~60% with the H24-JH probe as compared to the hybridization signal with the IGHJ6 (data not shown).

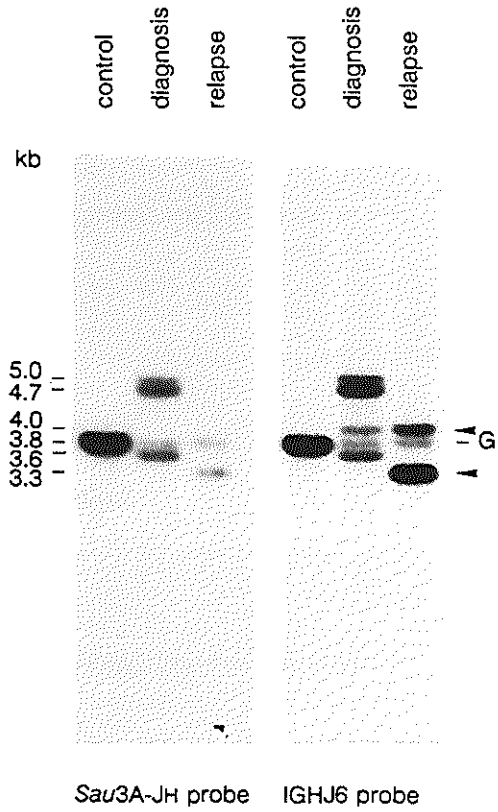

Figure 5. Comparison of JH probes for Southern blot analysis of IgH genes in one patient at diagnosis and relapse. The *Bgl*II filter was successively hybridized with the *Sau*3A-JH probe and the IGHJ6 probe. Upon hybridization with the IGHJ6 probe three distinct and one weak rearranged band were seen at diagnosis, whereas only one distinct and one weak rearranged band were detected at relapse. The weak band at diagnosis was identical to the weak band at relapse, indicating a clonal relationship between the diagnosis and relapse cell sample. Upon hybridization with the *Sau*3A-JH probe this clonal relationship was not detectable.

(Figure 5). Also by use of the H24-JH probe several of the weak rearranged bands were missed.

Several precursor B-ALL, with subclone formation, were analyzed at diagnosis and subsequent relapse. Although we have found major differences in IgH gene rearrangement patterns by use of the IGHJ6 probe, a clonal relationship could be proven between the leukemic cells at diagnosis and relapse, in the majority of patients. However this was not always possible when the *Sau*3A-JH probe was used, as is illustrated by Figure 5.

Detection limit of the Southern blot technique

In a dilution experiment, in which cells from a mature B-cell leukemia with two rearranged IgH alleles (including one involving the JH6 gene segment) were diluted in PB-MNC from a healthy individual, the Southern blot detection limit of the *Sau*3A-JH probe appeared to be 40-50% in case of a JH6 rearrangement and 10% if the rearrangement involved a further upstream JH gene segment. However, the detection limit of the IGHJ6 probe was 5%, independent of the rearranged JH gene segment (Figure 6).

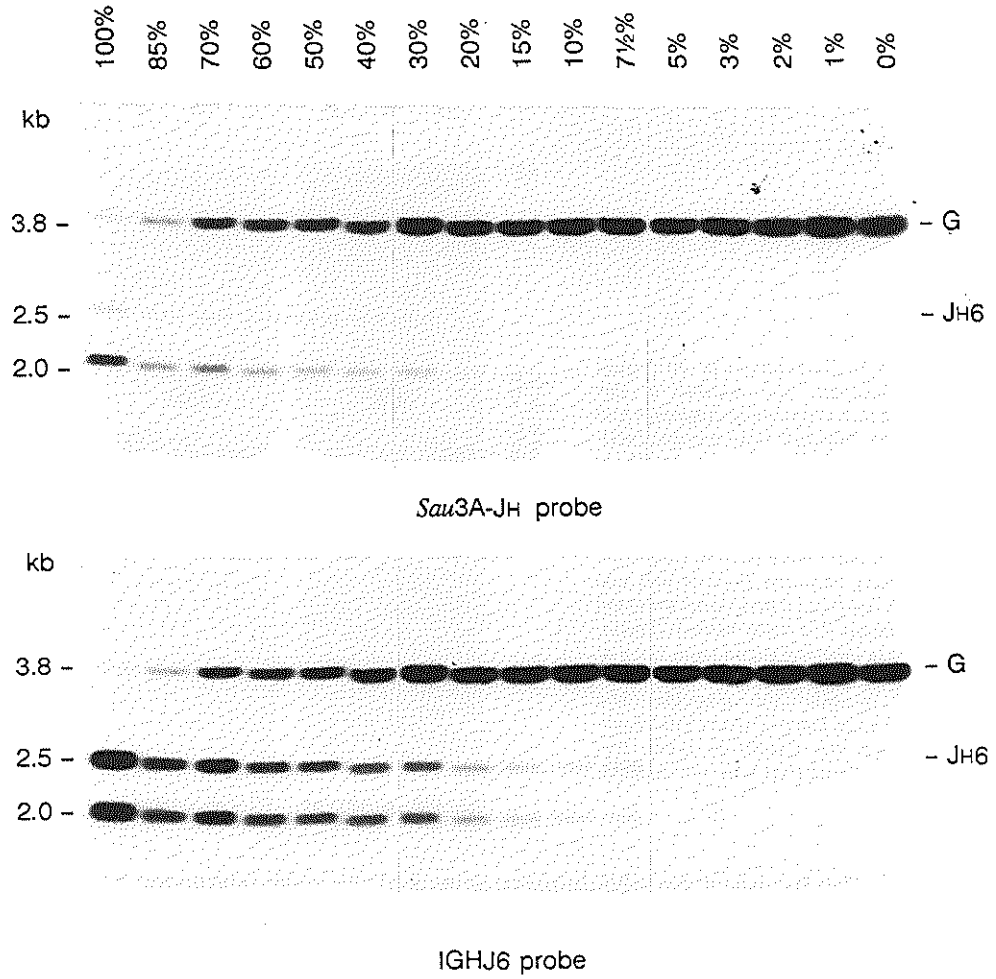

Figure 6. Dilution experiment in which B-CLL cells with two rearranged IgH alleles were diluted in PB-MNC from a healthy individual. The *Bgl*II filter was successively hybridized with the *Sau*3A-JH probe (upper pannel) and IGHJ6 probe (lower pannel). In this dilution experiment the two IgH gene rearrangements of the B-CLL were still detectable in the 5% dilution mixture upon hybridization with the IGHJ6 probe. Upon hybridization with the *Sau*3A-JH probe one rearranged IgH gene band was visible down to the 10% dilution mixture, but the other rearranged band (representing a JH6 gene rearrangement) was only visible down to the 40-50% dilution mixture.

DISCUSSION

To improve further the detection of clonal IgH gene rearrangements by routinely performed Southern blot analysis for diagnostic purposes, we carefully determined the precise restriction map of the JH and Cμ region of the IgH locus (Figure 1) and evaluated relevant combinations of restriction enzymes with JH and Cμ probes.

According to our longstanding experience with Southern blot analysis of rearrange-

ments in Ig and TcR genes, restriction enzymes which result in small germline restriction fragments give better results than enzymes which result in large germline restriction fragments (12). This is mainly due to the fact that limited separation or comigration of germline restriction fragments and rearranged restriction fragments, is not frequently seen when they are small (<10 kb). However the chance of insufficient separation and comigration increases progressively with the size of the restriction fragments, especially when they are >15 kb (12). Our data indicate that rearrangements in the JH region are optimally detectable by use of *Bgl*II, *Bam*HI/*Hin*dIII, *Xba*I, or *Bam*HI/*Sac*I digests in combination with a JH probe, whereas usage of *Bam*HI or *Eco*RI digests in combination with a Cμ or a JH probe should be avoided.

Polymorphisms of the restriction sites of the four optimal restriction enzyme digests are rare, if they occur at all (Table 2). Furthermore, the obtained restriction fragments do not contain the VNTR region, which is located ~1.8 kb upstream of the JH region (Figure 1). Nevertheless, we feel that appropriate Southern blot analysis should always include two different restriction enzyme digests per probe and that a third restriction enzyme digest should be used in case of doubt, e.g. suspicion of a polymorphism. The latter problem can be prevented by using granulocyte or fibroblast DNA from the patient as a control (12).

Although Cμ probes are not optimal for detection of IgH gene rearrangements, they might be useful for studying IgH class switching in mature B-cell malignancies by demonstrating deletion of the Cμ region or by hybridization of the Cμ probe to the same restriction fragment as the JH probe. For the latter purpose the IGHMU probe presented here can be used in combination with both *Bam*HI and *Eco*RI digests, whereas the traditional Cμ probes (e.g. IGHM2) are only informative in *Bam*HI digests (Figure 1).

In a large series of B-cell leukemias IgH gene rearrangement patterns were determined by successive hybridization of Southern blot filters with the *Sau*3A-JH, H24-JH, and IGHJ6 probes (6,16,17). The IGHJ6 probe resulted in distinct rearranged IgH gene bands of comparable density in all chronic B-cell leukemias and in a large part of the precursor B-ALL. In 32% (14/44) of rearranged IgH alleles in precursor B-ALL, and in 30% (26/87) of rearranged IgH alleles in chronic B-cell leukemias, the rearranged bands were weaker upon hybridization with the H24-JH probe, and faint or absent upon hybridization with the *Sau*3A-JH probe. We assumed that these weak or faint rearranged bands represented rearrangements to the JH6 gene segment, because a large part of the H24-JH probe and the main part of the *Sau*3A-JH probe recognize JH gene sequences, which are deleted upon rearrangement to the JH6 gene segment. This implies that the hybridization signal is restricted to the remaining 3′ part of the probe, which is 1.1 kb in case of the H24-JH probe, and only 230 bp in case of the *Sau*3A-JH probe. In all patients our assumption was proven to be correct by use of the IGHJ5 probe, which shows deletion of the JH5 gene segment if rearrangement to the JH6 gene segment has occurred. The frequent usage of the JH6 gene segment is in agreement with data published in the literature (28,29).

Densitometry of the rearranged bands in Figure 4 revealed that the hybridization signals of the JH6 rearrangements were only ~5% with the *Sau*3A-JH probe and ~60% with the H24-JH probe as compared to the hybridization signal with the IGHJ6 (data not shown). This relative decrease in density of JH6 rearranged bands is in line with the restriction of the hybridization to the 3′ part of the *Sau*3A-JH and H24-JH probes (Figure 1).

The frequency of biclonality or oligoclonality as determined by Southern blot analysis of IgH genes in precursor B-ALL is reported to be 15-30% (11,21,30-32), whereas we detected subclone formation in 38% of 60 non-random (consecutive) precursor B-ALL (33). It is obvious from the data presented here, that this discrepancy is probably due to the use of different probes and different restriction enzymes, resulting in larger (comigrating) restriction fragments. In several precursor B-ALL with subclone formation analyzed at diagnosis, and subsequent relapse, we found major differences in IgH gene rearrangement patterns by use of the IGHJ6 probe. However, in the majority of patients a clonal relationship could be proven between the leukemic cells at diagnosis and relapse (34), while this was not always possible when the *Sau*3A-JH probe was used.

These data indicate that due to its position, the *Sau*3A-JH probe is not suitable for optimal detection of each IgH gene rearrangement and that the H24-JH probe will result in bands of different density, depending on the rearranged JH gene segment. The latter might result in low density bands of JH6 rearrangements being erroneously interpreted as subclone formation (Figure 4). Therefore, application of these probes will lead to: (a) underestimation of the frequency of IgH gene rearrangements in B-cell malignancies, (b) underestimation and misinterpretation of subclone formation in precursor B-ALL, and (c) misinterpretation of clonal relationship and clonal evolution between diagnosis and relapse in precursor B-ALL. The above mentioned false-negative results of Southern blot analyses with the *Sau*3A-JH probe are probably caused by the low sensitivity of this probe. This was proven by use of a dilution experiment in which B-CLL cells with two rearranged IgH alleles (including one involving the JH6 gene segment) were diluted in PB-MNC from a healthy individual. The Southern blot detection limit of the *Sau*3A-JH probe appeared to be 40-50% in case of a JH6 rearrangement, and 10% if the rearrangement involved a further upstream JH gene segment (Figure 6). However, the detection limit of the IGHJ6 probe was 5%, independent of the rearranged JH gene segment (Figure 6). These data indicate that the detectability of clonal IgH gene rearrangements in lymphoproliferative diseases by use of the *Sau*3A-JH probe is highly dependent on the involved JH gene segment, as well as the relative frequency of the tumor cells.

We conclude that IgH gene rearrangements are optimally detectable in routinely performed Southern blotting by use of JH probes, which are complementary to the 3′ flanking sequences of the JH gene region (such as our IGHJ6 probe), in combination with at least two appropriate restriction enzyme digests (e.g. *Bgl*II and *Bam*HI/*Hin*dIII), which produce small germline bands and which are not affected by polymorphisms. It can be anticipated that these rules most probably also apply for optimal detection of rearrangements of other Ig and TcR genes.

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CHAPTER 2.3

DETECTION OF IMMUNOGLOBULIN KAPPA LIGHT-CHAIN GENE REARRANGEMENT PATTERNS BY SOUTHERN BLOT ANALYSIS*

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ABSTRACT

Immunoglobulin (Ig) light-chain (IgL) gene rearrangements occur in a sequential order during normal B-cell differentiation with Igκ gene rearrangements prior to Igλ gene rearrangements. Therefore, Igκ producing B-cells usually retain Igλ genes in germline configuration, whereas the Igκ genes are generally deleted on one or both alleles in most Igλ producing B-cells. The deletion processes in the Igκ locus are mediated via rearrangement of the kappa deleting element (Kde), which is located ~24 kb downstream of the constant (C)κ gene segment. Kde rearrangements can delete the Cκ region (including the Igκ enhancer) or the complete joining (J)κ-Cκ region via rearrangements to a heptamer recombination signal sequence in the Jκ-Cκ intron (intron RSS) or via rearrangement to a variable (V)κ gene segment, respectively.

To improve the Southern blot detection of clonal Igκ gene rearrangements and deletions in B-lineage malignancies, we developed a new set of optimal Jκ, Cκ, and Kde probes, and made a detailed restriction map of the Jκ, Cκ, and Kde region. Extensive Southern blot studies revealed that rearrangements in the Jκ gene region are optimally detectable by use of a Jκ probe in combination with at least two appropriate restriction enzymes i.e. *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, and/or *Sac*I. Jκ gene rearrangements are also detectable with the Cκ probe in *Bgl*II and *Bam*HI digests, if no deletion of the Cκ region has occurred. The two different types of Kde-mediated Jκ and/or Cκ gene deletions are easily detectable with the Kde probe in *Bgl*II, *Hin*dIII and/or *Eco*RI digests. This is in contrast to the inaccurate information obtained with the Jκ and Cκ probes, because these probes can detect deletions only in the form of decreased densities of Jκ and/or Cκ germline bands in the absence of rearranged bands.

Our detailed analysis of 217 B-lineage leukemias revealed that 62% (69/111) of precursor B-cell acute lymphoblastic leukemias (ALL) had rearranged and/or deleted Igκ genes. All 53 Igλ$^+$ chronic B-cell leukemias contained Igκ gene deletions; in 75% this concerned biallelic Jκ and/or Cκ gene deletions. Virtually all Igκ gene deletions appeared to

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* Leukemia, in press.

be mediated via Kde rearrangements, while only 1.5% of the Igκ gene deletions were mediated via an alternative deletion mechanism which involved the Jκ region.

INTRODUCTION

During B-cell differentiation Ig heavy-chain (IgH) gene rearrangements precede Igκ gene rearrangements, which in turn precede Igλ gene rearrangements (1-3). However, Igλ gene rearrangements in the presence of germline Igκ genes have been found in several human cell lines and leukemia patients (4-7), suggesting that rearrangement of Igκ genes on one or both alleles is not obligatory for Igλ gene rearrangements in human cells (8,9). Nevertheless, Igκ producing B-cells usually retain Igλ genes in germline configuration, whereas the Igκ genes are generally deleted on one or both alleles in most Igλ producing B-cells (10,11). It is assumed that the Igκ gene deletions are needed to exclude transcription and protein expression of (aberrant) pre-existing Vκ-Jκ gene rearrangements in order to prevent potential double IgL expression.

The deletion processes in the Igκ locus are mediated via a site-specific rearrangement of the so-called Kde, which is located ~24 kb downstream of the Cκ gene segment (12-15). Two types of Kde-mediated Igκ gene deletions can occur; i.e. rearrangement of Kde via heptamer-nonamer RSS at the 5´ side of Kde either to the intron RSS, thereby deleting the Cκ region (including the Igκ enhancer), or to heptamer-nonamer RSS at the 3´ side of a Vκ gene segment, thereby deleting the Jκ and Cκ regions (Figure 1A) (12-15). An alternative recombination mechanism resulting in deletion of only Jκ gene segments has also been described (Figure 1B) (16-19). Feddersen et al. postulated a two-step deletion of the Jκ region: first, rearrangement of a Jκ gene segment to the intron RSS, thereby only retaining the joined RSS (the so-called signal joint), and second, rearrangement of a Vκ gene

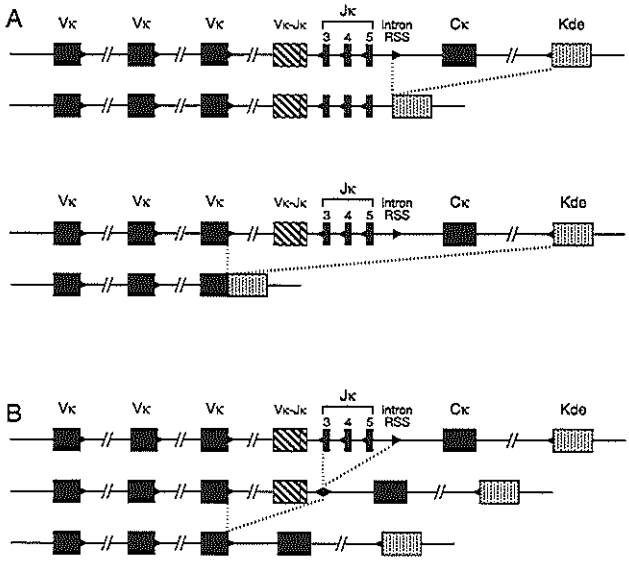

Figure 1. Schematic diagrams of Igκ gene deletions mediated by Kde rearrangements (A) and by an alternative deletion mechanism (B). **(A)** Two types of Kde-mediated Igκ gene deletions can occur: Kde rearrangement either to the intron RSS (upper diagram) or to the RSS of a Vκ gene segment (lower diagram). **(B)** The alternative deletion mechanism results in deletion of Jκ gene segments. First, rearrangement of a Jκ gene segment to the intron RSS occurs, retaining the signal joint and second, a Vκ gene segment rearranges to the still present RSS of the Jκ gene segment.

segment to the still present RSS of the rearranged Jκ gene segment (Figure 1B) (19).

Southern blot analysis represents a powerful tool for detection of clonal rearrangements of Ig genes (20). Since B-cell malignancies are clonal proliferations of immature and mature B-cells, Ig gene analysis has been widely used for diagnostic purposes (21-24). In addition to IgH gene rearrangement studies, Southern blot analysis of Igκ genes has been used to provide additional information concerning differentiation lineage or clonality of cell populations (24-34). Usage of Jκ and Cκ probes, generally allows the detection of clonal Igκ gene rearrangements. However, detection of Igκ gene deletions appears to be difficult, especially in cell samples with small clonal B-cell populations, because Igκ gene deletions are only detected by the absence of a hybridization signal when Jκ or Cκ probes are used. Since the majority of Igκ gene deletions is mediated via Kde rearrangements, usage of an optimal Kde probe will allow the detection of small clonal B-cell populations with Igκ gene deletions (20).

To improve the detection of clonal Igκ gene rearrangements and/or deletions by routinely performed Southern blot analysis, we developed new Jκ, Cκ, and Kde probes. Based on sequence data from the literature and our extensive Southern blot analysis of cell samples with germline Igκ genes, we also made a detailed restriction map and determined the occurrence of restriction fragment length polymorphisms (RFLP's) in the Jκ, Cκ and Kde regions. Finally, we studied the Igκ gene rearrangement patterns and the occurrence of the different types of Igκ gene deletions in a large series of 217 B-lineage leukemias.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) or bone marrow (BM) samples were obtained from 111 precursor B-ALL patients and 106 chronic B-cell leukemias (chronic lymphocytic leukemias (CLL), hairy cell leukemias, and prolymphocytic leukemias). The series of 106 chronic B-cell leukemias consisted of 53 $Ig\kappa^+$ leukemias and 53 $Ig\lambda^+$ leukemias. Mononuclear cells (MNC) were isolated by Ficoll-Paque centrifugation (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). The percentage of leukemic cells in all samples was at least 80%, as determined by immunophenotyping of the MNC (35). The MNC samples were frozen and stored in liquid nitrogen. Control DNA was obtained from the non-hematopoietic cell line Hela and granulocytes from healthy volunteers. The granulocytes were obtained from cell-pellets after Ficoll-Paque centrifugation.

Southern blot analysis

DNA was isolated as previously described (20,36). Fifteen μg of DNA were digested with the appropriate restriction enzymes (Pharmacia). The restriction fragments were size-separated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany). The membranes were hybridized with ^{32}P-random oligonucleotide labeled probes (20).

Restriction map and polymorphisms

DNA from granulocytes and the Hela cell line was used for digestion with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sac*I, *Xba*I and combinations of them (Pharmacia), to determine the precise position of the restriction sites in the Jκ, Cκ and Kde regions. In addition, granulocyte DNA from 75 healthy volunteers and/or DNA from PB-MNC of 88 patients with germline Igκ genes were used to determine the occurrence of RFLP's.

Construction of IGKJ, IGKC, and IGKDE DNA probes

The IGKJ (Jκ), IGKC (Cκ) and IGKDE (Kde) probes were obtained by cloning the purified polymerase chain

TABLE 1. Oligonucleotide primers used in construction of Igκ DNA probes.

Probe name	Code	Relative position[a]	Sequence[b] 5′ 3′	Reference[c]
IGKJ[d]	Jκ-p5′	+ 84	ctataagCTTCTGAAATTTGGGTCTGATGGC	36
	Jκ-p3′	+ 974	actggaatTCAGGAAAGCTGGCTACGGCAG	X67858
IGKC	Cκ-p5′	+ 10	tgttgaaTTCTTTCCTCAGGAACTGTGGCTG	37
	Cκ-p3′	+ 497	ACCACAGGTGCAAAGcTTCACTTTAT	37
IGKDE	Kde-p5′	+ 42	GGGCGACTCCTCATGAGTCTGC	13
	Kde-p3′	+ 533	TCGAGAGCTCTCAGCCCATGATGGCG	13

a. The position of the 3′ end of the oligonucleotide primers is indicated downstream (+) of the closest joining site (= start of RSS). In case of the IGKJ probe, the RSS of the Jκ5 gene segment, and in case of the IGKDE probe, the RSS of Kde. In case of the IGKC probe the position of the 3′ end of the oligonucleotide primers is indicated downstream (+) of the 5′ exon splice site of the Cκ exon.
b. The sequences in lower case characters represent the aspecific nucleotides, which generate restriction sites (underlined).
c. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references and EMBL databank accession number.
d. The IGKJ5 probe is a 540 bp *Hin*dIII/*Sac*I subclone of the IGKJ probe.

reaction (PCR) amplification products of granulocyte DNA from a healthy volunteer. The oligonucleotide primers with aspecific tails containing *Eco*RI, *Hin*dIII, *Pst*I, or *Sac*I restriction sites for cloning are given in Table 1. All oligonucleotide primers were synthesized according to published sequences of the Jκ, Cκ, and Kde regions (13,37,38, Whitehurst et al., EMBL databank accession no. X67858) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA, USA) with the solid-phase phosphotriester method and used without further purification.

PCR was essentially performed as previously described (36,39). An 1.0 μg sample of granulocyte DNA, 12.5 pmol of the upstream and downstream oligonucleotide primers and one unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used in each PCR of 100 μl. The PCR products were digested with the appropriate restriction enzymes and size-separated by 1.0% agarose gel electrophoresis. After recovery from the agarose gel using Millipore Ultrafree-MC filters (Millipore Corporation, Bedford, MA, USA) the PCR products were cloned, using pUC19 as cloning vector (36). All three probes were partly sequenced from both sides (at least 100 bp) to confirm their position in the Jκ, Cκ, and Kde regions (Figure 2). All sequence reactions were performed with the T7-sequencing kit (Pharmacia) following the manufacturer's instructions using ^{35}S-radiolabeling, and run in denaturing 8% polyacrylamide sequence gels.

The IGKJ5 probe was obtained from the IGKJ probe by use of *Hin*dIII and *Sac*I digestion and subsequent

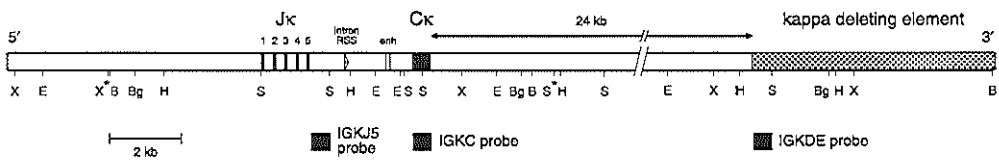

Figure 2. Restriction map of the human Igκ gene, i.e. the Jκ and Cκ region and Kde region, located ~24 kb downstream of the Cκ region. The location of relevant *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S), and *Xba*I (X) restriction sites are indicated. Also the location of the intron RSS as well as the Igκ enhancer (enh) are depicted (13,36,37,39, Whitehurst et al, EMBL databank accession no. X67858). The solid bars represent the three Igκ DNA probes: the IGKJ5, IGKC, and IGKDE probes. The asteriks indicates two polymorphic restriction sites (*Xba*I and *Sac*I).

cloning in the pUC19 vector (Table 1) (36). The IGKJ5 probe recognizes 540 bp of the 3′ flanking sequences of the Jκ region. The IGKC probe recognizes 527 bp of the Cκ region. The IGKDE probe recognizes 507 bp of the 5′ part of Kde sequences (Figure 2 and Table 1). The latter probe does not cross-hybridize with sequences on the long arm of chromosome 2 (locus 2q1.1) as described for other Kde probes (12,15).

Southern blot dilution experiment

Cells from a $Ig\lambda^+$ CLL with biallelic Cκ gene deletions were diluted in PB-MNC from a healthy volunteer, resulting in mixtures with the following leukemic cell content: 100%, 80%, 60%, 40%, 20%, 10%, 5% and 0% of B-CLL cells. DNA was isolated from all mixtures, digested with *Bgl*II and subjected to Southern blotting followed by successive hybridization with the ^{32}P-labeled IGKC and IGKDE probes.

RESULTS

Restriction map of the Jκ, Cκ, and Kde region

Sequence data from the literature (13,37,38,40, Whitehurst et al., EMBL databank accession no. X67858) and extensive Southern blot analysis of control DNA were used to determine the precise position of the relevant restriction sites. The obtained detailed restriction map of the germline Jκ, Cκ, and Kde regions is presented in Figure 2, whereas the sizes of the relevant germline restriction fragments are summarized in Table 2.

TABLE 2. Genomic probes and restriction enzymes for detection of Igκ gene rearrangements and deletions.

DNA probe[a]	Size of insert in bp (cloning sites)	Restriction enzymes used for digestion of genomic DNA	Size of germline restriction fragments in kb[b]		Allelic frequencies of RFLP's	
IGKJ5	540	*Sac*I	**1.9**		0%	(0/150)
	(*Hin*dIII/*Sac*I)	*Hin*dIII	5.1		0%	(0/298)
		*Eco*RI	9.3		0%	(0/102)
		*Xba*I	9.9	(12.5)	7%	(8/112)
		*Bgl*II	10.8		0%	(0/300)
		*Bam*HI	11.8		0%	(0/118)
IGKC	527	*Sac*I	**0.5 & 5.1**[c] (3.7)		11%	(17/148)
	(*Eco*RI/*Hin*dIII)	*Hin*dIII	5.9		0%	(0/142)
		*Eco*RI	2.7		0%	(0/114)
		*Xba*I	9.9	(12.5)	7%	(8/112)
		*Bgl*II	10.8		0%	(0/300)
		*Bam*HI	11.8		0%	(0/120)
IGKDE	507	*Sac*I	11.4		0%	(0/114)
	(*Pst*I-*Sac*I)	*Hin*dIII	2.7		0%	(0/148)
		*Eco*RI	10.7	(9.4)	0.7%	(1/144)
		*Xba*I	3.9		0%	(0/110)
		*Bgl*II	7.6		0%	(0/140)
		*Bam*HI	17.3		0%	(0/104)

a. The precise positions of the probes are presented in Figure 2.

b. The sizes of the *Sac*I germline restriction fragments, based on published sequence data, are presented in bold numbers (36,37, and Whitehurst et al., EMBL databank accession no. X67858). The numbers in parentheses represent the sizes of the polymorphic germline restriction fragments.

c. Two germline restriction fragments (0.5 kb and 5.1 kb) are obtained with the IGKC probe in *Sac*I digests (Figure 2).

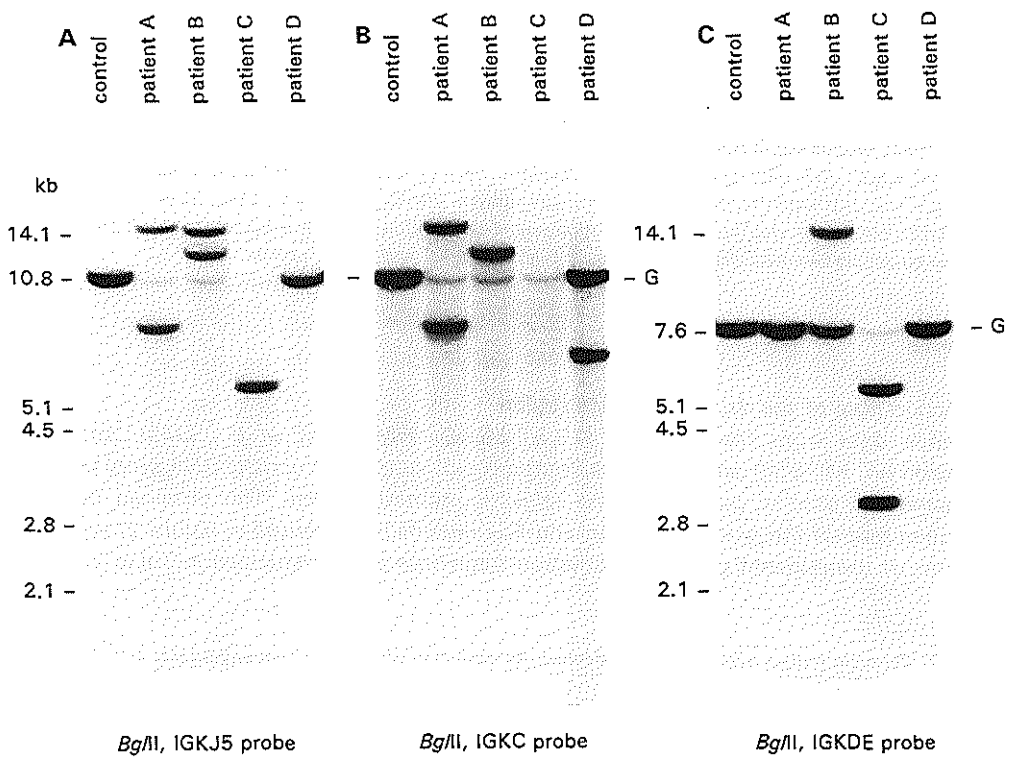

Figure 3. Southern blot analysis of four patients, who were selected for their Igκ gene rearrangement and/or deletion patterns. Control DNA and DNA from three chronic B-cell leukemias samples (lane two, three, and four) and one precursor B-ALL (lane five) were digested with *Bgl*II. The DNA filter was successively hybridized with the ^{32}P-labeled IGKJ5, IGKC, and IGKDE probes. The sizes (in kb) of the germline bands (G) and several molecular mass markers are indicated. The configuration of the Igκ genes of the four patients was: patient A, Vκ to Jκ on both alleles; patient B, Vκ to Jκ and Kde to the intron RSS: patient C, Kde to the intron RSS and Kde to Vκ; patient D, alternative Jκ gene deletion on one allele and the other allele in germline configuration.

Probes and restriction enzymes

We developed new Igκ DNA probes (IGKJ5, IGKC, and IGKDE) for optimal detection of Igκ gene rearrangements and Igκ gene deletions, according to the same criteria as previously described (Figure 2 and Table 2) (20,41,42). The IGKJ5 (Jκ) probe optimally detects Igκ gene rearrangements in *Sac*I, *Hin*dIII, *Eco*RI, *Xba*I, *Bgl*II or *Bam*HI digests, which result in germline bands of 1.9-11.8 kb (Figures 2, 3 and Tables 2, 3). The IGKC (Cκ) probe can only detect Igκ gene rearrangements in *Bam*HI or *Bgl*II digests, which contain the complete Jκ-Cκ region. The IGKDE (Kde) probe easily detects all Igκ gene deletions, which are mediated via rearrangement of Kde either to the intron RSS, or to the RSS of a Vκ gene segment. When rearrangement of the Kde to the intron RSS occurs, the IGKDE probe and IGKJ5 probe recognize an identical restriction fragment in *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII or *Xba*I digests (Table 3). Rearrangement of Kde to the intron RSS can also be proven with the IGKDE probe in *Sac*I digests, which results in an invariable 0.95 kb restriction fragment indepen-

TABLE 3. Hybridization patterns of Igκ gene rearrangements found with Igκ DNA probes.

Type of Igκ gene rearrangement or deletion	Restriction enzyme[a]	Igκ DNA probes		
		IGKJ5	IGKC	IGKDE
Vκ to Jκ	*Bam*HI, *Bgl*II, and *Xba*I	R	R_{id}	G
	*Eco*RI, and *Hin*dIII	R	G	G
	*Sac*I	R	G	G
Kde to intron RSS (Cκ gene deletion)	*Bam*HI, *Bgl*II, and *Xba*I	R	D	R_{id}
	*Eco*RI, and *Hin*dIII	R	D	R_{id}
	*Sac*I	R	D	R (0.95 kb)[b]
Kde to Vκ (Jκ-Cκ gene deletion)	*Bam*HI, *Bgl*II, and *Xba*I	D	D	R
	*Eco*RI, and *Hin*dIII	D	D	R
	*Sac*I	D	D	R
Vκ/Jκ to intron RSS[c] (Jκ gene deletion)	*Bam*HI, *Bgl*II, and *Xba*I	D	R	G
	*Eco*RI, and *Hin*dIII	D	G	G
	*Sac*I	D	G	G

Symbols used: R, rearranged band; R_{id}, rearranged band, which is identical to the rearranged band detected with the IGKJ5 probe; G, germline band; D, deletion of the involved gene segment.

a. The restriction enzymes *Bam*HI, *Bgl*II, and *Xba*I and the restriction enzymes *Eco*RI, and *Hin*dIII are combined because they give comparable hybridization patterns.

b. In case of a recombination between Kde and the intron RSS, the hybridization pattern of the IGKDE probe shows an invariable 0.95 kb rearranged band in *Sac*I digests.

c. In this Igκ gene deletion mechanism only Jκ gene segments are deleted (see Figure 1B) (19).

dent of the preexisting Vκ-Jκ gene rearrangement. Hybridization patterns of the three Igκ DNA probes in several restriction enzyme digests were determined for the various types of Igκ gene rearrangements and deletions, as summarized in Table 3.

Polymorphisms

Our studies on polymorphisms in the Jκ, Cκ, and Kde region revealed a polymorphic *Xba*I restriction site located 4.3 kb upstream of the Jκ gene region. This RFLP showed a polymorphic germline band of 12.5 kb instead of 9.9 kb, with an allelic frequency of 7% (Table 2 and Figures 2, 4). Therefore, *Xba*I is not recommended to use in Southern blot analysis of Igκ gene rearrangements and or deletions. Another RFLP, not important for Igκ gene rearrangement studies and previously reported in the literature (43,44), was found in the *Sac*I restriction site located 3.5 kb downstream of the Cκ region. This RFLP showed a polymorphic germline band of 3.7 kb instead of 5.1 kb, with an allelic frequency of 11%, comparable with the allelic frequencies (9-12%) found in the literature (Table 2 and Figures 2, 4) (43,44). In the Kde region a rare *Eco*RI RFLP (polymorphic germline band of 9.4 kb instead of 10.7 kb) was found with an allelic frequency of 0.7% (Table 2).

Igκ gene rearrangement patterns in B-lineage leukemias

DNA samples from a group of 217 B-lineage leukemias were examined for the presence of rearranged and/or deleted Igκ genes. The results are summarized in Table 4. Rearrangements and/or deletions of the Igκ genes were found in 62% of precursor B-ALL cases and

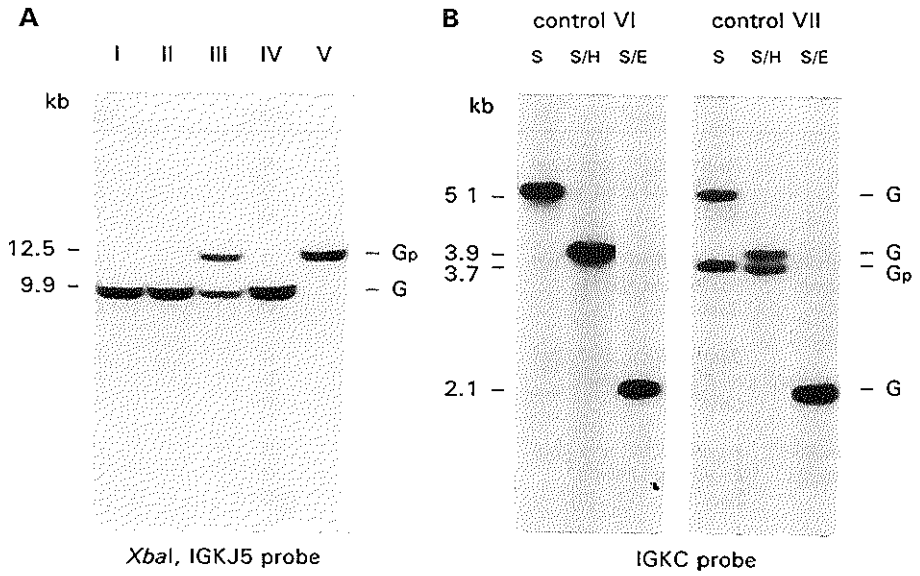

Figure 4. Southern blot analysis of two RFLP's in the Jκ-Cκ region. (**A**) DNA from five healthy volunteers (control I-V) was digested with *Xba*I. The DNA filter was hybridized with the ^{32}P-labeled IGKJ5 probe. Controls III and V contained a RFLP on one and both alleles, respectively, which was caused by a polymorphic *Xba*I restriction site, located 4.3 kb upstream of the Jκ region. (**B**) DNA from two healthy volunteers (control VI and VII) was digested with *Sac*I (S), a combination of *Sac*I and *Hin*dIII (S/H), and a combination of *Sac*I and *Eco*RI (S/E). The DNA filter was hybridized with the ^{32}P-labeled IGKC probe. Control VII contained a RFLP, caused by a polymorphic *Sac*I restriction site, located 3.5 kb downstream of the Cκ region.

in 100% of mature B-cell leukemia cases. Rearrangement of Kde occurred in 50% of precursor B-ALL cases on one or both alleles, in 32% of Igκ$^+$ chronic B-cell leukemias on one allele, and in 100% of Igλ$^+$ chronic B-cell leukemias on one allele (13 cases) or both alleles (40 cases).

The allelic distribution of the different types of Igκ gene rearrangements and deletions is summarized in Table 5. In precursor B-ALL patients, more than half of the Igκ alleles (54%) was found to be in germline configuration. Deletions of Igκ gene segments occurred in 38% (85/222) of alleles. The majority of deletions, 95% (81/85), was due to rearrangement of Kde: in 31% (25/81) to the intron RSS and in 69% (56/81) to the RSS of a Vκ gene segment. In two precursor B-ALL patients, an alternative deletion of the Jκ region was found on one allele, while the Cκ region was still present (Figures 1B and 3). In Igκ$^+$ chronic B-cell leukemias, only 24% of the Igκ alleles remained in germline configuration. More than half of the Igκ alleles (60%) contained Vκ-Jκ gene rearrangements. In 16% of the Igκ alleles, a Kde-mediated deletion was found, more or less equally divided over both types of Kde-mediated Igκ gene deletions. In Igλ$^+$ chronic B-cell leukemias, the majority of Igκ alleles (88%;93/106) contained Igκ gene deletions. In 98% (91/93) of these alleles the deletions were due to recombination of Kde: in 55% (50/91) to the intron RSS and in 45% (41/91) to the RSS of a Vκ gene segment. In only 1% (one allele) an alternative deletion was found as described above.

Our data indicate that Igκ gene deletions in precursor B-ALL are more frequently me-

TABLE 4. Igκ gene rearrangement patterns in B-lineage leukemias.

B-lineage leukemia type	Jκ-Cκ-Kde region germline	Jκ-Cκ region Rearrangement	Jκ-Cκ region Deletion	Jκ-Cκ region Deletion	Kde region rearrangement
	all Igκ probes (G/G)	IGKJ5 probe (R/G, R/R or D/R)	IGKJ5 probe (D/G or D/D)	IGKC probe (D/G or D/D)	IGKDE probe (R/G or R/R)
precursor B-ALL	38% (42/111)	30% (33/111)	32% (36/111)	50% (56/111)	50% (56/111)
$Igκ^+$ chronic B-cell leukemia	0% (0/53)	100% (53/53)	0% (0/53)	32% (17/53)	32% (17/53)
$Igλ^+$ chronic B-cell leukemia	0% (0/53)	83% (44/53)	17% (9/53)	100% (53/53)	100% (53/53)

Symbols used: R, rearranged allele; G, germline allele; D, deletion of the involved gene segment.

diated via rearrangement of Kde to a Vκ gene segment than via rearrangement of Kde to the intron RSS (69% vs 31%). On the other hand in chronic B-cell leukemias we found a nearly equal distribution in $Igκ^+$ chronic B-cell leukemias and a slight reversed preference in $Igλ^+$ chronic B-cell leukemias (45% vs 55%).

In two precursor B-ALL and one $Igλ^+$ chronic B-cell leukemia no hybridization signals were detected on one allele with all three Igκ DNA probes, indicating deletion of the complete Jκ-Cκ-Kde region e.g. by loss of chromosome 2. Unfortunately, no cytogenetic data were available in these cases.

Detectability of small malignant B-cell populations by use of Igκ DNA probes

The value of an optimal Kde probe for detection of Igκ gene deletions of small malignant B-cell populations, was evaluated in a dilution experiment (Figure 5). Hybridization of the DNA filter with the IGKC probe revealed a decreased density of the Cκ germline bands,

TABLE 5. Allelic frequencies of different types of Igκ gene rearrangements and deletions in B-lineage leukemias.

B-lineage leukemia type	Igκ gene in germline configuration	Vκ to Jκ rearrangement	Igκ gene deletion: Kde to intron RSS	Igκ gene deletion: Kde to Vκ	Igκ gene deletion: Vκ/Jκ to intron RSS	Igκ gene deletion: Jκ-Cκ-Kde deletion[a]
precursor B-ALL	54% (119/222)	8% (18/222)	11% (25/222)	25% (56/222)	1% (2/222)	1% (2/222)
$Igκ^+$ chronic B-cell leukemia	24% (25/106)	60% (64/106)	8% (8/106)	8% (9/106)	0% (0/106)	0% (0/106)
$Igλ^+$ chronic B-cell leukemia	5% (5/106)	8% (8/106)	47% (50/106)	39% (41/106)	1% (1/106)	1% (1/106)

a. No hybridization signals were detected with all three Igκ probes, indicating deletion of the complete Jκ-Cκ-Kde region, e.g. by loss of chromosome 2.

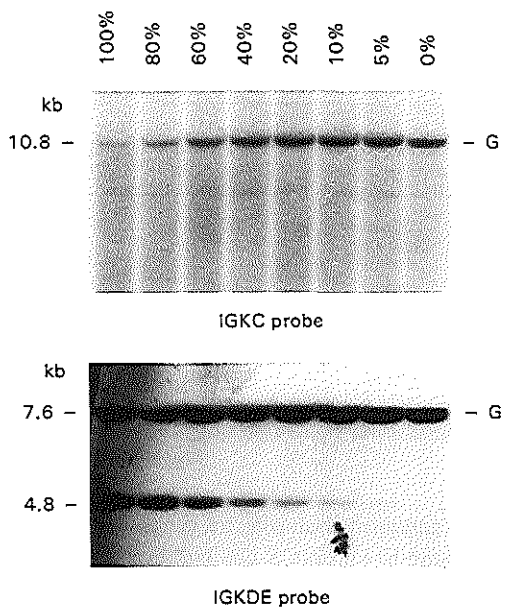

Figure 5. Dilution experiment in which B-CLL cells with two Kde-mediated Igκ gene deletions were diluted in PB-MNC from a healthy individual. The *Bgl*II filter was successively hybridized with the IGKC probe (upper panel), and IGKDE probe (lower panel). In this dilution experiment the Kde rearrangements were still detectable in the 5% dilution mixture (unfortunately, one of the Kde rearrangements comigrated with the germline band (7.6 kb) of the Kde probe). Upon hybridization with the IGKC probe a decreased density of the Cκ germline bands was found down to the 40-50% dilution mixture.

which could not be interpreted reliably when less than 40-50% of tumor cells were present. Upon hybridization with the IGKDE probe two rearranged bands were observed, which remained detectable down to the 5% dilution step (Figure 5).

DISCUSSION

To further improve the detection of clonal Igκ gene rearrangements and deletions by routinely performed Southern blot analysis for diagnostic purposes, we developed three new Igκ DNA probes (IGKJ5, IGKC, and IGKDE), determined the precise restriction map of the Jκ, Cκ, and Kde regions of the Igκ locus (Figure 2), and evaluated relevant combinations of restriction enzymes with the IGKJ5, IGKC, and IGKDE probes.

According to our experience with Southern blot analysis of rearranged Ig and TcR genes, optimal detection of clonal rearrangements can generally be obtained with J probes, which are located just 3´ of the J gene segments, in combination with restriction enzymes which result in small non-polymorphic germline restriction fragments (20,41,42). The small size of the restriction fragments reduces the chance of co-migration of germline bands and rearranged bands (20,42).

Our data indicate that rearrangements in the Jκ region are easily detectable by use of *Sac*I, *Hin*dIII, *Eco*RI, *Bgl*II, or *Bam*HI digests in combination with the IGKJ5 probe (Figures 2, 3 and Table 3). If no Cκ gene deletion has occurred, these rearrangements are also detectable by use of the IGKC probe in *Bam*HI or *Bgl*II digests, because their germline restriction fragments contain the complete Jκ-Cκ region (Figures 2, 3 and Table 3). Kde-mediated Igκ gene deletions are easily detectable with the IGKDE probe in *Bgl*II, *Hin*dIII, or

*Eco*RI digests.

Discrimination between the two types of Kde-mediated deletions is possible by successive hybridization with the IGKDE and IGKJ5 probes. In case of Cκ gene deletion (rearrangement of Kde to the intron RSS) the IGKJ5 and IGKDE probes will recognize the same rearranged restriction fragment, whereas in case of Jκ-Cκ gene deletion (rearrangement of Kde to a Vκ gene segment) the IGKDE probe will recognize a rearranged band, not detectable with the IGKJ5 probe (Figure 3 and Table 3). The third type of Igκ gene deletion, (i.e. deletion of Jκ gene segments without deletion of the Cκ gene segment) is rare and can be detected by successive hybridization with the IGKJ5 and IGKC probes in *Bgl*II or *Bam*HI digests, resulting in a rearranged band with the IGKC probe which is not detectable with the IGKJ5 probe (Figures 1B, 2, and 3 and Table 3). RFLP's of the relevant restriction sites (*Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, and *Sac*I) are extremely rare in the Jκ, Cκ, and Kde region (Table 2) (43,44).

The value of an optimal Kde probe for detection of Igκ gene deletions by Southern blot analysis was demonstrated in a dilution experiment (Figure 5). Since Kde-mediated deletions are recognized as Kde rearrangements by hybridization with the IGKDE probe, a detection limit of ~5% could be reached (Figure 5). This is in contrast to hybridization with the IGKJ5 or IGKC probes, which result in germline bands with decreased density in case of Igκ gene deletions. It appeared that identification of Igκ gene deletions with Jκ or Cκ probes is only possible, if the cell samples contain a high tumor load (>40-50%) (Figure 5). Therefore, we wish to emphasize that an optimal Kde probe (e.g. the IGKDE probe) should be used for reliable detection of Igκ gene deletions. Application of this probe also allows additional clonality studies in $Ig\lambda^+$ B-cell malignancies, because they generally have monoallelic or biallelic Kde gene rearrangements.

Igκ gene rearrangement patterns were studied in a series of 217 B-lineage leukemias by use of our three Igκ DNA probes (Tables 4 and 5). Rearrangements and/or deletions of the Igκ genes were found in 62% of 111 precursor B-ALL and in 100% of 106 chronic B-cell leukemias. The frequencies of Igκ gene rearrangements and deletions in precursor B-ALL are essentially higher than previously reported (6,24,26,29,32,33,45). This is most probably due to the optimal choice of restriction enzymes and probes, especially the use of the IGKDE probe (20,42). In the total group of B-lineage leukemias, the far majority of Igκ gene deletions occurred via Kde rearrangements, but in 1.5% of alleles with Igκ gene deletions (in two precursor B-ALL and one $Ig\lambda^+$ chronic B-cell leukemia) only the Jκ gene segments were deleted. This alternative deletional mechanism has previously been described by Feddersen et al., who found this type of deletion in three out of 65 B-lineage leukemias and lymphomas (18,19).

A striking difference in frequency of the two types of Kde mediated Igκ gene deletions was found between our groups of precursor B-ALL and chronic B-cell leukemias. Kde rearrangements to Vκ gene segments were found in 69% (56/81) of Kde-mediated deletions in the precursor B-ALL group, while these were found in only 46% (50/108) of Kde-mediated deletions in the chronic B-cell leukemia group. The latter was comparable with the 40% found by Feddersen et al. in their group of mature B-cell leukemias and lymphomas (38 multiple myelomas and eight CLL) (18). Preference for Vκ-Kde rearrangements can be explained by the fact that rearrangements via complete heptamer-nonamer RSS (in case of Vκ-Kde rearrangement) occur more easily than via an incomplete heptamer RSS, as present

in the Jκ-Cκ intron (46). Therefore, the lower frequency of Vκ-Kde rearrangements in mature B-cells as compared to immature B-cells might be caused by a smaller number of available germline Vκ gene segments due to (continuing) Vκ-Jκ rearrangements (47), and/or the lower accessibility of Vκ gene segments to rearrangements in mature B-cells.

Our analysis of the large group of chronic B-cell leukemias provided evidence for the hierarchical model of ordered IgL gene rearrangements (10,11), because in all $Ig\lambda^+$ chronic B-cell leukemias one or two Igκ gene rearrangements and/or deletions were detected, and only a minority (~ 6%) of $Ig\kappa^+$ chronic B-cell leukemias contained rearranged Igλ genes (unpublished results). Some reports describe the occurrence of Igλ gene rearrangements in the presence of germline Igκ genes in a few cases of B-lineage ALL (4-6). This would be in favour of the stochastic rearrangement model (8,9), but it should be noted that this phenomenon has only been found in immature B-cells and not in mature B-cells. Probably these ALL originated from a small pool of "unusual" precursor B-cells, which rearranged their Igλ genes prior to Igκ genes or this phenomenon is related to the malignant transformation of the immature B-cell.

The regulatory signals which are responsible for the model of ordered IgL gene rearrangements in B-cells are not known. It has been suggested that deletion of Igκ genes, particularly the Igκ enhancer sequences, may allow progression from Igκ to Igλ gene rearrangement, either by elimination of inhibitory sequences or by encoding a transacting factor that can induce Igλ gene rearrangement (12-15). However, several Igκ gene targeting studies in mouse (by deleting Cκ and/or Igκ enhancer sequences) demonstrate that rearrangements in the Igλ locus can occur in the absence of Igκ gene rearrangements or Kde-mediated Igκ gene deletions (48-50). These data suggest that Igκ gene deletion is not a prerequisite for the progression of Igκ to Igλ gene rearrangements. It is more plausible that Igκ gene deletions play a role in deleting non-functional Igκ genes or in deleting functionally rearranged Igκ genes, which code for auto-reactive Ig molecules (51,52). Moreover, Igκ gene deletions will avoid double IgL expression on the cell surface of B-cells (7,53).

We conclude that all types of Igκ gene rearrangements and deletions are optimally detectable in routinely performed Southern blotting with the three presented Igκ DNA probes (IGKJ5, IGKC, and IGDKE probes) in combination with at least two appropriate restriction enzymes (i.e. *Hin*dIII, *Bgl*II, *Eco*RI, *Sac*I, and/or *Bam*HI). Hybridization of the three probes to *Hin*dIII filters results in the same germline bands as in *Bam*HI/*Hin*dIII filters. Nevertheless, we recommend to use the three probes in combination with *Bgl*II digests and *Bam*HI/*Hin*dIII double digests (instead of *Hin*dIII single digests), because these two digests also allow optimal analysis of IgH gene rearrangements with the IGHJ6 probe (42). Our extensive analyses in a series of 217 B-lineage leukemias show that Igκ gene rearrangements and deletions occur in higher frequencies than reported previously (especially in case of precursor B-ALL), and that Igκ gene deletions are virtually always mediated via Kde rearrangements.

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IMMUNOGLOBULIN AND T-CELL RECEPTOR GENE REARRANGEMENTS IN PRECURSOR B-ALL AT DIAGNOSIS

CHAPTER 3.1

ANTIGEN SPECIFIC RECEPTOR GENES: CLINICAL APPLICATIONS

Introduction

In general, acute lymphoblastic leukemias (ALL) consist of clonal cells, which are regarded as malignant counterparts of normal immature B- and T-cells (1-5). Immunoglobulin (Ig) and T-cell receptor (TcR) gene rearrangements occur early during lymphoid differentiation (see Chapter 2.1) (6-8). Therefore, cells of immature lymphoid malignancies will have identically rearranged Ig or TcR genes (2,7,9). Based on this concept, the various precursor B-ALL and T-ALL have been used to study the Ig and TcR gene rearrangements during lymphoid differentiation (2,9-16). On the other hand, analysis of Ig and TcR genes with the Southern blot technique and polymerase chain reaction (PCR) technique can be used for diagnostic studies (2,9,12,16,17-25).

Secondary gene rearrangements

In order to obtain antigen specific receptors on their cell membrane, B- and T-cells try to assemble functional Ig and TcR molecules by recombination of their Ig and TcR genes, respectively (6-8). These rearrangements occur in a hierarchical order (7,26-28). Because these rearrangement processes are highly complex, non-functional and aberrant Ig or TcR genes rearrangements occur frequently (6,8,29). To partly overcome these problems, each B- and T-cell has two Ig or TcR alleles on which the Ig and TcR genes are located, respectively (7).

As confirmed in ALL studies most B- and T-cells have biallelic Ig or TcR rearrangements (2,9,16,17). In addition to the biallelic rearrangements, secondary gene rearrangements can occur in order to rescue precursor B- and T-cells, with two non-functionally rearranged Ig or TcR genes (30-40). The various secondary rearrangements and their occurence in the different antigen receptor gene rearrangements are summarized in Table 1 and shown in Figure 1. D-JH replacements in B-cells replace preexisting out-of-frame DJH

TABLE 1. Secondary rearrangements (replacements).

	Ig genes			TcR genes			
	IgH	Igκ	Igλ	TcR-α	TcR-β	TcR-γ	TcR-δ
D-J replacements[a]	+[b]	–	–	–	(+)	–	(+)
V-J replacements	–	+	(+)	+	(+)	+	–
V replacements	+	–	–	–	+	(+)	–

a. Abbreviations used: V, variable gene segment; D, diversity gene segment; J, joining gene segment.
b. Symbols used: +, secondary rearrangement reported to occur; (+), secondary rearrangement can occur, but is not reported so far; –, this form of secondary rearrangement is impossible in the involved Ig or TcR gene locus.

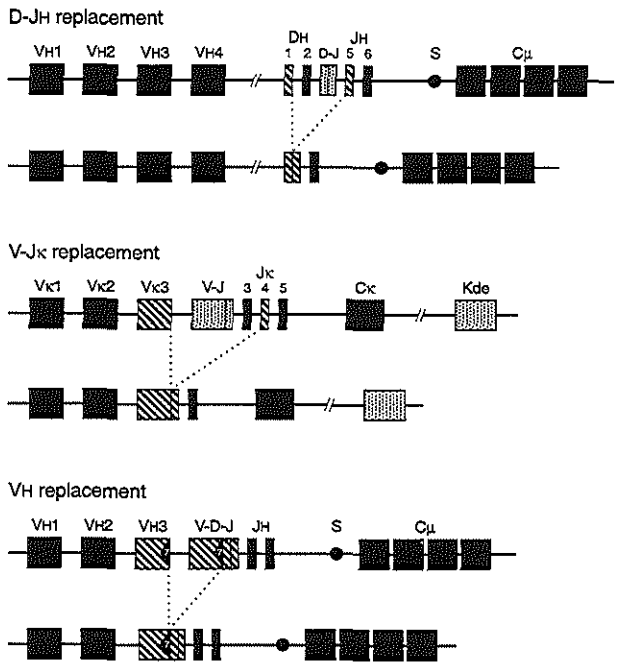

Figure 1. Schematic diagram of secondary rearrangements. **Upper panel:** An example of a D-JH replacement. A DH1-JH5 rearrangement replaces a preexisting out-of-frame DJH complex. **Middle panel:** An example of a V-Jκ replacement. A Vκ3-Jκ4 rearrangement replaces a preexisting out-of-frame or self-reactive V-Jκ rearrangement. **Lower panel:** An example of a VH to V-D-JH rearrangement. The VH4 gene segment is replaced by the VH3 gene segment via a rearrangement process in which heptamer (indicated as 7) joining sequences are involved (31).

complexes by joining an upstream DH to a downstream JH (Figure 1). In case of V-J replacement, a comparable process will take place (Figure 1). These replacements may occur repeatedly in the same Ig or TcR locus as long as it carries unrearranged V, D, or J gene segments. V replacements represent a third type of secondary rearrangements, in which a rearranged V gene segment is replaced by a new upstream V gene segment. This rearrangement is mediated by a heptamer recombination signal sequence (RSS) found in the 3´ region of VH, Vβ, and Vγ genes (Figure 1) (31). So far no Vγ replacements have been reported (31). The internal heptamer RSS are not present in the V gene segments of IgL and TcR-α/δ loci (31).

Diagnostic applications of Ig and TcR gene analysis.

Traditionally, Southern blot analysis of Ig and TcR genes is used for determination of clonality in a part of the lymphoproliferative disorders. Since the discovery of the PCR technique in 1985, its value has been proven in the diagnosis of clonal lymphoproliferative disorders, especially in combination with denaturing gradient-gel electroforeses (DGGE) or single-strand confirmation polymorphism (SSCP) analysis (19-25,41,42). The main advantages of the PCR based technique compared to the Southern blot technique in the assessment of clonality are: it is much less time-consuming; there is no requirement for radioactive isotopes; it requires less starting material, and it offers improved sensitivity for detection of clonality (0.2-1%) if combined with DGGE or SSCP analysis (25,41,42).

The PCR technique is highly sensitive during follow-up when patient-specific probes are used for detection of low numbers of tumor cells. Therefore, all possible precautionary

TABLE 2. Comparison of two techniques used in Ig and TcR gene rearrangement studies at diagnosis or during follow-up.

	Southern blotting	PCR analysis
amount of DNA per test	10-15 μg	0.1-1 μg
time consumption[a]	1 week	6 hours
sensitivity	~5%	0.2-1% (at diagnosis)[b] 0.01-0.0001% (during follow-up)[c]
radioactivity	yes	not essential
false-positive (contamination)	no	yes[d]
false-negative	–	15-30%

a. The time consumption mentioned is independent of the time necessary for DNA extraction, rehybridization of DNA filters, or extra PCR analysis in case of negative results.
b. The detection limit of the PCR technique is 0.2-1% if combined with DGGE or SSCP analysis.
c. The detection limit of the PCR technique is 0.01-0.0001% during follow-up when patient-specific probes are used for detection of low numbers of tumor cells.
d. False positive results due to contamination of small amounts of DNA (PCR products) from other patients have important implications for the detection of MRD by PCR analysis during follow-up.

measures should be taken to prevent cross-contamination of PCR products between patient samples in PCR-mediated minimal residual disease (MRD) studies, causing false-positive results (43,44). False-negative results obtained with the PCR technique in 15-30% of monoclonal cases can be explained by either technical problems (e.g. *Taq* polymerase inhibition and bad primer annealing), substrate problems (e.g. germline configuration, deletion of the involved gene segments, point mutations in the primer template sequences), incomplete rearrangements (D-J instead of V-D-J rearrangements), or rare V gene usage (23,45). The latter cause can be overcome by developing more primers which will recognize these V gene segments. The comparison between the two techniques is shown in Table 2.

The various clinical applications of Ig and TcR gene analysis by the Southern blot and PCR technique are summarized in Table 3. They can be divided in three phases of disease stages. First, analysis of cell samples at diagnosis; second, analysis of cell samples during and after treatment; and third, analysis of cell samples at relapse. The most often used diagnostic application is the discrimination between polyclonal and monoclonal lymphoproliferative diseases at presentation. It should be noted that monoclonality not necessarily implies a malignancy (Table 3) (9,46-51). In addition, using the presence of cross-lineage Ig or TcR gene rearrangements in acute non-lymphoblastic leukemia clonality can be proven (9,52). Other clinical applications at diagnosis are: the detection of two or more subclones and proof or exclusion of the common clonal origin of two malignant lymphoid cell populations (9,53-58). During and after treatment the detection of MRD is possible with a detection limit of ~5% in case of the Southern blot technique and a detection limit of 0.01-0.0001% in case of the PCR technique, thereby using leukemia-specific junctional region probes (59-63). Diagnostic applications at relapse are determination of identical Ig and/or TcR gene rearrangements (clonal relation), determination of differences in Ig and/or TcR gene rearrangements (clonal evolution), or diagnosis of secondary malignancies at relapse (64-69).

TABLE 3. Diagnostic applications of Ig and TcR gene rearrangement analysis[a].

At diagnosis

1. Discrimination between polyclonal and monoclonal lymphoid cell populations
   Caution: Monoclonality does not necessarily imply malignancy (e.g. large granular lymphocytic leukemia and monoclonal or oligoclonal lymphoproliferations in primary and secondary immunodeficiencies).

2. Assignment or exclusion of the differentiation lineage of a malignancy.
   Caution: cross-lineage Ig and TcR gene rearrangements can occur.

3. Detection of two or more sublones within one malignancy.

4. Proof of exclusion of the common clonal origin of two malignant lymphoid cell populations.
   Caution: One should try to discriminate between two independent lymphoid malignancies and subclone formation within a malignancy.

During and after treatment

5. Detection of low numbers of malignant cells.
   Caution: The detection limit of the Southern blot technique is ~5% and of the PCR technique 0.01-0.0001%.

At relapse

6. Analysis of lymphoid malignancies at diagnosis and subsequent relapses.
   - Determination of identical rearranged Ig and TcR genes (clonal relation).
   - Determination of differences in Ig and TcR gene rearrangements (clonal evolution).
   - Diagnosis of secondary leukemia.

a. Adapted from reference 9.

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CHAPTER 3.2

MULTIPLE REARRANGED IMMUNOGLOBULIN GENES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA OF PRECURSOR B-CELL ORIGIN*

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SUMMARY

Sixty precursor B-cell acute lymphoblastic leukemia (ALL) patients were analyzed for the configuration of their immunoglobulin (Ig) genes. Rearrangements and/or deletions of the Ig heavy-chain (IgH), Ig kappa-chain (Igκ), and Ig lambda-chain (Igλ) genes were detected in 98, 48, and 23% of cases, respectively. Although these percentages suggest the presence of a hierarchical order in IgH and Ig light-chain (IgL) gene rearrangements during B-cell differentiation, no correlation was found between the immunophenotype of the precursor B-ALL, and the arrangement patterns of their IgH and IgL genes.

Multiple rearranged IgH gene bands, generally differing in density, were found in 27 (45%) of the precursor B-ALL in various restriction enzyme digests. Cytogenetic data were used to determine whether the presence of more than two rearranged IgH gene bands was caused by hyperdiploidy of chromosome 14 or other chromosome 14 aberrations. The combined cytogenetic and IgH gene data allowed the precursor B-ALL to be divided into three groups: a monoclonal group (n=36; 60%), a biclonal group (n=16; 27%), and an oligoclonal group (n=8; 13%). In five biclonal ALL, biclonality at the Igκ gene level was also found. Such subclone formation was not detected at the Igλ gene level. As the detection limit of the Southern blot technique is 2-5%, it might well be that small subclones remained undetected, implying that the frequency of subclone formation at the IgH gene level in precursor B-ALL is probably higher than 40%.

It has been suggested that precursor B-ALL with multiple IgH gene rearrangements have a higher tendency to relapse. Although higher relapse rates were found in the oligoclonal group (53%) and in the combined bi-/oligoclonal group (33%) compared with

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the monoclonal group (20%), the log rank trend test showed no significancy.

The occurrence of multiple subclones in precursor B-ALL as found by IgH gene analyses will severely hamper the detection of minimal residual disease (MRD) using the polymerase chain reaction (PCR) mediated amplification of "tumor-specific" IgH gene junctional regions, because it cannot be predicted which detectable (or undetectable) subclone will cause MRD and/or relapse. Therefore it can be expected that the PCR technique will frequently produce false-negative results during the follow-up of precursor B-ALL.

INTRODUCTION

Leukemias and malignant lymphomas are generally thought to represent clonal expansions from a single transformed cell. This concept is supported by cytogenetic studies (1,2), glucose-6-phosphate dehydrogenase enzyme analyses (3), restriction fragment length polymorphism studies (4), and the detection of identical Ig idiotypes (5,6). Analyses of rearrangements in Ig and T-cell receptor (TcR) genes have also been used for identification of clonal expansions of malignant (precursor) B- and T-cells (7-11). Rearrangement of variable (V), (diversity (D),) and joining (J) gene segments of Ig and TcR genes generates unique DNA sequences at the junctional region of these gene segments. This junctional region is different in each lymphocyte or lymphocyte clone and is known as complementarity determining region III for Ig genes (12). Therefore, junctional regions of rearranged Ig and TcR genes in leukemias and malignant lymphomas can be regarded as "tumor-specific" markers (13-17). Based on this assumption, several workers have suggested that the PCR can be used for the amplification of "tumor-specific" junctional regions of rearranged IgH genes to detect MRD in precursor B-ALL (14,16,17).

Rearrangements of IgH genes occur in about 98% of precursor B-ALL, whereas rearrangements and/or deletions of IgL genes (Igκ or Igλ) are reported to occur in 5-40% of precursor B-ALL (18-22). Although the IgL gene rearrangements seem to occur in a hierarchical order during normal B-cell differentiation with Igκ gene rearrangements prior to Igλ gene rearrangements (22-24), this is not supported by precursor B-ALL studies, since IgL gene rearrangements are detectable in different subtypes of precursor B-ALL at variable frequencies (22,25).

Several studies indicate that multiple rearranged IgH genes may occur in precursor B-ALL, as detected by the use of a JH probe in various restriction enzyme digests (26-32). The reported frequencies of these multiple IgH gene rearrangements vary from 15-30% (26-32). Kitchingman et al. (27) suggested that the presence of multiple rearranged bands is due to the presence of subclones (bi-/oligoclonality) and may be related to a poor prognosis in childhood ALL. The latter was not confirmed by Katz et al. (32), but the median follow-up in their study was only 14 months.

This paper reports a detailed study of the configuration of the IgH and IgL genes in 60 consecutive, well-characterized cases of precursor B-ALL, in which the frequency of multiple rearranged Ig gene bands was carefully determined and the prognostic significance of biclonality and oligoclonality associated with IgH gene rearrangement was evaluated.

MATERIALS AND METHODS

Cell samples

Lymphoblasts were obtained at initial diagnosis from 60 of 87 consecutive children with precursor B-ALL, who presented at the Sophia Children's Hospital, Rotterdam, The Netherlands. The availability of sufficient cells for DNA extraction was the only selection criterion, which appeared not to be related to age or other established poor prognostic factors except of white blood cell (WBC) count. The median WBC count of the 27 non-selected precursor B-ALL was 4.8×10^9/l (range $1.0\text{-}36.8 \times 10^9$/l), but 20×10^9/l (range $2.8\text{-}268 \times 10^9$/l) for the 60 selected precursor B-ALL, indicating that the WBC count at least partly influenced the availability of sufficient cells for this study. The 60 patients were treated according to seven different therapy protocols. All cell samples were obtained after informed consent according to the guidelines of the Medical Ethics Committee of the University Hospital/Erasmus University, Rotterdam. For three samples the cell bank of the Dutch Childhood Leukemia Study Group (DCLSG), The Hague, was relied upon.

Mononuclear cells (MNC) were isolated by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) from peripheral blood and bone marrow samples. In one case the malignant cells were derived from a lymphnode biopsy. These MNC samples were subjected to a detailed immunological marker analysis (see later) and the remaining MNC were stored in liquid nitrogen.

Immunological marker analysis

The MNC of the precursor B-ALL patients were analyzed for the following: nuclear expression of terminal deoxynucleotidyl transferase (TdT) (Supertechs, Bethesda, MD, USA); cell membrane expression of the B-cell markers CD9 (BA-2; Hybritech, San Diego, CA, USA), CD10 (VIL-A1; Dr. W. Knapp, Vienna, Austria), CD19 (B4; Coulter Clone, Hialeah, FL, USA), CD20 (B1; Coulter Clone), CD22 (Leu-14; Becton Dickinson, San Jose, CA, USA), and CD37 (Y29/55; Dr. H. K. Forster, Hoffman-La Roche, Basel, Switzerland); cytoplasmic expression of the Ig heavy-chain μ (weak Cyl$g\mu$) (Kallestad Laboratories, Austin, TX, USA); membrane expression of the T-cell markers CD2 (T11; Coulter Clone), CD3 (Leu-4; Becton Dickinson), CD5 (Leu-1; Becton Dickinson) and CD7 (3A1; American Type Culture Collection, Rockville, MD, USA); membrane expression of the myeloid cell markers CD13 (My7; Coulter Clone), CD14 (My4; Coulter Clone), CD15 (VIM-D5; Dr. W. Knapp), and CD33 (My9; Coulter Clone); HLA-DR antigen (L243; Becton Dickinson) and the precursor marker CD34 (BI-3C5; SeraLab, Crawley Down, UK). The immunofluorescence stainings for TdT, Cyl$g\mu$ and the cell membrane markers were performed as described previously (33) and evaluated with Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) and/or FACScan (Becton Dickinson). A leukemia was considered to be a precursor B-ALL if the malignant cells were positive for TdT, CD19, and HLA-DR (null ALL), or for TdT, CD10, CD19, and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR, and Cyl$g\mu$ (pre-B-ALL).

Southern blot analysis

DNA was isolated from frozen MNC as described previously (34,35). Control DNA was obtained from granulocytes or other cell samples with germline Ig genes. DNA (20 μg) was digested with the appropriate restriction enzymes (Pharmacia). The restriction fragments were size-separated in 0.7% agarose gels and blotted onto Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described previously (35).

IgH gene rearrangements were detected with a ^{32}P-random oligonucleotide labeled J$_H$ probe (35). The J$_H$ probe was a 0.95-kb *Bst*EII fragment which recognizes sequences just 3′ of the J$_H$ gene segments (35). The configuration of the Igκ genes was analyzed with a Jκ and a constant (C)κ probe. The Jκ probe was a 0.55-kb *Hae*III fragment which recognizes sequences just 3′ of the Jκ gene segments (35). The Cκ probe was a 2.5-kb *Eco*RI fragment (35). All 60 DNA samples were analyzed by use of the J$_H$, Jκ and Cκ probes in *Bgl*II digests, as well as in *Bam*HI/*Hin*dIII and *Bam*HI/*Sac*I double digests. If necessary for confirmation, *Eco*RI and/or *Pst*I digests were also used. Deletions of the Cμ gene segment were detected using the 1.2-kb *Eco*RI Cμ fragment in *Bgl*II and *Bam*HI/*Hin*dIII digests (35). The configuration of the Igλ genes was analyzed with the Cλ3 probe (a 0.8-kb *Bgl*II/*Eco*RI fragment) (35). Fifty-seven DNA samples were analyzed with this probe in *Eco*RI/*Hin*dIII double digests and three DNA samples in an *Eco*RI digest (35).

Recombinant plasmids were handled under PZEK1 containments according to the National Institutes of Health guidelines for research involving recombinant DNA molecules.

Cytogenetic analysis

Cytogenetic studies of the cell samples were performed according to standard procedures, including in some cases the methotrexate treatment of cultures (36). In each case multiple metaphase spreads were studied for the number of chromosomes and for the presence of structural chromosome abnormalities. The karyotypes were established according to the ISCN (1985) (37).

Statistical data

The Kruskal-Wallis test was performed to determine the statistical significance of the WBC count in the 60 precursor B-ALL (38). The Kaplan-Meier plot was used to analyze the relationship between the number of subclones and the disease-free survival in the 60 precursor B-ALL (39). Statistical significance of these data was determined with the log rank trend test (38).

RESULTS

Immunological marker analysis

Immunological marker analysis of the 60 precursor B-ALL revealed that two were null ALL, 40 were common ALL, and 18 were pre-B-ALL. The results of the analyses are summarized in Table 1. Most of the precursor B-ALL were negative for the T-cell and myeloid markers tested, but some expressed the T-cell marker CD7 or the myeloid markers CD13 or CD33.

TABLE 1. Immunophenotype of the 60 precursor B-ALL patients.

precursor B-ALL type	Immunological markers[a]										
	TdT	HLA-DR	CD34	CD9	CD10	CD19	CD20	CD22	CD37	Cylgμ[b]	Smlg
null ALL (n = 2)	+	+	+	+	−	+	−	−	± (1/2)	−	−
common ALL (n = 40)	+	+	± (15/19)	± (29/37)	+	+	± (16/38)	± (23/32)	± (2/37)	−	−
pre-B-ALL (n = 18)[b]	± (17/18)	+	± (3/8)	+	± (16/18)	+	± (7/18)	± (5/10)	± (2/13)	+	−

a. Symbols used: (+) expression (>50% of MNC) in all leukemias; (±) a part of the leukemias was positive (the number of positive leukemias/tested leukemias is indicated in parentheses); (−) no expression (<15% of MNC) in all leukemias. Percentage positivity between 15-50% was not found.

b. A leukemia was considered to be a pre-B-ALL if more than 15% of the leukemic cells expressed Cylgμ.

Southern blot analysis of immunoglobulin gene rearrangements

DNA samples from the 60 precursor B-ALL were examined for the presence of rearranged IgH and IgL genes. Rearrangements and/or deletions of the IgH genes, Igκ genes, and Igλ genes were found in 98% (59 cases), 48% (29 cases), and 23% (14 cases) of the precursor B-ALL, respectively (Figures 1-3 and Table 2). No clear correlation was found between the immunophenotype of the precursor B-ALL and the rearrangement patterns of the IgH and IgL genes (Table 2). In one common ALL, IgH gene analysis only revealed a

TABLE 2. Summary of Ig gene rearrangements in the 60 precursor B-ALL patients.[a]

	IgH genes		Igκ genes			Igλ genes
	Rearrangement	Deletion	Rearrangement	Deletion		Rearrangement
Precursor B-ALL type	JH probe[b] (R/G, R/R, or R/D)[c]	JH and Cμ probe (D/D)	Jκ probe[d] (R/G, R/R, or R/D)	Jκ probe (D/D or D/G)	Cκ probe (D/D or D/G)	Cλ probe (R/G or R/R)
null ALL (n = 2)	100% (2/2)	0% (0/2)	0% (0/2)	0% (0/2)	0% (0/2)	50% (1/2)[e]
common ALL (n = 40)	90% (36/40)	8% (3/40)	38% (15/40)	18% (7/40)	43% (17/40)	28% (11/40)[e]
pre-B-ALL (n = 18)	100% (18/18)	0% (0/18)	28% (5/18)	11% (2/18)	28% (5/18)	11% (2/18)
Total	93% (56/60)	5% (3/60)	33% (20/60)	15% (9/60)	37% (22/60)[f]	23% (14/60)
	98% (59/60)		48% (29/60)			

a. In case of subclone formation, the Ig gene rearrangement patterns of the major clone have been used in this table.
b. Twenty-seven patients had three or more rearranged IgH genes, generally differing in density. In 24 patients this was due to subclone formation and in the other three patients hyperdiploidy of chromosome 14 caused one or two additional rearranged IgH gene bands.
c. Symbols used: (R) rearranged allele; (G) germline allele; (D) deletion of the studied gene segment.
d. Two patients had three rearranged Igκ gene bands of different density, two patients had two rearranged Igκ gene bands of obvious different density, and one patient had two weak rearranged Igκ genes and a strong germline band, which was not due to non-leukemic cells. All five belonged to the group of biclonal ALL, as determined by IgH gene analysis and cytogenetics.
e. Rearranged Igλ genes but germline Igκ genes were found in one null ALL and two common ALL.
f. All patients had at least one rearranged and/or deleted Jκ gene.

germline band, which was not due to contaminating non-leukemic cells, as the leukemic cell load was 93% in this patient. This common ALL also had germline IgL genes. In three common ALL, the JH and Cμ gene segments were deleted on both alleles. In one of these cases this was partly caused by the loss of one chromosome 14. In the other two cases several C gene segments of the IgH gene locus were still present (data not shown) and no translocation or other karyotypic changes involving chromosome 14 were detectable, which suggests that the JH and Cμ gene segments had been selectively deleted.

Rearrangements in the Jκ gene region occured in 20 cases (33%) of precursor B-ALL on one or both alleles, whereas in nine cases (15%) deletion of the Jκ gene region on one allele (with the other allele in germline configuration), or both alleles was found. In 22 cases (37%) deletion of the Cκ gene segments on one allele (10 cases) or both alleles (12 cases) was found. In these cases deletion of the Jκ gene segment was found in 50% of the Igκ gene alleles with a deleted Cκ gene segment. However, in one ALL the Jκ gene segments were deleted on both alleles, while the Cκ gene segment was still present on one allele. In most cases with rearranged Igλ genes, the Igκ genes were deleted and/or rearranged, but rearranged Igλ genes with germline configuration of both the Igκ gene loci were found in three precursor B-ALL patients (one null ALL and two common ALL). This implies that IgL gene rearrangements were found in 53% of cases. Further details concerning the rearrangement patterns of the IgH and IgL genes are summarized in Table 2.

Multiple rearranged IgH gene bands, generally differing in density, were found in 27 precursor B-ALL. In 18 patients (30%) three or four rearranged IgH gene bands were found, and in nine patients (15%) more than four rearranged IgH gene bands were detected. The presence of genetic polymorphisms, such as are due to the hypervariable polymorphic region at the 5′ side of the JH gene locus (40-42), were excluded by using different combinations of restriction enzymes (*Bgl*II, *Bam*HI/*Hin*dIII and *Bam*HI/*Sac*I). Detailed analysis of the Igκ genes revealed that in five ALL with multiple IgH gene bands, two or more

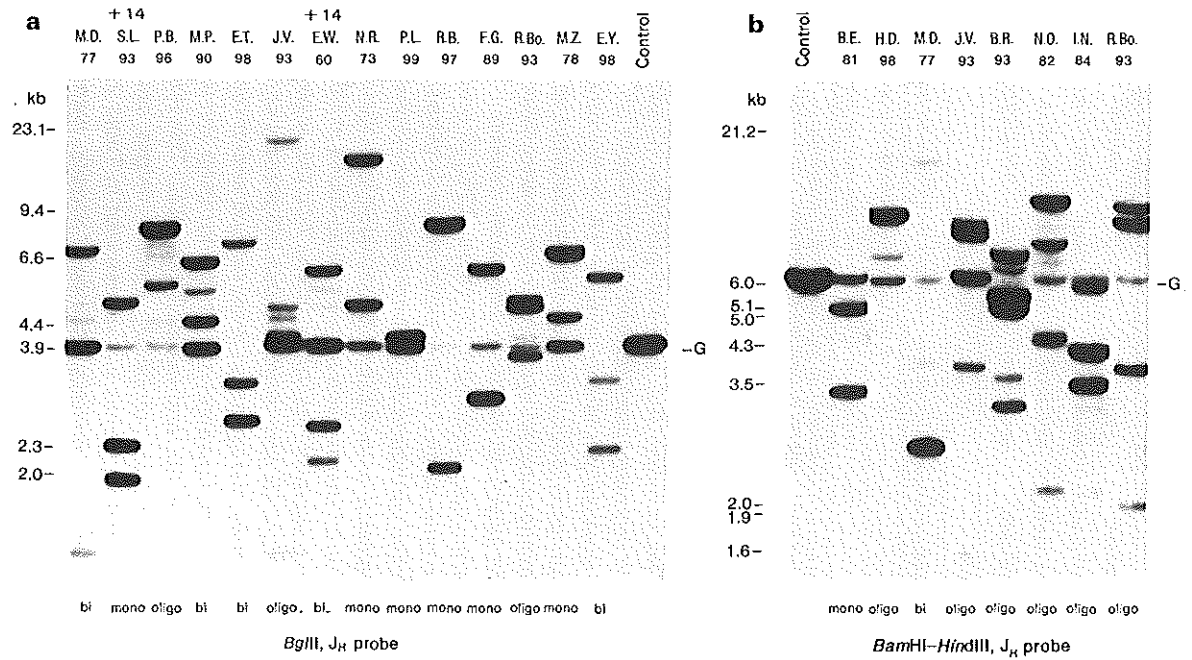

Figure 1. Southern blot analysis of the IgH genes in several precursor B-ALL. Control DNA and DNA from precursor B-ALL were digested with *Bgl*II (A) and a combination of *Bam*HI and *Hin*dIII (B), size-separated and blotted onto nylon membrane filters which were hybridized with the ^{32}P-labeled JH probe. The sizes (in kb) of the germline bands (G) and several molecular weight markers are indicated. The leukemic cell load is given in percentages below the patient's initials. In 13 of the presented precursor B-ALL cases more than two rearranged IgH gene bands were detected, which differed in density in most instances. In two patients (SL and EW) trisomy 14 (+ 14) was the cause of one of the extra bands. Based on the combined IgH gene and cytogenetic data it was possible to conclude whether the precursor B-ALL were monoclonal (mono), biclonal (bi), or oligoclonal (oligo). Detailed information concerning the biclonal and oligoclonal leukemias is given in Tables 3 and 4.

rearranged Igκ gene bands of different density were present, as determined in at least two restriction enzyme digests (Figure 2 and Table 3). No such multiple rearranged bands were found upon Igλ gene analysis (Figure 3). The assumption that multiple rearranged IgH or Igκ gene bands are caused by the occurrence of two or more subclones with differently rearranged Ig genes, is only feasible if the presence of extra copies of chromosome 14 (which contains the IgH gene locus in band 14q32) and chromosome 2 (which contains the Igκ gene locus in band 2p12) has been excluded by cytogenetic analysis.

Cytogenetic analysis

Karyotypic analysis at diagnosis was performed in 57 of the 60 patients. Five patients had a hypodiploid karyotype (<46 chromosomes), 16 patients had a hyperdiploid karyotype (>46 chromosomes), and eight patients had a translocation (e.g. t(1;19), t(4;11), and t(9;22)). In the other 28 patients no clonal aberrations were found. One patient with a hypodiploid karyotype had lost one copy of chromosome 14, whereas IgH gene analysis demonstrated a complete deletion of the JH and Cμ gene loci on both alleles. Another patient with a hypodiploid karyotype had lost one copy of chromosome 14 in a part of the leukemic cells, which correlated with the finding that IgH gene analysis showed one clear rearranged IgH gene band and one weak rearranged IgH gene band. Five patients with a hyperdiploid karyotype had one extra copy of chromosome 14 and one patient had two extra copies of this chromosome. In four of these six precursor B-ALL patients, the presence of more than two rearranged IgH gene bands could be correlated with extra copies of chromosome 14. This allowed the conclusion that three patients had a monoclonal ALL instead of a biclonal ALL (e.g. patient S.L. in Figure 1) and that one patient (patient E.W.) had a biclonal ALL instead of an oligoclonal ALL (Figure 1). The other two patients with a hyperdiploidy of chromosome 14 had no additional rearranged IgH gene bands. In the ALL with more than two rearranged Igκ genes (see earlier), no additional copies of chromosome 2 were detected.

On the basis of the combined cytogenetic and IgH gene data, the precursor B-ALL (n=60) were divided in three groups: a monoclonal group (n=36; 60%), a biclonal group (n=16; 27%), and an oligoclonal group (n=8; 13%) (Tables 3 and 4).

Clinical and biological characteristics of patients in the monoclonal, biclonal, and oligoclonal groups

The median age in the monoclonal group was 4.8 years (range 1.3-15.0), in the biclonal group 3.7 years (range 1.6-12.2), and in the oligoclonal group 5.3 years (range 0.2-12.4). The median WBC count at diagnosis was 14.6 x 10^9/l (range 2.8-198.7 x 10^9/l), 18.4 x 10^9/l (range 8-88.2 x 10^9/l), and 74.1 x 10^9/l (range 17.5-268 x 10^9/l) for the monoclonal, biclonal and oligoclonal groups, respectively. A significant p-value of 0.03 in the WBC count data was found with the Kruskal-Wallis test, demonstrating a relationship between bi-/oligoclonality and high WBC count. Additional clinical and laboratory characteristics at diagnosis of the 16 biclonal and eight oligoclonal precursor B-ALL patients are given in Tables 3 and 4.

Of the 36 monoclonal ALL patients one was diagnosed as null ALL, 27 as common ALL, and eight as pre-B-ALL. Of the 16 biclonal ALL, 11 patients were diagnosed as common ALL and five patients as pre-B-ALL. Of the eight oligoclonal ALL one patient was

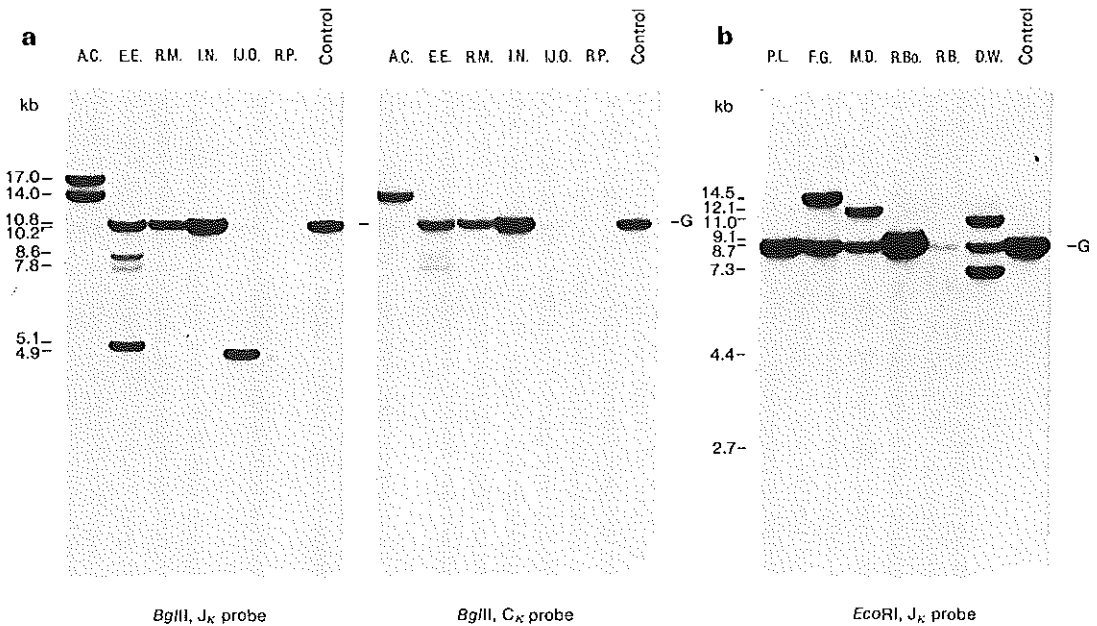

Figure 2. Southern blot analysis of the Igκ genes in several precursor B-ALL. Control DNA and DNA from precursor B-ALL patients were digested with *Bgl*II (**A**) and *Eco*RI (**B**), size-separated, and blotted onto nylon membrane filters which were hybridized with the ^{32}P-labeled Jκ probe (*Bgl*II and *Eco*RI filters) and subsequently rehybridized with the ^{32}P-labeled Cκ probe (*Bgl*II filter). The sizes (in kb) of the germline bands (G) and rearranged bands are indicated. The patients are indicated with their initials. **A:** *Bgl*II filter with DNA from six precursor B-ALL hybridized with the Jκ and Cκ probe. The Jκ gene locus was rearranged in four patients (AC, EE, IN and IJO), deleted on one allele in one patient (patient IJO) and deleted on both alleles in another patient (RP). In four patients (AC, EE, IJO and RP) a deletion of the Cκ gene segment was found on one or both alleles. In patient EE three rearranged bands of different density are visible, indicating biclonality. **B:** *Eco*RI filter with DNA from six other precursor B-ALL patients. Four patients (FG, MD, RBo and DW) had rearrangements of the Jκ gene locus. In patient MD three rearranged bands of different density and one germline band due to non-leukemic cells (~23%) are visible. Patient RBo had one rearranged Igκ gene allele (Table 4), which appeared as a 9.1 kb band in the *Eco*RI digest; this band almost co-migrated with the germline band. In patient DW two rearranged and one germline band are present, the latter being caused by the presence of non-leukemic cells (~27%). In patient RB the Jκ gene segments were deleted on both alleles. For more detailed description see Tables 3 and 4.

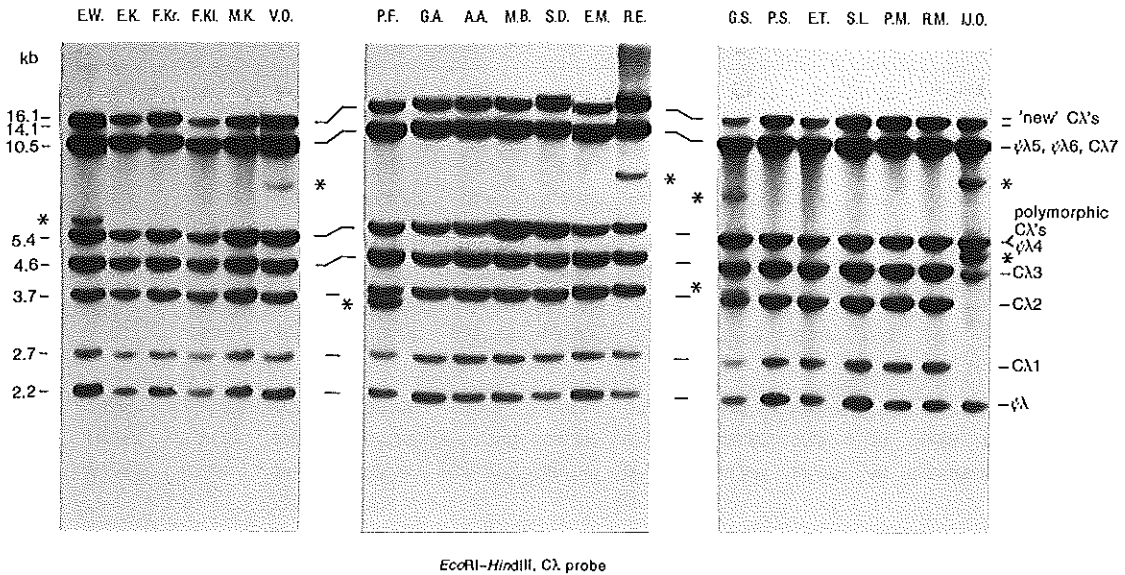

Figure 3. Southern blot analysis of the Igλ genes in 20 precursor B-ALL patients. Control DNA and DNA from precursor B-ALL patients were digested with a combination of *Eco*RI and *Hin*dIII, size-separated, and blotted onto nylon membrane filters which were hybridized with the ^{32}P-labeled Cλ probe. The sizes (in kb) of the germline bands are indicated. The patients are indicated with their initials. Six presented precursor B-ALL had one or two rearranged bands (indicated by asterisks).

TABLE 3. Clinical characteristics and Southern blot analysis of 16 biclonal precursor B-ALL patients.[a]

Patients	GA	AA	MB	RBh	SD	MD	EE	NK	MP	CR	ET	AV	VO	EW	DW	EY
Clinical characteristics																
Age (years)/sex[b]	2.8/M	1.6/F	3.8/M	4.8/M	2.3/M	7.9/M	4/F	5.9/F	2.4/M	3.7/M	2.2/M	1.9/F	12.2/M	2/F	1.8/M	4.7/M
WBC ($\times 10^9$/l)	8.3	12.9	25.9	10	88.2	9.7	24.0	9.6	76	62	16.7	18.4	16.5	27	24.1	8
Duration of remission (months)	66+	80+	23+	27+	17	61+	58+	80+	88+	65+	5	28+	32+	54+	39	31+
Sample[c]	BM	BM	BM	BM	PB	PB	BM	PB	BM	BM	BM	BM	BM	PB	PB	LN
Leukemic cells (%)[d]	92	98	90	96	91	77	98	84	90	97	98	94	99	60	73	98
Southern blot analysis																
IgH genes (JH probe)[e]																
*Bgl*I	G^W,3R	2R,2R^W	G^W,3R	2R,R^W	G^W,3R	G,R,2R^W	2R,2R^W	G^W,3R	G,2R,$R^{W[f]}$	2R,R^W	3R	G,2R,R^W	2R,2R^W	G,3R,2R^W	G,2R,2R^W	2R,R^W
*Bam*HI/*Hin*dIII	G^W,3R	2R,2R^W	G^W,3R,R^W	2R,R^W	G^W,2R	G^W,R,2R^W	2R,2R^W	G^W,3R	G^W,3R,R^W	2R,R^W	3R	G,2R	2R,2R^W	G,3R,2R^W	G,2R,1R^W	2R,R^W
*Bam*HI/*Sac*I	G^W,3R	2R,2R^W	G^W,3R,R^W	NT[g,h]	G^W,3R	NT[h]	NT[h]	G^W,3R	G,2R[f]	2R,2R^W	3R	G,2R	2R,R^W	G,3R,2R^W	NT	2R,R^W
Igκ genes (Jκ probe)																
*Bgl*I	G	G	G	R,R^W	G^W,R,R^W	G,R,2R^W	G,R,2R^W	G,R	G^W,R,D	G,2R^W	G,D	G	G	G,R	G,R,R	G
*Bam*HI/*Hin*dIII	G	G	G	R,R^W	G^W,R,R^W	G,R,2R^W	G,R,2R^W	G,R	G^W,R,D	G,2R^W	G,D	G	G	G,R	G,R,R	G
*Bam*HI/*Sac*I	G	G	G	R,R^W	NT[h]	NT[h]	NT[h]	G,R	G^W,R,D	G,2R^W	G,D	G	G	G,R	NT	G
Igκ genes (Cκ probe)																
*Bgl*I	G	G	G	R,D	G^W,D,R^W	G,D	G,D,2R^W	G,R	G^W,R,D	G,D	G,D	G	G	G	G,R,D	G
*Bam*HI/*Hin*dIII	G	G	G	G,D	G,D	G,D	G,D	G	G,D	G,D	G,D	G	G	G	G,D	G
Igλ genes (Cλ probe)																
*Eco*RI/*Hin*dIII	G	G	G	G	G	G,R[i]	G,R	G	G[i]	G	G	G,R	G,R	G,R	G	G

a. Biclonality was defined as the presence of two subclones, as deduced from the detection of three or four rearranged bands and/or a strong germline band, which could not be attributed to the presence of non-leukemic cells (leukemic cells >90%). The rearranged bands often differed in density and were not attributable to hyperdiploidy of chromosome 14 or other chromosome 14 aberrations, except of patient EW (see text).
b. (M) male; (F) female.
c. (BM) bone marrow; (PB) peripheral blood; (LN) lymph node.
d. The leukemic cell load was determined by the percentage of TdT, CD10, and CD19.
e. In most cases the rearrangement patterns were confirmed with *Eco*RI digests and in a few cases with *Pst*I digests.
Symbols used: (G) allele in germline configuration; (G^W) weak germline band; (R) rearranged allele; (R^W) weak rearranged band; (D) deletion of the involved gene (segment).
f. The G-band in *Bgl*I and *Bam*HI/*Sac*I digests of patient MP probably represents a rearrangement, because in the *Bam*HI/*Hin*dIII and in an *Eco*RI digest an extra rearranged band and a weak germline band were detected.
g. (NT) not tested.
h. The rearrangement pattern could be confirmed in an *Eco*RI digest.
i. In this patient the Igλ genes were analyzed with the Cλ probe in an *Eco*RI digest.

TABLE 4. Clinical characteristics and Southern blot analysis of eight oligoclonal precursor B-ALL patients[a].

Patients	RBo	PB	HD	RH	IN	NO	BR	JV
Clinical characteristics								
Age (years)/sex[b]	8.1/F	9.3/M	5.3/M	0.2/M	3.2/M	0.9/F	12.4/M	1.5/F
WBC ($\times 10^9$/l)	155	268	37	202.2	20.9	74.1	17.5	20.2
Duration of remission (months)	14	9	48^+	6	75^+	56	54^+	66^+
Sample[c]	PB	BM	BM	PB	BM	PB	BM	PB
Leukemic cells (%)[d]	93	96	98	95	84	84	93	93
Southern blot analysis								
IgH genes (JH probe)[e]								
*Bgl*II	G^W,3R,R^W	G^W,2R,$4R^W$	G,2R,$4R^W$	G,R,$4R^W$	G^W,2R,$4R^W$	G^W,R,$5R^W$	4R,$2R^W$	G,R,$5R^W$
*Bam*HI/*Hin*dIII	G^W,3R,R^W	G^W,2R,$4R^W$	G,2R,$4R^W$	G,R,$2R^W$	G^W,2R,$4R^W$	G^W,R,$5R^W$	4R,$2R^W$	G,R,$5R^W$
*Bam*HI/*Sac*I	G^W,3R,R^W	G^W,2R,$4R^W$	G,2R,$4R^W$	NT[f]	G^W,2R,$3R^W$	G^W,R,$5R^W$	NT	G,$5R^W$
Igκ genes (Jκ probe)								
*Bgl*II	G,R	G	G,D	G	G	G	G	G
*Bam*HI/*Hin*dIII	G,R	G	G,D	G	G	G	G	G
*Bam*HI/*Sac*I	G,R	G	G,D	NT	G	G	NT	G
Igκ genes (Cκ probe)								
*Bgl*II	G,D	G	D,D	G	G	G	G	G
*Bam*HI/*Hin*dIII	G,D	G	D,D	G	G	G	G	G
Igλ genes (Cλ probe)								
*Eco*RI/*Hin*dIII	G	G	G,R	G	G	G	G	G

a. Oligoclonality was defined as the presence of more than two subclones as deduced from the detection of five or more rearranged bands and/or a strong germline band which could not be attributed to the presence of non-leukemic cells (leukemic cells >90%), or four rearranged bands which could not be paired due to differences in density (e.g. patient RBo).
b. (M) male; (F) female.
c. (BM) bone marrow; (PB) peripheral blood.
d. The leukemic cell load was determined by the percentage of TdT, CD10 and CD19.
e. In most cases the rearrangement patterns were confirmed with *Eco*RI digests and in a few cases with *Pst*I digests. Symbols used: G, allele in germline configuration; (G^W) weak germline band; (R) rearranged allele; (R^W) weak rearranged band; (D) deletion of the involved gene (segment).
f. (NT) not tested.

diagnosed as null ALL, two patients as common ALL, and five patients as pre-B-ALL. The frequencies of pre-B-ALL in the monoclonal, biclonal, and oligoclonal groups are 23% (8/36), 33% (5/16), and 63% (5/8), respectively, suggesting a correlation between oligoclonality and pre-B-ALL.

Disease-free survival in the monoclonal, biclonal, and oligoclonal groups

In the monoclonal group seven patients developed a relapse; only one was diagnosed as a high risk patient because of high WBC count ($>50 \times 10^9$/l). Six other high risk patients did not relapse. In the biclonal group three relapses occurred. One of these three patients was diagnosed as a high risk patient. In the oligoclonal group four relapses occurred. All four were diagnosed as high risk patients. Figure 4 shows the relationship between the

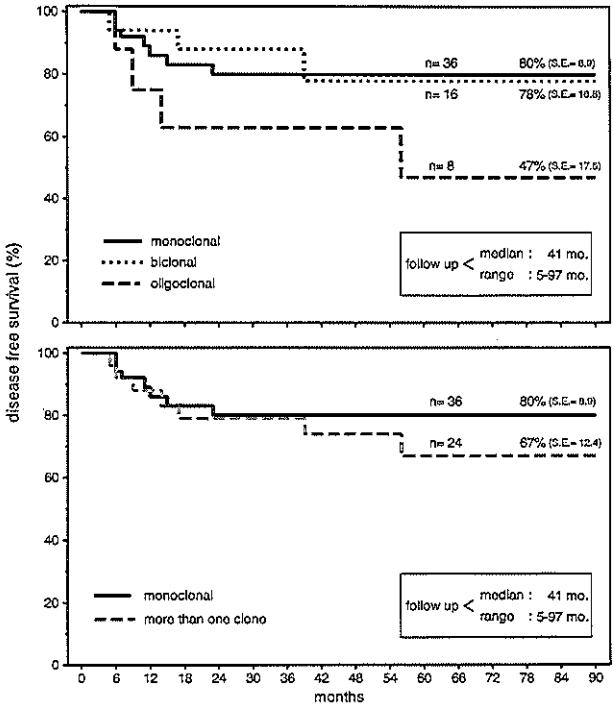

Figure 4. Kaplan-Meier plots concerning the relationship between the number of clones in precursor B-ALL and disease-free survival. Median follow-up: 41 months (range 5-97). **A.** The monoclonal group (n = 36; 80%, SE = 8.9) is indicated with the thick line, the biclonal group (n = 16; 78%, SE = 16.8) with the dotted line, and the oligoclonal group (n = 8; 47%, SE = 17.5) with the broken line. **B.** The monoclonal group (n = 36; 80%, SE = 8.9) is indicated with the thick line and the combined bi-/oligoclonal group (n = 24; 67%, SE = 12.4) with the broken line. Although the oligoclonal group tends to have a higher relapse rate, no significant differences were found between the groups in the log rank trend test.

number of clones in precursor B-ALL and disease-free survival in two Kaplan-Meier plots. The median follow-up time is 41 months (range 5-97). The disease-free survival rate of the three different groups is 80% (S.E. 8.9) for the monoclonal group, 78% (S.E. 16.8) for the biclonal group, and 47% (S.E. 17.5) for the oligoclonal group (Figure 4A). The log rank trend test shows a p-value of 0.19. When the biclonal and oligoclonal groups are combined the disease-free survival rate is 67% (S.E. 12.4) with a p-value of 0.5 (Figure 4B).

DISCUSSION

Immunoglobulin gene rearrangements

Sixty precursor B-ALL were analyzed for the configuration of their Ig genes. Rearrangements and/or deletions of the IgH, Igκ, and Igλ genes were found in 98%, 48%, and 23% of the precursor B-ALL, respectively. The percentages of IgL gene rearrangements are higher than reported elsewhere (18-22), probably due to our choice of restriction enzymes and probes (35). Although the frequency of IgH and IgL gene rearrangements suggests the presence of a hierarchical order in Ig gene rearrangements during B-cell differentiation (22-24), no correlation could be found between the immunophenotype and Ig gene rearrangement pattern of the precursor B-ALL. In addition, it is assumed that Igκ genes rearrange prior to Igλ genes (23,43), but in this series of precursor B-ALL three ALL had rearranged Igλ genes with germline Igκ genes. This implies that IgL gene rearrangements were found in 53% of cases. In four common ALL no rearranged IgH gene bands were detected. The

IgH genes were germline in one patient, whereas in the other three a complete deletion of the JH and Cμ gene locus was found on both alleles. An unusual deletion of the Igκ genes was found in one precursor B-ALL in which Jκ gene segments were deleted on both alleles, while the Cκ gene segment was still present on one allele. These aberrant rearrangements and deletions are not found in normal mature B-lymphocytes (23,43,44). Nevertheless, it might well be that these rearrangement and deletion processes also occur in normal precursor B-cells, thereby leading to cell abortion, which would be in line with the fact that only a small part of precursor B-cells reach the mature B-cell stage (45,46).

Multiple rearranged Ig gene bands

Several studies indicate that multiple rearranged IgH gene bands occur in 15-30% of all precursor B-ALL, as detected by a JH probe in multiple restriction enzyme digests (26-32). In 45% (27 cases) of this series of precursor B-ALL, more than two rearranged IgH gene bands were found, which generally differed in density.

Cytogenetic data were used to determine whether the presence of more than two rearranged IgH gene bands was caused by hyperdiploidy of chromosome 14 or translocations with a chromosomal breakpoint situated in or near the JH gene locus (1,2,47,48). In five precursor B-ALL patients trisomy 14, and in one patient tetrasomy 14 was found. In two precursor B-ALL with trisomy 14, only two rearranged IgH gene bands were detected, which indicates that no additional rearrangements had occurred in the IgH gene on the extra chromosome 14, or that a deletion of the IgH gene locus had occurred on one chromosome 14. In the other four precursor B-ALL with hyperdiploidy of chromosome 14, more than two rearranged IgH gene bands were found, which could be attributed to the presence of additional copies of chromosome 14. These data suggest that cytogenetic events (e.g. duplications of chromosomes) can occur prior to or after rearrangement processes. The combined cytogenetic and IgH gene data allowed the division of the precursor B-ALL into three groups: a monoclonal group (n = 36; 60%), a biclonal group (n = 16; 27%) (Table 3), and an oligoclonal group (n = 8; 13%) (Table 4). This heterogeneity at the DNA level (biclonality and oligoclonality) was not clearly associated with immunophenotypic heterogeneity (data not shown). As the detection limit of the Southern blot technique is 2-5% (35), it might well be that small subclones remained undetected, implying that the frequency of subclone formation at the IgH gene level in precursor B-ALL is probably higher than 40%.

In five biclonal ALL, two or more rearranged Igκ gene bands of different density were found (Figure 2 and Table 3). The absence of additional copies of chromosome 2 in these five biclonal ALL allowed the conclusion that both clones had differently rearranged Igκ genes. Such subclone formation was not detected at the Igλ gene level. To our knowledge, this is the first report on the finding that subclone formation at the IgH gene level can be accompanied by subclone formation at the Igκ gene level in precursor B-ALL.

Biclonality and oligoclonality at the Ig gene level can be attributed to somatic mutations or to continuing rearrangement processes in the original clone (30,49,50), such as D to JH rearrangements and VH to D-JH rearrangements, or secondary rearrangements such as D-JH replacements and VH replacements (30,51-53). It is improbable that somatic mutations play a role in the subclone formation in precursor B-ALL, as most multiple rearranged bands were detectable in all restriction enzyme digests, whereas somatic

mutations will be unlikely to affect all restriction sites (49,50). Bird et al. (30) analyzed a biclonal ALL and an oligoclonal ALL by molecular cloning and sequencing of the junctional regions. Their results indicate that continuing rearrangements and/or secondary rearrangements most probably cause subclone formation. It is predictable that these processes of continuing rearrangements and/or secondary rearrangements probably represent physiological mechanisms, which also occur during normal B-cell differentiation.

Biclonality and oligoclonality might be associated with a higher relapse rate

Kitchingman et al. (27) suggested that precursor B-ALL with multiple IgH gene rearrangements have a higher tendency to develop a relapse, probably because these ALL may be more resistant to cytostatic therapy. This was not confirmed by other workers (31,32). According to the Kaplan-Meier plots, a 33% higher relapse rate was found here in the oligoclonal group compared to the monoclonal group (median follow-up: 41 months) (Figure 4A). The combined biclonal and oligoclonal group had a 13% higher relapse rate than the monoclonal group (Figure 4B). Although the oligoclonal precursor B-ALL seems to have a higher tendency to develop a relapse, the log rank trend test did not reveal significant differences.

Katz et al. (32) suggested that multiple rearranged bands in precursor B-ALL have a poorer outcome together with other risk factors. In this study a clear relationship was found between biclonality and oligoclonality on one hand, and high WBC count on the other. A correlation between oligoclonality and the pre-B immunophenotype (CyIgμ^+) was also found. The latter finding may be due to a higher chance for a functionally rearranged IgH gene in ALL with multiple IgH gene rearrangements.

The 60 precursor B-ALL patients were treated according to seven different therapy protocols. Therefore an additional study needs to be performed on a larger series of patients receiving identical treatment to determine whether the occurrence of multiple IgH gene rearrangements (biclonality and oligoclonality) represents an independent risk factor for relapse or a risk factor coherent with other high risk factors.

Minimal residual disease

Recent reports indicate that the PCR technique may be useful for the detection of MRD in lymphoid malignancies via the amplification of the junctional regions of rearranged Ig and TcR genes (13-17). Several workers claim that MRD in precursor B-ALL is detectable using PCR mediated amplification of "tumor-specific" IgH gene junctional regions (14,16,17). However, this application of the PCR technique in precursor B-ALL will be severely hampered by the high frequency of subclone formation at the IgH gene level, because most subclones will have their own specific junctional region (30), and because it is unpredictable which detectable (or undetectable) subclone will cause MRD and/or relapse. Therefore, it can be anticipated that the PCR technique will frequently cause false-negative results during the follow-up of precursor B-ALL (22,54).

CONCLUSION

The results of this study indicate that biclonality and oligoclonality at the IgH gene level occur at a high frequency (at least 40%) in precursor B-ALL at diagnosis and that this can be accompanied by subclone formation at the IgL gene level. The follow-up data suggest that patients with an oligoclonal ALL may have a higher tendency to relapse than patients with a monoclonal ALL, although no significancy was found. The occurrence of multiple IgH gene rearrangements in precursor B-ALL has important implications for the detection of MRD using the PCR mediated amplification of "tumor-specific" IgH gene junctional regions, because it can be foreseen that the subclone formation at the IgH gene level will frequently lead to false-negative results.

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CHAPTER 3.3

DIFFERENCES IN IMMUNOGLOBULIN HEAVY-CHAIN GENE REARRANGEMENT PATTERNS BETWEEN BONE MARROW AND BLOOD SAMPLES IN CHILDHOOD PRECURSOR B-ACUTE LYMPHOBLASTIC LEUKEMIA AT DIAGNOSIS*

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SUMMARY

Bone marrow (BM) and corresponding peripheral blood (PB) samples from 30 patients with precursor B-acute lymphoblastic leukemias (precursor B-ALL) were analyzed for the configuration of their immunoglobulin (Ig) heavy-chain (IgH) and Ig kappa-chain (Igκ) genes. Rearrangements and/or deletions of the IgH and Igκ genes were detected in 100% and 47% of patients in this series of precursor B-ALL, respectively. Multiple rearranged IgH gene bands, generally differing in density, were found in 10 precursor B-ALL samples. This multi-band pattern is most probably caused by subclone formation due to continuing rearrangement processes. In five of the 10 bi-/oligoclonal cases (50%) differences in IgH gene rearrangement patterns between BM and PB samples were observed, which could be interpreted as the presence of an extra subclone in two cases and differences in the size of the subclones in three cases. In the 20 monoclonal precursor B-ALL, no dissimilarities in IgH gene rearrangement patterns between BM and the corresponding PB samples were found. Differences in Igκ gene rearrangement patterns between BM and PB were not observed in this series of precursor B-ALL, which is in line with the finding that no multiple Igκ gene rearrangements were detectable.

In all five cases, the extra subclones or the relatively larger sized subclones were found in the BM samples, suggesting that subclone formation in precursor B-ALL occurs in the tissue compartment from which the precursor B-ALL cells are thought to originate. This phenomenon will lead to underestimation of subclone formation, if only IgH gene analysis of PB samples is performed. In addition, it will hamper the detection of minimal residual disease (MRD) by the polymerase chain reaction (PCR)-mediated amplification of "leukemia-

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specific" IgH gene junctional regions, because it is unpredictable which subclone will cause MRD and/or relapse.

INTRODUCTION

The different types of ALL are generally regarded as malignant counterparts of precursor B- and T-cells (1-5). Comparable to normal precursor B-cells, precursor B-ALL are thought to originate from BM, while most T-ALL probably arise from cortical thymocytes (1-4). Therefore, analysis of ALL such as the rearrangement pattern of Ig and T-cell receptor genes has provided important insights into early human lymphoid differentiation (5-11).

Rearrangements of IgH genes occur in about 98% of precursor B-ALL (5,6,11-14), whereas rearrangements and/or deletions of the Igκ and Ig lambda-chain (Igλ) genes are found in ~45 and ~20% of precursor B-ALL, respectively (13,14). Several studies have indicated that multiple rearranged IgH genes may occur in precursor B-ALL, as detected by use of a joining (J)H probe in various restriction enzyme digests (14-21). This multi-band pattern of rearranged IgH genes occurs at high frequency (at least 40%) in precursor B-ALL at diagnosis (14,22). Such multiple rearrangements have also been found at the Igκ gene level (6% of precursor B-ALL), but not at the Igλ gene level (14). Most probably these multiple Ig gene rearrangements are caused by subclone formation (biclonality and oligoclonality) due to continuing rearrangement processes (14,19). It might be speculated that these precursor B-ALL subclones differ in biological behaviour, such as their dissemination pattern from BM to PB or to other extramedullary sites. This led us to determine whether differences in IgH and Igκ gene rearrangement patterns occur between BM and PB from the same precursor B-ALL patient. For this purpose we have analysed both BM and PB samples from 30 patients at diagnosis.

MATERIALS AND METHODS

Cell samples

Lymphoblasts from BM and PB were obtained at initial diagnosis from 30 children with precursor B-ALL, who presented at the Sophia Children's Hospital, Rotterdam. Mononuclear cells (MNC) were isolated by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) from BM and PB samples in each case. These MNC samples were subjected to a detailed immunological marker analysis according to standard protocols (for detailed description, see Chapter 3.2 and reference 23). The remaining MNC were stored in liquid nitrogen. A leukemia was considered to be a precursor B-ALL if the malignant cells were positive for terminal deoxynucleotidyl transferase (TdT), CD19 and HLA-DR (null ALL), or for TdT, CD10, CD19 and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR, and cytoplasmic Ig heavy-chain μ (pre-B-ALL) (3,24).

Southern blot analysis

DNA was isolated from frozen MNC as described previously (25,26). Control DNA was obtained from granulocytes or other cell samples with germline Ig genes. Fifteen μg of DNA were digested with the appropriate restriction enzymes (Pharmacia). Completeness of digestion was checked with λ DNA and plasmid DNA in parallel digests as described previously (26). The restriction fragments were size-separated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described (26).

To take care that the BM and PB lanes of each patient contained comparable amounts of DNA, the DNA content of the digested samples was checked before loading into the agarose gel (26). This was further verified by staining of the agarose gel with ethidium bromide.

IgH gene rearrangements were detected with a ^{32}P-random oligonucleotide labeled JH probe (26). The JH probe was a 0.95-kb *Bst*EII fragment which recognizes sequences just 3′ of the JH gene segments (26). The configuration of the Igκ genes was analyzed with a Jκ probe. The Jκ probe was a 0.55-kb *Hae*III fragment which recognizes sequences just 3′ of the Jκ gene segments (26). All 30 DNA samples were analyzed by use of the JH and Jκ probes in *Bgl*II digests, as well as in a combination of *Bam*HI and *Hin*dIII digests.

RESULTS AND DISCUSSION

Immunological marker analysis of the 30 precursor B-ALL revealed that two were null ALL, 16 were common ALL and 12 were pre-B-ALL. Except for differences in blast cell counts, no discrepancies were found in morphology or immunological marker expression between BM and corresponding PB samples of the precursor B-ALL patients.

DNA from BM and PB samples of the 30 precursor B-ALL was examined for the presence of rearranged IgH and Igκ genes. Rearrangements and/or deletions of the IgH and Igκ genes were found in 100% (all 30 cases) and 47% (14 cases) of the precursor B-ALL, respectively. Multiple rearranged IgH gene bands, generally differing in density, were found in 10 precursor B-ALL. Six were biclonal ALL and four were oligoclonal ALL (for detailed description, see Chapter 3.2). Subclone formation at the Igκ gene level was not found in the present series of precursor B-ALL.

In the 20 monoclonal precursor B-ALL no differences in IgH and Igκ gene rearrangement patterns between BM and corresponding PB samples were found. However, in five out of the 10 bi-/oligoclonal cases (50%) differences in IgH gene rearrangement patterns between BM and PB samples were observed. Two patients (one biclonal and one oligoclonal) showed weak additional bands in their BM sample (Figure 1A) and in three other patients (one biclonal and two oligoclonal) obvious differences in density of the rearranged bands were found (Figure 1B), indicating that the number or size of the subclones in BM and PB differed in these five patients. Differences in blast cell counts between BM and PB could only partly explain these detected differences in rearrangement patterns. The five cases with differences in rearrangement patterns were pre-B-ALL, corresponding with the fact that this phenotype is associated with a high frequency of subclone formation (14). In the 10 bi-/oligoclonal ALL no differences in Igκ gene rearrangement patterns between BM and PB were detectable, which is in line with the absence of multiple Igκ gene rearrangements in this series of precursor B-ALL.

Interestingly, in all five cases with differences in rearrangement patterns between BM and PB the extra subclones or the larger sized subclones were found in the BM samples, suggesting that subclone formation in precursor B-ALL (due to continuing Ig gene rearrangements) occurs in the tissue compartment from which precursor B-ALL cells originate. This would imply that dissemination of the different subclones occurs independently and may be related to the phase of subclone formation, i.e. the phase of the continuing rearrangement process. Therefore, the differences described here in IgH gene rearrangement patterns

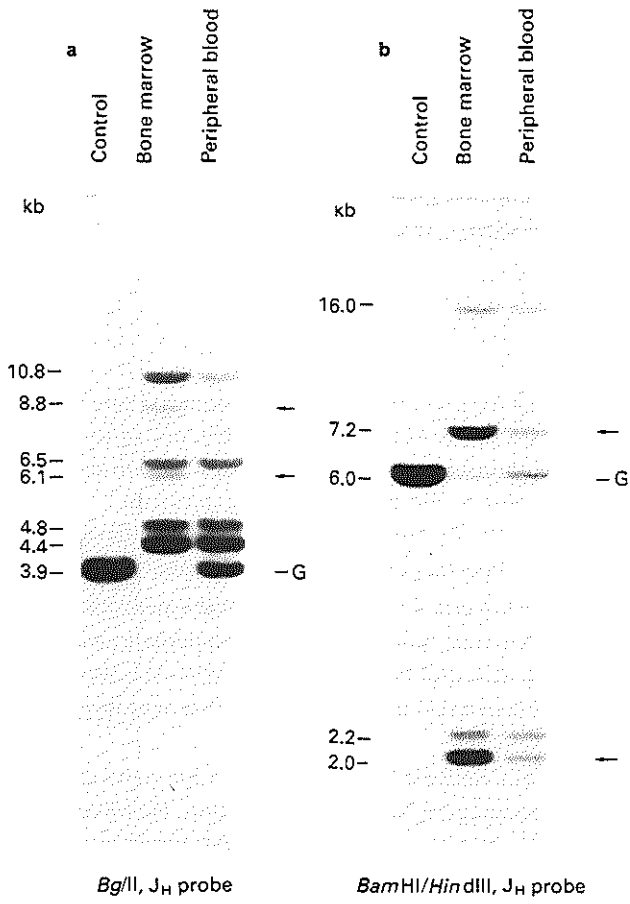

Figure 1. Southern blot analysis of IgH gene rearrangements in bone marrow (BM) and peripheral blood (PB) samples from two precursor B-ALL patients at diagnosis. Control DNA and DNA from the leukemic cell samples were digested with *Bgl*II (**A**) or a combination of *Bam*HI and *Hin*dIII (**B**), size separated and blotted onto nylon membrane filters which were hybridized with the ^{32}P-labeled J_H probe. The sizes (in kb) of the germline bands (G) and rearranged bands are indicated. **A:** In both BM and PB lanes four clearly rearranged bands are visible, while two faint additional bands are visible in the BM lane (arrows). **B:** In both BM and PB lanes four rearranged bands are visible. Two rearranged bands (16.0 and 2.2 kb) have similar densities in the BM lane and to the PB lane, whereas two other rearranged bands (7.2 and 2.0 kb) have an obvious higher density in the BM lane than in the the PB lane (arrows).

between BM and PB in precursor B-ALL at diagnosis will lead to an underestimation of subclone formation if only PB samples are analyzed.

Moreover, variability in the occurrence of subclones with differently rearranged IgH genes will hamper the detection of MRD by use of the PCR-mediated amplification of "leukemia-specific" IgH gene junctional regions (27-29), as it is unpredictable which subclone will cause MRD and/or relapse (30).

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CHAPTER 3.4

CROSS-LINEAGE T-CELL RECEPTOR GENE REARRANGEMENTS IN CHILDHOOD PRECURSOR B-ACUTE LYMPHOBLASTIC LEUKEMIA*

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ABSTRACT

A large series of 116 precursor B-cell acute lymphoblastic leukemia (ALL) patients was analyzed for cross-lineage rearrangements and/or deletions in the T-cell receptor (TcR)-β, TcR-γ, and TcR-δ genes. In 108 cases all three TcR genes were analyzed. In 94% (101/108) of the precursor B-ALL patients one or more genes were rearranged. TcR-β gene rearrangements were found in 36% (40/110), TcR-γ gene rearrangements in 57% (62/108), TcR-δ gene rearrangements in 50% (58/116), and deletions in 41% (47/116) of the cases.

TcR-β gene rearrangements were monoallelic complete V(D)Jβ gene rearrangements in 54% of cases or incomplete Dβ-Jβ2 gene rearrangements in 37% of cases. TcR-γ genes most frequently (78%) contained monoallelic rearrangements to Jγ1 gene segments and at least 80% of TcR-δ gene rearrangements represented incomplete Vδ2-Dδ3 or Dδ2-Dδ3 gene rearrangements.

Identification of TcR gene rearrangements is needed for subsequent polymerase chain reaction (PCR) analysis of TcR gene junctional regions for detection of minimal residual disease (MRD) during follow-up of ALL patients. In precursor B-ALL this especially concerns TcR-γ and TcR-δ gene rearrangements. In at least 78% of precursor B-ALL the junctional region of these TcR gene rearrangements can be identified and used as target for PCR-mediated MRD detection.

INTRODUCTION

The enormous diversity of immunoglobulin (Ig) and TcR molecules is generated during B- and T-cell differentiation by a series of ordered rearrangements of variable (V), diversity (D), and joining (J) gene segments (1,2). The germline configuration of the various TcR

* Submitted for publication.

genes (TcR-α, TcR-β, TcR-γ, and TcR-δ) is shown in Figure 1. Ig and TcR gene rearrangements are different in each lymphoid cell, but identical in each clone of lymphoid cells. Analysis of Ig and TcR genes has become an important tool for determining clonality in lymphoid malignancies. In addition, immunophenotyping and immunogenotyping studies have provided evidence for the idea that leukemias and malignant lymphomas represent clonal expansions of hematopoietic (precursor) cells, arrested at distinct stages of maturation (3-8). Initially, Ig and TcR gene rearrangements were regarded as B-lineage and T-lineage specific markers, respectively (4-6). However, the finding of cross-lineage expression of "lineage-specific" immunological markers and cross-lineage Ig and TcR gene rearrangements has disputed this view (9-11).

Cross-lineage rearrangements of Ig heavy-chain (IgH) genes have been observed in 10-15% of T-ALL and in ~ 5% of mature T-cell leukemias and lymphomas (12-14), but cross-lineage rearrangements of Ig light-chain (IgL) genes in malignant T-cell proliferations are extremely rare (<1%) (14,15). On the other hand TcR-β, TcR-γ, and TcR-δ gene rearrangements and/or deletions have been found in ~30%, ~50%, and ~80% of precursor B-ALL, respectively (13,14,16-46). Cross-lineage TcR gene rearrangements in chronic B-cell leukemias and B-cell lymphomas are found less frequently (<5%) (14,20,25,31,36,38,47-54). Most TcR gene rearrangements in precursor B-ALL are monoallelic and incomplete (14,17,22,23,30,31,38,40,41,54-59). Furthermore, transcription of rearranged TcR-β or TcR-γ genes in precursor B-ALL is rare (1-5%) (18,23,25,34,35,60). In the far majority of precursor B-ALL TcR-β gene rearrangements

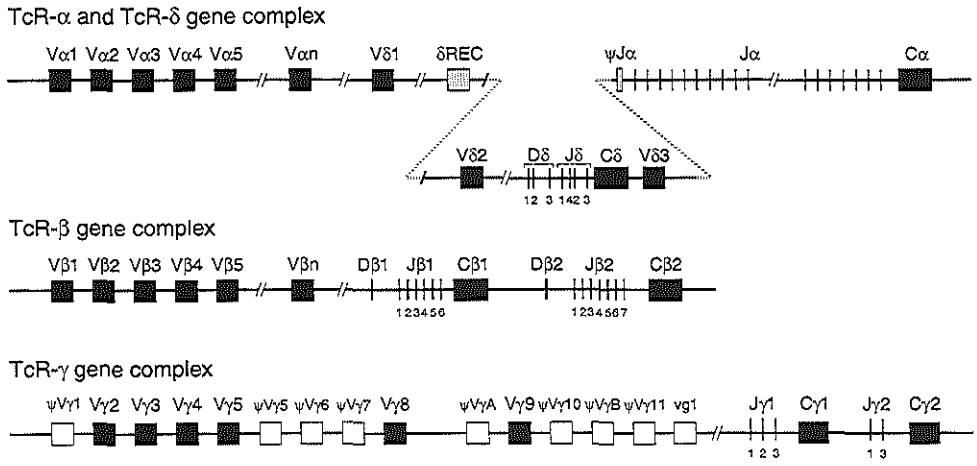

Figure 1. Schematic diagram of human TcR genes. The TcR-α gene complex consists of >50 V gene segments, a remarkably long stretch of ~55 functional J gene segments, and one C gene segment. The TcR-β gene complex consists of >70 V gene segments and two C gene segments, both of which are preceded by one D and six or seven J gene segments. The TcR-γ gene complex consists of a restricted number of V gene segments (six functional V gene segments and nine pseudo genes) and two C gene segments, each preceded by two or three J gene segments. The major part of the TcR-δ gene complex is located between the Vα and Jα gene segments and consists of six V, three D, and four J gene segments and one C gene segment. The δREC and ψJα gene segments play a role in TcR-δ gene deletions, which precede TcR-α gene rearrangements. Pseudo genes (ψ) are indicated with open symbols. Recently, a new functional Jδ gene segment (Jδ4) is found and located between the Jδ1 and Jδ2 gene segments (81,82).

occur in the TcR-Jβ2 locus and TcR-γ gene rearrangements occur in the TcR-Jγ1 locus (14,17,22,23,31,40,55,57), whereas in T-ALL the TcR-β1 and TcR-β2 loci are used in comparable frequencies and the TcR-γ2 locus is used in the majority of TcR-γ gene rearrangements (14,23,31,40). In precursor B-ALL the far majority of TcR-δ gene rearrangements are incomplete Vδ2-Dδ2 and Dδ2-Dδ3 rearrangements, whereas in T-ALL complete Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements dominate (41,42,54,56,58,59,61,62). Moreover, cross-lineage TcR gene rearrangements in precursor B-ALL seem to occur in the same hierarchical order as found during normal T-cell differentiation (first, TcR-δ gene rearrangements, followed by TcR-γ gene rearrangements, and subsequently TcR-β gene rearrangements). IgL gene rearrangements and (Vδ-)Dδ-Jδ rearrangements seem to be more lineage-specific, because they are rarely found in T-lineage or B-lineage cells, respectively. Apparently, the final steps in Ig and TcR gene rearrangements are more tightly controlled in a lineage-specific way. The occurrence of cross-lineage rearrangements has been explained by the finding that B- and T-cells use a common recombinase system for gene rearrangement (63), but this does not explain the difference in frequency of cross-lineage rearrangements between immature and mature leukemias and the occurrence of Ig and TcR gene rearrangements in acute myeloid leukemias (14,64).

Junctional regions of rearranged Ig and TcR genes in leukemias and lymphomas can be regarded as 'tumor-specific' markers, because they are formed by joining of different V, (D), and J gene segments as well as by deletion and random insertion of nucleotides at the junctions of the gene segments (1,2,65-67). Therefore, each junctional region of rearranged Ig and TcR genes is different in each leukemia and lymphoma. Currently, it has been proven that in precursor B-ALL the PCR can be used for amplification of 'tumor-specific' junctional regions of cross-lineage rearranged TcR-γ and TcR-δ genes to detect MRD (66-75).

The aim of our study was to determine the precise frequency of cross-lineage TcR gene rearrangements in childhood precursor B-ALL, to evaluate the occurrence of preferential TcR gene rearrangements, and to estimate the number of precursor B-ALL patients with identifiable cross-lineage rearrangements, which are therefore suitable targets for PCR-mediated MRD detection. In 116 well-characterized cases of precursor B-ALL the configuration of the TcR-β, TcR-γ, and/or TcR-δ genes was analyzed and compared with almost all previously published childhood precursor B-ALL cases. The detection of rearrangements in the TcR-α locus by Southern blotting is difficult due to the long stretch of Jα gene segments (about 85 kb) (Figure 1). The occurrence of TcR-α gene rearrangements in precursor B-ALL can therefore only be assumed based on TcR-δ gene deletions In this manuscript, when TcR-δ gene rearrangements are described, rearrangements in the TcR-δ/α locus are ment.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) or bone marrow (BM) samples from children with precursor B-ALL (116 cases) were obtained at initial diagnosis. Most cases (n = 99) were diagnosed at the Sophia Children's Hospital, Rotterdam. Their cell samples were collected by the Department of Immunology, Erasmus University Rotterdam. The other 17 children were treated in different Dutch hospitals and their cell samples were collected by the Dutch Childhood Leukemia Study Group (DCLSG), The Hague. The age distribution of the 116 precursor B-ALL was

from 2 months till 16 years and 6 months (median 4 years and 7 months). Seventeen children were younger than 2 years and six of them were infants (age <1 year).

The diagnosis of precursor B-ALL was made according to the FAB classification (76), based on cytomorphology of BM smears stained with May-Grünwald Giemsa and cytochemistry (periodic acid-schiff). Each diagnosis was confirmed by the laboratory of the DCLSG.

Mononuclear cells (MNC) were isolated from PB or BM samples by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). The freshly obtained MNC samples were subjected to a detailed immunological marker analysis according to standard protocols (for detailed description, see references 7,8,77). Remaining MNC were stored in liquid nitrogen. A leukemia was considered to be a precursor B-ALL, if the malignant cells were positive for terminal deoxynucleotidyl transferase (TdT), CD19 and HLA-DR (null ALL), for TdT, CD10, CD19 and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR and cytoplasmic Ig heavy chain μ (Cyl$g\mu$) (pre-B-ALL) (8,78). Immunological marker analysis of the 116 precursor B-ALL revealed that five were null ALL, 80 were common ALL, and 31 were pre-B-ALL.

Southern blot analysis

DNA was isolated from frozen MNC as described previously (79,80). Control DNA was obtained from granulocytes or a non-hematopoietic cell line with germline TcR genes. Fifteen μg of DNA were digested with the appropriate restriction enzymes (Pharmacia). The restriction fragments were size-separated in 0.7% agarose gels and transferred onto Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) (80).

TcR-β gene rearrangements were analyzed with the ^{32}P-random oligonucleotide labeled TCRBD1U (Dβ1 upstream), TCRBD1 (Dβ1 downstream), Jβ1, TCRBD2U (Dβ2 upstream), TCRBD2 (Dβ2 downstream), TCRBJ2 (Jβ2) and TCRBC (constant (C)β) probes in *Eco*RI and/or *Hin*dIII digests and for confirmation in *Bgl*II digests (80 and Breit et al. unpublished results). The configuration of the TcR-γ genes was analyzed by use of the Jγ1.3 and Jγ2.1 probes in *Eco*RI digests and for confirmation in *Kpn*I and/or *Bgl*II digests, and by use of the Jγ1.2 probe in a *Bgl*II digest (80). The configuration of the TcR-δ genes was analyzed by use of TCRDV1 (Vδ1), TCRDV2 (Vδ2), TCRDV3 (Vδ3), TCRDRE (δ_{REC}), TCRDD1 (Dδ1), TCRDD3 (Dδ3), TCRDJ1 (Jδ1), TCRDJ2 (Jδ2), TCRDC4 (Cδ), and TCRAPJ (ψJα) probes in *Eco*RI, *Hin*dIII and *Bgl*II digests (59).

The used combinations of probes and restriction enzymes allow identification of the various types of incomplete (D-Dβ, D-Jβ, V-Dβ) and complete (V(-D)-Jβ) TcR-β gene rearrangements in the TcR-Jβ1 and TcR-Jβ2 locus (80). In addition, they allow the precise identification of rearrangements of the various Vγ gene segments to the Jγ1 (Jγ1.1, Jγ1.2, and Jγ1.3) or Jγ2 (Jγ2.1 and Jγ2.3) gene segments (80). Finally, they allow the detection of the various (preferential) types of TcR-δ gene rearrangements and deletions (59,80).

RESULTS

TcR-β gene rearrangements

DNA samples from 110 precursor B-ALL at diagnosis were examined for the presence of rearranged TcR-β genes. In the total group of precursor B-ALL we found in 36% (40/110) of cases a rearranged TcR-β gene on one or both alleles (Table 1). In all five null ALL no TcR-β gene rearrangements were detected. In common ALL and pre-B-ALL, TcR-β gene rearrangements were detected in 42% (31/74) and 29% (9/31), respectively (Table 1). The allelic frequencies of TcR-β gene rearrangements are summarized in Table 2. Germline configuration was found in 74% (163/220) of TcR-β alleles. The upstream and downstream Dβ probes allowed the detection of the incomplete V-Dβ, D-Dβ, and D-Jβ gene rearrangements (Figure 2A). No rearrangements were detected with the TCRBD1 and Jβ1 probes; only rearrangements in the TcR-β2 locus were detected. The majority concerned complete V(D)Jβ2 rearrangements (54%) or incomplete Dβ-Jβ2 rearrangements (37%) (Table 2).

TABLE 1. Frequencies in precursor B-ALL of cross-lineage TcR gene rearrangements[a].

		TcR gene rearrangements and/or deletions			
				TcR-δ	
precursor B-ALL	TcR (G/G)	TcR-β (R/G or R/R)	TcR-γ (R/G or R/R)	(R/G, R/R or D/R)	(D/G or D/D)
null ALL	20% (1/5)	0% (0/5)	20% (1/5)	40% (2/5)	20% (1/5)
common ALL	3% (2/72)	42% (31/74)	61% (44/72)	53% (42/80)	41% (33/80)
pre-B-ALL	13% (4/31)	29% (9/31)	55% (17/31)	45% (14/31)	42% (13/31)
total group of precursor B-ALL	6% (7/108)	36% (40/110)	57% (62/108)	50% (58/116)	41% (47/116)

Symbols used: R, rearranged allele; G, allele in germline configuration; D, deletion of the Cδ gene segment.
a. Number of patients with germline or rearranged cross-lineage TcR genes per total group of precursor B-ALL patients in parentheses.

TABLE 2. Allelic frequencies of TcR-β gene rearrangements in precursor B-ALL.

TcR-β gene configuration	null ALL (n=10)	common ALL (n=148)	pre-B-ALL (n=62)	total group of precursor B-ALL (n=220)
Germline configuration	100% (10)	70% (103)	81% (50)	74% (163)
Rearrangement	0% (0)	30% (45)	19% (12)	26% (57)
Rearrangements[a]				
Dβ1-Jβ1 Vβ-Dβ1 Vβ(-Dβ)-Jβ1	0% (0)	0% (0/45)	0% (0/12)	0% (0/57)
Dβ1-Dβ2	0% (0)	4% (2/45)	0% (0/12)	4% (2/57)
Dβ1-Jβ2	0% (0)	9% (4/45)	8% (1/12)	9% (5/57)
Dβ2-Jβ2	0% (0)	31% (14/45)	17% (2/12)	28% (16/57)
Vβ-Dβ2	0% (0)	2% (1/45)	17% (2/12)	5% (3/57)
Vβ-(Dβ)-Jβ2[b]	0% (0)	53% (24/45)	58% (7/12)	54% (31/57)

a. In our group of precursor B-ALL no TcR-β1 gene rearrangement were detected.
b. Southern blot analysis does not allow identification of the Dβ gene segment(s) in complete Vβ(-Dβ)-Jβ2 gene rearrangements.

TcR-γ gene rearrangements

DNA samples from 108 precursor B-ALL at diagnosis were examined for the presence of rearranged TcR-γ genes. In the total group of precursor B-ALL we found TcR-γ gene

rearrangements on one or both alleles in 57% (62/108) of cases (Table 1). The distribution of the TcR-γ gene rearrangements in the three precursor B-ALL subgroups was: 20% (1/5) in null ALL, 61% (44/72) in common ALL, and 55% (17/31) in pre-B-ALL (Table 1). The allelic frequencies of TcR-γ gene rearrangements are summarized in Table 3. TcR-γ genes in germline configuration were found in 50% of alleles. The majority (79%; 84/107) of TcR-γ gene rearrangements occurred to Jγ1 gene segments (Figure 2B). In 60% of these Jγ1 gene rearrangements a V gene segment of the Vγl family was used. Strikingly, the Jγ1 gene rearrangements in pre-B-ALL occurred to the Vγll (Vγ9) gene segment in 57% of cases, whereas in common ALL Vγ9-Jγ1 gene rearrangements were found in only 22% of cases. The only TcR-γ gene rearrangements in null ALL concerned biallelic VγlV-Jγ1 gene rearrangements. The majority of Jγ2 gene rearrangements (91%) involved V gene segments of the Vγl family (Table 3 and Figure 2B).

TcR-δ gene rearrangements

DNA samples from 116 precursor B-ALL were analyzed for the presence of TcR-δ gene rearrangements and/or deletions. In the total group of precursor B-ALL patients TcR-δ gene rearrangements were found in 50% (58/116) and TcR-δ gene deletions in 41% (47/116), equally distributed over the three different precursor B-ALL subgroups (Table 1). The allelic

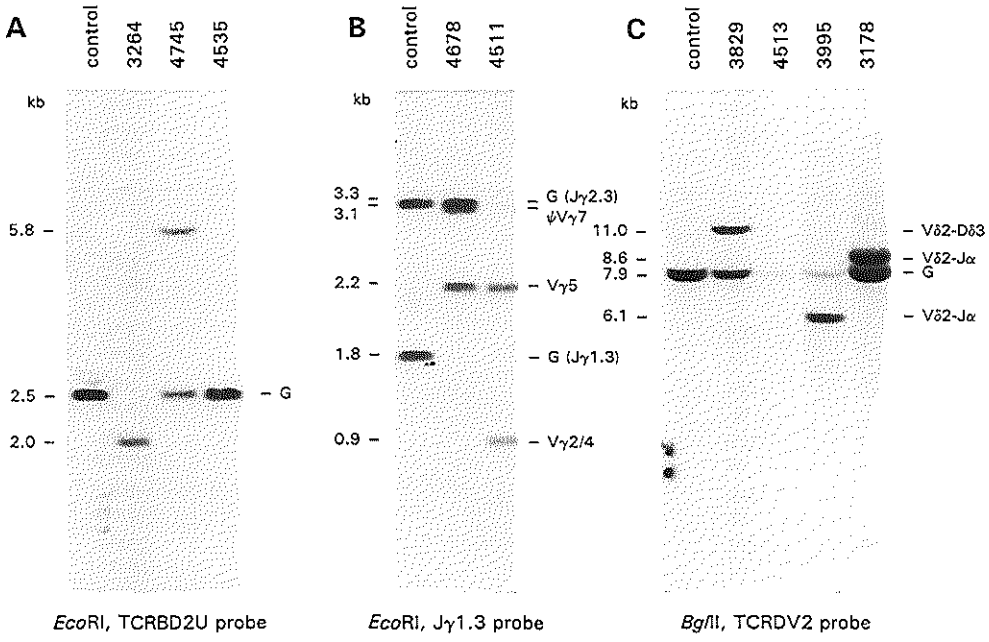

Figure 2. Southern blot analysis of TcR genes in several precursor B-ALL patients. Control DNA and DNA from precursor B-ALL patients were digested with the appropriate restriction enzymes, size-separated, and blotted onto nylon membrane filters, which were hybridized with ^{32}P-labeled probes. (A) TcR-β gene analyses using *Eco*RI with the TCRBD2U probe. (B) TcR-γ gene analyses using *Eco*RI with the Jγ1.3 probe. (C) TcR-δ gene analyses using *Bgl*II with the TCRDV2 probe. The germline bands (G) and several TcR gene rearrangements in the TcR-δ/α locus are indicated.

TABLE 3. Allelic frequencies of TcR-γ gene rearrangements in precursor B-ALL.

TcR-γ gene configuration[a]	null ALL (n=10)	common ALL (n=144)	pre-B-ALL (n=62)	total group of precursor B-ALL (n=216)
Germline configuration	80% (8)	46% (66)	56% (35)	50% (109)
Jγ1 gene rearrangements	20% (2)	41% (59)	37% (23)	39% (84)
Jγ2 gene rearrangements	0% (0)	13% (19)	6% (4)	11% (23)
Jγ1 gene rearrangements				
Vγ I	0% (0/10)	71% (42/59)	35% (8/23)	60% (50/84)
Vγ II	0% (0/10)	22% (13/59)	57% (13/23)	31% (26/84)
Vγ III	0% (0/10)	0% (0/59)	4% (1/23)	1% (1/84)
Vγ IV	20% (2/10)	7% (4/59)	4% (1/23)	6% (5/84)
Jγ2 gene rearrangements				
Vγ I	0% (0)	95% (18/19)	75% (3/4)	91% (21/23)
Vγ II	0% (0)	0% (0/19)	0% (0/4)	0% (0/23)
Vγ III	0% (0)	0% (0/19)	0% (0/4)	0% (0/23)
Vγ IV	0% (0)	5% (1/19)	25% (1/4)	9% (2/23)

a. The Jγ1 locus consists of Jγ1.1, Jγ1.2, and Jγ1.3 gene segments and the Jγ2 of Jγ2.1 and Jγ2.3 gene segments. The Vγ I family consists of Vγ1, Vγ2, Vγ3, Vγ4, Vγ5, ψVγ5, Vγ6, ψVγ7, and Vγ8 gene segments; the Vγ II, Vγ III, and Vγ IV families contain the Vγ9, ψVγ10, and ψVγ11 gene segments, respectively. The VγA, VγB and Vγ12 (Vg1) gene segments are pseudogenes and no rearrangement of these three Vγ gene segments was found in our series of TcR-γ gene rearrangements.

frequencies of TcR-δ gene rearrangements and deletions are summarized in Table 4. TcR-δ genes in germline configuration were found in 22% (51/232) of alleles. In 72% of rearranged alleles a Vδ2-Dδ3 rearrangement and in 9% a Dδ2-Dδ3 rearrangement was found (Figure 2C). The remaining TcR-δ gene rearrangements (in which the Cδ gene segment was still present) were all detected by use of the Jδ1 probe, but could not be identified precisely. They are probably caused either by rearrangements of a Vα gene segment to the Dδ3-Jδ1 region or by translocations or other chromosomal aberrations in the TcR-δ locus (59). Indications of TcR-δ gene rearrangements to the recently discovered Jδ4 gene segment, located between the Jδ1 and Jδ2 gene segments were not found (Figure 1) (81,82). Based on rearrangements of Vδ and Dδ gene segments in the absence of the Cδ gene region, we concluded that in 36% (36/102) of the TcR-δ gene deletions a Vδ2 or Dδ2 gene segment was probably rearranged to a Jα gene segment (Figure 2C). Based on our extensive TcR-δ Southern blot analyses, we assumed that in the majority of TcR-δ gene deletions a Vα-Jα gene rearrangement had occurred, because upon hybridization

TABLE 4. Allelic frequencies of TcR-δ gene rearrangements or deletions in precursor B-ALL.

TcR-δ gene configuration	null ALL (n=10)	common ALL (n=160)	pre-B-ALL (n=62)	total group of precursor B-ALL (n=232)
Germline configuration	60% (6)	19% (31)	23% (14)	22% (51)
TcR-δ gene rearrangements	20% (2)	36% (58)	31% (19)	34% (79)
TcR-δ gene[a] deletions	20% (2)	44% (71)	47% (29)	44% (102)
TcR-δ gene rearrangements				
Vδ2-Dδ3	100% (2/2)	71% (41/58)	74% (14/19)	72% (57/79)
Dδ2-Dδ3	0% (0/2)	10% (6/58)	5% (1/19)	9% (7/79)
other[b]	0% (0/2)	19% (11/58)	21% (4/19)	19% (15/79)

a. Based on rearrangements of Vδ2 or Dδ2 gene segments in the absence of the Cδ gene region, we assumed that in these TcR-δ gene deletions (35%; 36/102) a Vδ2 or Dδ2 gene segment was rearranged to a Jα gene segment. A Vα-Jα rearrangement was assumed in 60% (61/102) based on the fact that no hybridization signal was obtained when all TcR-δ and ψJα probes were used for hybridization, except for germline hybridization signals with Vδ or δREC probes in some cases. Translocations or other chromosomal aberrations occuring in the TcR-δ/α locus could not be ecxluded.

b. Rearranged TcR-δ bands (in which the Cδ locus is present) probably caused either by rearrangements of Vα gene segments to the Dδ3-Jδ1 locus or by translocations or other chromosomal aberrations in the TcR-δ locus.

with all TcR-δ and ψJα probes no hybridization signal due to deletion of the invoved TcR-δ and ψJα gene segments was observed, except for germline hybridization signals with Vδ or δREC probes in some cases. Still, translocations or other chromosomal aberrations occurring in the TcR-α/δ locus cannot be excluded. In four precursor B-ALL patients (five alleles) an unique hybridization pattern was found in which Vδ, δREC, and Dδ1 probes resulted in germline hybridization signals, whereas all other TcR-δ probes and the ψJα probe showed no hybridation signal due to the deletion of these DNA gene segments. In these precursor B-ALL cases, it cannot be excluded that potential targets for rearrangements are present in the Dδ2-Dδ3 region or that chromosome aberrations in the TcR-δ/α locus are present.

Combinations of cross-lineage TcR gene rearrangements

The different combinations of cross-lineage TcR gene rearrangements in our total group of precursor B-ALL are summarized in Table 5. In the five null ALL no combinations of cross-lineage TcR gene rearrangements were found, whereas in common ALL 35% of cases had all three TcR genes rearranged or deleted. Twenty-nine percent of precursor B-ALL cases showed TcR-δ rearrangements and/or deletions, with germline TcR-β and TcR-γ genes. In contrast, TcR-β and/or TcR-γ gene rearrangements without TcR-δ gene rearrangements and/or deletions were observed in only 3% of cases. Similarly, 28% of precursor B-ALL cases had a rearranged TcR-γ gene with a germline TcR-β gene, while only 8% showed TcR-β gene rearrangements without TcR-γ gene rearrangements. These data indicate that TcR-δ gene rearrangements and/or deletions occur most frequently and that virtually all TcR-β and TcR-γ rearrangements coincide with TcR-δ gene rearrangements and/or deletions.

TABLE 5. Combination of TcR gene rearrangements in childhood precursor B-ALL from our data and data reviewed from the literature.

References	Cross-lineage TcR gene rearrangements and/or deletions							
	G	β	γ	δ	$\beta + \gamma$	$\beta + \delta$	$\gamma + \delta$	$\beta + \gamma + \delta$
Hara,1988 (30) (n=29)	10% (3)	0% (0)	0% (0)	34% (10)	0% (0)	3% (1)	24% (7)	28% (8)
Dyer,1989 (13) (n=12)	0% (0)	0% (0)	0% (0)	25% (3)	0% (0)	8% (1)	17% (2)	50% (6)
Felix,1990 (39) (n=52)	15% (8)	4% (2)	0% (0)	25% (13)	0% (0)	6% (3)	29% (15)	21% (11)
Fey,1990 (40) (n=7)	0% (0)	0% (0)	0% (0)	43% (3)	0% (0)	14% (1)	29% (2)	14% (1)
This study (n=108)	6% (7)	2% (2)	1% (1)	29% (31)	0% (0)	6% (6)	27% (29)	30% (32)
total group of precursor B-ALL (n=208)	9% (18)	2% (4)	<1% (1)	29% (60)	0% (0)	6% (12)	26% (55)	28% (58)

Symbols used: G, germline configuration; β, TcR-β gene rearrangement; γ, TcR-γ gene rearrangement; δ, TcR-δ gene rearrangement and/or deletion.

DISCUSSION

A large group of 116 precursor B-ALL patients was analyzed for the configuration of their cross-lineage TcR gene rearrangements. Rearrangements and/or deletions of the TcR-β, TcR-γ, and TcR-δ genes were found in 36%, 57%, and 91%, respectively. Since the first reports on cross-lineage TcR gene rearrangements in precursor B-ALL (16-20), the configuration of TcR-β, TcR-γ, and TcR-δ genes of a large group of precursor B-ALL patients has been published (13,14,21-46). To determine the frequency of rearrangements and deletions of the various cross-lineage TcR genes in childhood precursor B-ALL we reviewed the reports, which indicated that childhood cases were analyzed, or the reports which were performed in collaboration with a children's hospital (13,17,19,21,24,28,30,32-35,38-44). It was found that TcR-β gene rearrangements occur in 33% of precursor B-ALL, TcR-γ gene rearrangements in 44%, and TcR-δ gene rearrangements and deletions in 57% and 26%, respectively (Table 6). These percentages of TcR gene rearrangements and/or deletions are lower than in our series of childhood precursor B-ALL (Table 1). This discrepancy is probably due to our larger panels of TcR DNA probes (especially for the TcR-β and TcR-δ genes) and restriction enzymes in comparison with the published studies. Ig and TcR gene rearrangements are easily missed when only one DNA probe is used in combination with a restriction enzyme which results in large germline restriction fragments (59,80,83,84).

The combination of our TcR gene rearrangements in childhood precursor B-ALL and those reviewed from the literature are summarized in Table 5. Only four research groups analyzed and compared all three cross-lineage TcR genes in a total number of 100 cases (13,30,39,40). Theirs and our combined data show that TcR-δ gene rearrangements and/or deletions occurred in the majority of cases (~90%) and that 29% (60/208) of childhood

TABLE 6. Summary of TcR gene rearrangements in childhood precursor B-ALL reviewed from the literature[a].

	TcR gene rearrangements and/or deletions				
			TcR-δ		
precursor B-ALL	TcR-β (R/G or R/R)	TcR-γ (R/G or R/R)	(R/G, R/R or D/R)	(D/G or D/D)	references
null ALL	19% (11/62)	25% (16/64)	36% (9/25)	36% (9/25)	13,21,24-26, 33,35,38,41
common ALL	39% (49/125)	51% (72/140)	36% (5/14)	64% (9/14)	13,21,24,26, 32,35,40
pre-B-ALL	26% (11/42)	38% (15/40)	100% (4/4)	0% (0/4)	13,21,24,35,40
$CD10^+$ precursor B-ALL[b]	34% (94/273)	48% (142/294)	56% (53/94)	32% (30/94)	13,21,24-26,32, 33,35,38,40,41
total group of precursor B-ALL[c]	33% (151/462)	44% (207/470)	57% (143/253)	26% (66/253)	13,19,21,24-26,28,30, 32,33,35,38-41,43,44

Symbols used: R, rearranged allele; G, allele in germline configuration; D, deletion of the Cδ gene segment.

a. The data about TcR gene rearrangements in childhood precursor B-ALL are derived from reports, which indicated that only childhood cases were studied or which were performed in collaboration with a children's hospital (13,17,19,21,24-28,30,32-35,38-44). Several research groups have published data on TcR gene rearrangements in precursor B-ALL more than once. To avoid double counting, we have selected the most informative reports from each research group: the data from refs 24 and 30 were used, while data from ref. 34 were excluded; the data from refs. 17 and 21 were combined; data from refs. 28 and 39 were used, while data from ref. 27 were excluded.

b. This patient group contains all published childhood $CD10^+$ precursor B-ALL cases, including the above mentioned common ALL and pre-B-ALL cases.

c. This patient group contains all published childhood precursor B-ALL cases, including the above mentioned precursor B-ALL cases.

precursor B-ALL had TcR-δ gene rearrangements and/or deletions with germline TcR-γ and TcR-β genes (Table 5). In contrast, TcR-γ and/or TcR-β gene rearrangements without TcR-δ gene rearrangements and/or deletions were found in only 2% (5/208) of cases (Table 5). Similarly, 27% (56/208) of precursor B-ALL cases had rearranged TcR-γ genes with germline TcR-β genes, while only 8% (16/208) showed TcR-β gene rearrangements without TcR-γ gene rearrangements (Table 5). Apparently in precursor B-ALL, rearrangements in the TcR-δ/α locus occur first, followed by TcR-γ gene rearrangements, and subsequently by TcR-β gene rearrangements.

The frequencies of cross-lineage TcR gene rearrangements seem to be related to the maturation stages of B-cells. The frequency of TcR gene rearrangements is lower in immature precursor B-ALL (null ALL) as compared to the $CD10^+$ precursor B-ALL (common ALL and pre-B-ALL) (see Table 1 and 6) and no combinations of cross-lineage TcR gene rearrangements were found in null ALL. Unfortunately, conclusions are difficult to draw because only five null ALL were analyzed. When comparing our common ALL and pre-B-ALL subgroups, it is striking that in the "mature" ($Cy Ig\mu^+$) pre-B-ALL group a higher frequency of patients have all TcR genes in germline configuration (13% in pre-B-ALL versus 3% in common ALL). Cross-lineage TcR gene rearrangements are rare (<5%) in mature B-cell malignancies, such as chronic leukemias and lymphomas (14,20,25,31,36,38,47-54). These combined data suggest that the occurrence of cross-lineage TcR gene rearrangements is dependent on the activity of the V(D)J recombinase in the malignant cells. Apparently, in immature B-cells (null ALL) and more mature B-cells (pre-B-ALL, chronic B-cell leukemias, and B-cell lymphomas) V(D)J recombinase is less active, or the mature B-

cell malignancies have already passed the lineage commitment point so that TcR gene rearrangements are no longer possible.

The occurrence of cross-lineage TcR gene rearrangements in precursor B-ALL has been explained by the assumption that B- and T-cells use a common recombinase complex for gene rearrangements (63). It has been suggested that the cross-lineage TcR gene rearrangements in precursor B-ALL are related to a high activity of the recombinase enzymes in B-cell precursors (85,86). However, expression of the recombination activating genes (RAG1 and RAG2) is not higher in precursor B-ALL with cross-lineage TcR gene rearrangements as compared to precursor B-ALL without cross-lineage TcR gene rearrangements (87). A second explanation could be that TcR gene rearrangements only occur in early precursor cells and that the occurrence of TcR gene rearrangements in precursor B-cells stops the differentiation and maturation of these cells, because in normal precursor B-cells no cross-lineage TcR gene rearrangements were found (88), and they are rare in mature B-cell malignancies (<5%) (14). One could speculate that in case of precursor B-cells in the BM, as in case of cortical thymocytes in the thymus, a large fraction of proliferating, immature cells with cross-lineage gene rearrangements survive only when they become neoplastic (11). However, this would implicate that oncogenic transformation preferentially affects precursor B-cells with cross-lineage TcR gene rearrangements. A third explanation could be that cross-lineage TcR gene rearrangements in precursor B-ALL represent a genetic deregulation due to the continuing activity of the recombinase system after malignant transformation and the maturation arrest (9,10). We prefer the latter explanation, because this would explain the virtual absence of cross-lineage TcR gene rearrangements in normal precursor B-cells and in mature B-cell malignancies.

Several laboratories have published details about the different types of TcR gene rearrangements in precursor B-ALL(14,17,22,23,30,31,38,40-42,54-59). It appears that in precursor B-ALL most TcR-β gene rearrangements are monoallelic complete V(D)Jβ2 rearrangements, TcR-γ genes most frequently (78%) contain monoallelic rearrangements to the Jγ1 gene segments, and at least 80% of TcR-δ gene rearrangements represent incomplete Vδ2-Dδ3 or Dδ2-Dδ3 rearrangements (14,17,22,23,30,31,38,40-42,54-59). In general, our data confirm these findings, but we found a significantly higher frequency of incomplete TcR-β gene rearrangements (46% of all TcR-β gene rearrangements) (Table 2). This is probably due to our usage of four different Dβ probes, which allow identification of incomplete V-Dβ, D-Dβ, and D-Jβ gene rearrangements.

Due to their enormous diversity, junctional regions of rearranged Ig and TcR genes represent ideal PCR targets for the detection of MRD (65-67). In precursor B-ALL this especially concerns junctional regions of IgH, TcR-γ and TcR-δ genes. For this purpose the precise configuration of the gene rearrangements (i.e. the V,(D), and J gene segments) has to be identified at diagnosis. We determined the frequency of precursor B-ALL, which has an identifiable type of TcR-γ (57%) and/or TcR-δ (43%) gene rearrangement and found that in at least 78% (83/108) of precursor B-ALL one or more rearranged TcR-γ and/or TcR-δ genes can be used as targets for PCR-mediated MRD detection. The stability of these leukemia-specific TcR gene junctional regions was reasonably high as found by our study on 40 childhood ALL patients at diagnosis and relapse, which showed that in at least 75% of rearranged TcR-γ and/or TcR-δ alleles the rearrangement remained stable (89).

Our study indicates that cross-lineage TcR gene rearrangements and/or deletions in

childhood precursor B-ALL occur in high frequency, in a hierarchical order, and are incomplete in a large part of cases (TcR-β and TcR-δ genes). The occurrence of these cross-lineage TcR gene rearrangements in precursor B-ALL might be explained by a genetic deregulation due to the continuing activity of the recombinase system after malignant transformation. In at least 78% of precursor B-ALL these TcR gene rearrangements can be identified and used as targets for PCR-mediated MRD detection.

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PHENOTYPIC AND (IMMUNO)GENOTYPIC CHANGES IN ALL AT RELAPSE

CHAPTER 4.1

CHARACTERIZATION OF ACUTE LYMPHOBLASTIC LEUKEMIA AT DIAGNOSIS AND RELAPSE

Chemotherapy is highly effective in childhood acute lymphoblastic leukemia (ALL) (1). Nevertheless, 20-30% of children with ALL eventually develop a bone marrow (BM) and/or extramedullary relapse (1,2). As indicated in the General Introduction (section 1.6), there is still no adequate explanation of the biological basis for the failure of treatment in these patients. Apparently, a minority of leukemic cells are able to escape destruction in the present treatment protocols. This may be due to selection of subclones which have a different biological behaviour or due to development of resistance to the applied cytostatic drugs (see also Chapter 3). During the last two decades various studies, analysing data at diagnosis and relapse, have been performed to obtain insight into the mechanisms that influence the occurrence of relapse in ALL. These studies concern: comparative glucose-6-phosphate dehydrogenase and glucocorticoid receptor analyses (3,4), detection of morphological (5-7), immunophenotypic (8-10), and karyotypic shifts at relapse (11-19), comparative studies on immunoglobulin (Ig) idiotype (20), DNA-fingerprints (21), oncogene amplification or mutation (22,23), and analysis of Ig and T-cell receptor (TcR) genes at diagnosis and relapse (24-36).

The results of these comparative studies (especially analysis of immunophenotypic, karyotypic, and immunogenotypic shifts) have direct consequences for the detection of minimal residual disease (MRD) during and after therapy. Detection of MRD by several methods (e.g. immunological marker analysis and polymerase chain reaction (PCR) analysis) is not only important for analysing the cytoreduction of ALL cells caused by the applied therapy protocol, but also for early detection of relapse.

Comparative karyotypic analysis at diagnosis and relapse in 246 childhood ALL patients published in the literature showed in at least 33% (81/246) karyotypic shifts at relapse (Table 1; A→A$^+$ and A→B) (11,12,14,15,17,18). In 95% (77/81) of these shifts additional structural changes were found proving the clonal relation between the clones at diagnosis and relapse (Table 1). In a minority (5%; 4/81) of all studied childhood ALL cases, no clonal relation was found between karyotypes at diagnosis and relapse (Table 1). Karyotypic shifts from normal to abnormal or from abnormal to normal were not included in these calculations, because, as indicated in the General Introduction (section 1.3), finding of a normal karyotype at diagnosis or relapse does not always exclude chromosomal abnormalities. First, technical failures or bad leukemic metaphases may interfere with identification of the abnormal clone and second, a low percentage (25-50%) of lymphoblasts present in the BM of patients in relapse might influence the rate of identification of the leukemic clone.

Two studies reported only data of a complete change in karyotype at relapse (13,19). Cytogenetic evidence for a complete change in karyotype at relapse in 166 childhood ALL

TABLE 1. Cytogenetic findings of 246 childhood ALL at diagnosis and relapse as published in the literature.

number of ALL patients	Cytogenetic findings: no changes N→N	no changes A→A	changes N→A	changes A→N	changes A→A+	changes A→B	references
51	3	8	6	4	28	2	11
12	–	–	4	–	8	–	12
21	5	5	1	2	8	–	14
21	2	3	3	–	13	–	15
25	2	6	2	2	12	1	17
116	43	35	17	12	8	1	18
246	55	57	33	20	77	4	
(%)	(22)	(23)	(13)	(8)	(31)	(2)	

Abbreviations used: N, normal karyotype; A, abnormal karyotype; A+, additional chromosomal abnormalities at relapse; B, complete new abnormal karyotype at relapse.

was found in 10% (16/166) of cases and could be confirmed with changes in immunophenotype in 15 cases (13,19). The occurrence of karyotypic shifts without clonal relation could have important implications for the PCR-mediated MRD detection of leukemia-specific fusion regions of frequently occurring translocations (t(1;19), t(9;22), and t(4;11)) in ALL. In only one case (<1%) of all published childhood ALL cases (n=412), a t(1;19) was lost at relapse (13), confirming the stability of karyotype in ALL and its value for PCR-mediated MRD detection.

In Chapter 4.2, morphological and immunophenotypic analysis of a large group of 40 ALL patients (32 precursor B-ALL and 8 T-ALL) has been performed at diagnosis and relapse. The occurrence of morphological and immunophenotypic shifts will be discussed as well as their consequences for MRD detection with immunological marker analysis. In Chapter 4.3, comparative Ig and TcR gene analysis on DNA samples of 40 ALL patients (30 precursor B-ALL and 10 T-ALL) at diagnosis and relapse are described. Eighteen precursor B-ALL and six T-ALL of this group of patients are also described in Chapter 4.2. The occurrence and possible cause of immunogenotypic changes (clonal evolution) will be discussed, as well as the consequences for the PCR-MRD detection on patient-specific junctional regions of Ig and/or TcR genes.

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CHAPTER 4.2

IMMUNOPHENOTYPIC CHANGES BETWEEN DIAGNOSIS AND RELAPSE IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA*

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ABSTRACT

To get more insight into the phenotypic changes of childhood acute lymphoblastic leukemia (ALL) at presentation and at relapse, a detailed morphological and immunophenotypic study in 40 childhood ALL cases (32 precursor B-ALL and 8 T-ALL) was performed.

Monoclonal antibody (McAb) panels for non-lineage specific markers (terminal deoxynucleotidyl transferase (TdT), CD34, and HLA-DR), B-lineage markers (CD10, CD19, CD20, and CD22), T-lineage markers (CD1, CD2, CD3, CD4, CD5, CD7, and CD8), and cross-lineage myeloid markers (CD14, CD15, and CD33) were compared at diagnosis and at relapse. In cases of low blast counts ($\leq$70%) at relapse, double labeling for membrane markers and TdT were used in order to define the precise immunophenotype of the TdT^+ leukemic cells. An immunological marker-shift was defined as either a conversion from positive to negative and vice versa or a difference in positivity of $\geq$50%.

Morphological differences between diagnosis and relapse were detected in 34% of precursor B-ALL and 12% of T-ALL. Differences in immunological marker expression were found in 69% of precursor B-ALL and in 63% of T-ALL. The morphological shifts and marker-shifts were not correlated.

Marker-shifts were found in all markers tested in precursor B-ALL, except for HLA-DR. A shift in CD10 expression was observed only in relapses occurring 30 months or more after diagnosis. In four precursor B-ALL an intra-lineage shift was found at relapse (one common ALL to null ALL and three pre-B-ALL to common ALL or null ALL) and two precursor B-ALL cases were diagnosed as acute non-lymphocytic leukemia (ANLL) at relapse based on morphology and immunophenotype. In T-ALL, neither intra-lineage nor

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inter-lineage shifts were detected, although shifts were observed in all T-cell markers tested, except for the lineage specific CD3 and T-cell receptor (TcR) markers.

In conclusion, marker-shifts at relapse are frequently found in precursor B-ALL and T-ALL, in a small percentage leading to an intra-lineage shift (10%) or inter-lineage shift (5%). Therefore monitoring for minimal residual disease (MRD) by immunological marker analysis should include the usage of multiple marker combinations, preferentially in combination with polymerase chain reaction (PCR) analysis of rearranged immunoglobulin (Ig) and/or TcR genes.

INTRODUCTION

Although the prognosis of childhood ALL has improved during the last years (1-4), 20-30% of the patients relapse. To obtain insight into the mechanisms which influence the development of a relapse in childhood leukemia and for determining the therapy thereafter, we need to know the phenotypic and genotypic characteristics of the leukemic cells at diagnosis and at relapse.

Several comparative studies on childhood ALL at diagnosis and relapse report morphological changes (5-8) and immunophenotypic changes at relapse (8-12). Major immunophenotypic changes generally accompany a conversion of hematopoietic cell lineage: lymphoid to myeloid or vice versa, a so-called inter-lineage shift (6,13). This inter-lineage shift is either due to multi-lineage properties of the leukemic clone at diagnosis, or due to irradication of the original malignant clone and induction of a second independent neoplasm (14). However, most changes reported in the literature are minor immunophenotypic shifts with loss or acquisition of one or few antigens. In some cases this might lead to a change in immunophenotypic classification, a so-called intra-lineage shift, such as from pre-B-ALL to common ALL.

In precursor B-ALL marker-shifts generally concern loss or acquisition of CD10 antigen (common ALL antigen) (8,9,11,15-17), HLA-DR (8,9,11), TdT (11), or CD20 antigen (16), and sometimes loss of cytoplasmic immunoglobulin heavy-chain μ (Cyl$g\mu$) (12). In T-ALL only few shifts have been reported, which generally concerned CD2 antigen (E rosette receptor) (10) or TdT (12,16). However most reports did not study the expression of all relevant B-cell, T-cell, and myeloid markers at diagnosis and at relapse. Also the comparability of these studies is limited, because of major differences in McAb panels, application of different techniques, and differences in definition of immunophenotypic shifts.

The aim of this study was to get more insight into the phenotypic changes (morphological and immunological) of childhood ALL at presentation and at relapse, because such changes might influence their classification for treatment and might limit the immunophenotypic detection of MRD (12,18,19). Therefore we performed a detailed morphological and immunophenotypic study in 40 childhood ALL cases, i.e. 32 precursor B-ALL and eight T-ALL.

MATERIALS AND METHODS

Patients

In the Netherlands, bone marrow (BM) and peripheral blood (PB) samples of children suspected of having leukemia are send to the laboratory of het Dutch Childhood Leukemia Study Group (DCLSG) for confirmation and classification of diagnosis and relapse. Between 1989 and 1993, 40 relapses were registered including six second or third relapses (33 precursor B-ALL and 7 T-ALL), with adequate BM or PB samples for blast cell immunophenotyping at both diagnosis and relapse by comparable methods. This was 45% of all hematological relapses in childhood ALL in the Netherlands in this period. We added cell samples of 6 additional relapses (3 precursor B-ALL and 3 T-ALL), who relapsed before september 1989 and of whom sufficient mononuclear cells (MNC) from diagnosis and relapse cell samples were stored in liquid nitrogen.

Morphology and Cytochemistry

BM and PB smears at diagnosis and at relapse were stained by standard techniques including periodic acid-Schiff reagens, Sudan Black B, myeloperoxidase (MPO), a combined naphthol AS-D chloroacetate esterase and α-naphthyl acetate esterase. Classification followed French-American-British (FAB) criteria at diagnosis and at relapse.

Immunophenotyping

MNC were isolated from BM, PB, or pleural exudate by Ficoll-Paque centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) for 15 min. (room temperature (RT), 1250 g). The MNC were analysed on cytocentrifuge preparations (Shandon Cytospin II; Life Sciences International Limited, Runcorn, England) for nuclear expression of TdT as well as for intracellulair expression of Cylgμ, CD22 (Leu-14), MPO, and CD3 (Leu-4). Cell membrane immunophenotype was analysed for HLA-DR antigen and CD34 (BI-3C5); the B-cell markers CD9 (BA2), CD10 (VIL-A1), CD19 (B4), CD20 (B1), CD22 (Leu-14), CD24 (BA1); the T-cell markers CD1 (Leu-6), CD2 (T11), CD3 (Leu-4), CD4 (Leu-3a), CD5 (Leu-1), CD7 (3A1), CD8 (Leu-2a), TcR-$\alpha\beta$ (BMA031) and TcR-$\gamma\delta$ (11F2); and the myeloid markers CD14 (My4), CD15 (VIM-D5), CD33 (My9), CDw65 (VIM-2). The rabbit anti-TdT antiserum was purchased from Supertechs (Bethesda, MD, USA); the McAb of the Leu series, anti-HLA-DR, and 11F2 were obtained from Becton Dickinson (San Jose, CA, USA); 3A1 hybridoma was obtained from American Type Culture Collection (Rockville, MD, USA); BMA031 was kindly provided by Dr R. Kurrle (Behring, Marburg, Germany); B4, B1, and the My series were obtained from Coulter Clone (Hialeah, FL, USA); VIL-A1, VIM-2, and VIM-D5 were kindly provided by Dr W. Knapp (Vienna, Austria); BA1 was obtained from Hybritech (San Diego, CA, USA); BA2 and BI-3C5 from Seralab (Crawley Down, England); T11 and anti-MPO from the Central Laboratory of the Blood Transfusion Service (Amsterdam, the Netherlands).

Washing of cells was performed with phosphate buffered saline (PBS; pH 7.8) supplemented with 1% bovine serum albumine (BSA). Cytocentrifuge preparations were made with a cell concentration of 10^6/ml. For labeling of cells in suspension the cell concentration was adjusted to 10^7/ml.

In 20 cases at diagnosis, immunophenotyping was performed by immunoperoxidase stainings on cytocentrifuge preparations, except for detection of TdT and Cylgμ, for which immunofluorescence stainings were used. For all other analyses at diagnosis and relapse we used immunofluorescence stainings of cells in suspension (membrane antigens) or cytocentrifuge preparations (intracellular antigens). The immunofluorescence stainings were analyzed by fluorescence microscopy (Zeiss, Oberkochen, Germany) and/or a FACScan flow cytometer and Lysis-II software (Becton Dickinson).

Some antigens were difficult to detect by use of immunoperoxidase stainings; this especially concerned the CD24, CD33 and CD34 antigens. Therefore, in all cases where an immunophenotypic shift was found which could be due to differences in applied techniques, immunophenotyping was repeated by immunofluorescence stainings. See tables for specification of technique per cell sample.

Immunoperoxidase

Cytocentrifuge preparations were fixed in buffered formaldehyde acetone (pH 7.4; 4°C; 30 sec) rinsed in

water, washed with PBS (pH 7.4), incubated with 50 μl of the relevant McAb (RT; 60 min), washed and incubated with 50 μl peroxidase-conjugated rabbit anti-mouse antiserum with 2% pooled AB human serum added. After washing, staining with diaminobenzidine 0.5 mg/ml and 0.05 M imidazole was performed in 0.05 M Tris-HCl buffer (pH 7.6) and 0.02% H_2O_2 (RT; 10 min), followed by counterstaining with hematoxylin. At least 200 MNC were counted.

Immunofluorescence

50 μl of a cell suspension was incubated (4°C, 30 min) with 50 μl of the relevant McAb, washed, and subsequently incubated with 50 μl fluorescein isothiocyanate (FITC) conjugated goat anti-mouse-Ig antiserum (4°C, 30 min) and washed again. The labelled cell suspension was either mounted in glycerol with 1 mg *p*-phenyleendiamine per ml and analyzed on a fluorescence microscope, or measured and analyzed by use of a FACScan flow cytometer.

TdT detection

Cytocentrifuge preparations were fixed in methanol (4°C, 30 min), washed, incubated with 15 μl rabbit anti-TdT antiserum in a moist chamber (RT; 30 min) washed, subsequently incubated with 15 μl FITC-conjugated goat anti-rabbit-Ig antiserum and washed again. The preparations were mounted in glycerol with 1 mg *p*-phenyleendiamine per ml.

Double marker analyses

For double marker analysis of TdT and membrane markers, MNC in suspension were labeled with the relevant McAb and a tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse Ig antiserum. The labeled cells were spun onto slides, fixed, and subjected to TdT staining. At least 200 TdT positive cells were analyzed for expression of the tested membrane marker (20). Double marker immunophenotyping was done in all relapse cases with less then 70% blasts.

Definitions

A relapse was defined by the treatment protocol as having ≥ 20% blasts. Immunological markers were judged to be positive if expressed in ≥ 20% of the malignant cells. A leukemia was classified as a precursor B-ALL, if the malignant cells were positive for TdT, CD19, and HLA-DR (null ALL), or for TdT, CD10, CD19 and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR and Cylgμ (pre-B-ALL) (20). A T-ALL was defined by positivity for TdT, cytoplasmic CD3 (CyCD3), surface membrane CD3 (SmCD3), and CD7 (20).

An immunophenotypic shift was defined as a conversion from positive to negative and vice versa. Restriction to this rule would exclude major shifts in marker positivity (e.g. from 85% to 25%), because the cut-off point of marker positivity was defined at 20%. Therefore we also defined loss or acquisition of a marker, if the percentage positivity differed ≥50% between diagnosis and relapse. If the blast count at relapse was ≤70%, double marker analysis was used for defining positivity and for defining a shift. An intra-lineage shift was defined as a change in sub-classification within the precursor B-ALL or T-ALL groups (for instance from pre-B-ALL to common ALL). An inter-lineage shift was defined as a change from ALL to ANLL.

RESULTS

Morphological classification

In 11 of 32 precursor B-ALL and in 1 of 8 T-ALL a cytomorphological shift was observed. In 2 of these 12 cases, ALL at diagnosis shifted to ANLL at relapse (Figure 1), whereas the other changes represented shifts from L1 at diagnosis to L2 at relapse (n=5), or vice versa (n=5) (see Tables 1-3).

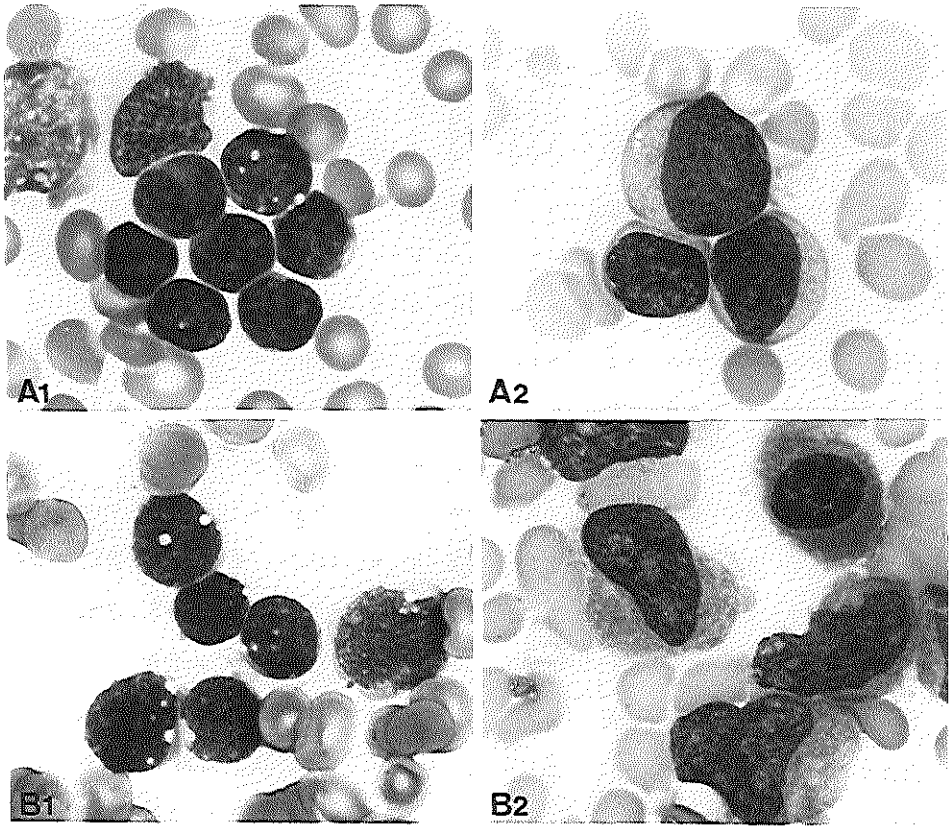

Figure 1. Cytomorphology of BM smears in two precursor B-ALL patients at diagnosis and relapse. **A:** According to the FAB classification precursor B-ALL patient 3797 had a L1 morphology at diagnosis (A1), which changed to M5 at relapse (A2). **B:** Precursor B-ALL patient 4522 had a L1 morphology at diagnosis (B1) and relapsed as a M4 (B2).

Immunological marker analysis of precursor B-ALL at diagnosis and relapse

The 32 precursor B-ALL at diagnosis were classified as null ALL (n = 1), common ALL (n = 21), and pre-B-ALL (n = 10).

In 22 of the 32 precursor B-ALL (69%) marker-shifts were found at relapse: the null ALL, 11 of 21 common ALL, and all 10 pre-B-ALL (Table 1). The remaining ten common ALL retained their original immunophenotype (Table 2). Shifts occured in nearly all tested markers except for HLA-DR. In approximately half of the patients with a marker-shift, this concerned only one marker, but up to five marker-shifts were found in patient 4522 and seven marker-shifts in patient 3797; both patients relapsed as ANLL (Table 1 and Figure 1).

The marker-shifts were found in the pan B-cell markers CD19 (3/32) and CD22 (6/32) as well as in other B-cell markers like CD9 (6/32), CD10 (5/32), and CD20 (5/32) (Table 4). Changes in Cylgμ expression were observed in five cases (5/32) (Table 4). Also non-lineage-restricted markers like TdT and CD34 as well as cross-lineage markers

TABLE 1. Immunophenotype of precursor B-ALL with immunophenotypic shifts[a].

					Non-lineage			B-lineage									T-lineage		Myeloid			
pat nr (sex, age)	D/R (time from diagnosis in months)	method/ cell sample	FAB	BL %	TdT	HLA DR	CD 34	CD 10	CD 19	CD 22 Cy	CD 22 Sm	CD 20	CD 9	CD 24	CyIgμ	SmIgμ	CD 3 Sm	CD 7	CD 14	CD 15	CD 33	Immuno-phenotype
2308	D	FC/PB	L1	86	92	89	0	84	91	–	64	5	85	88	86	3	3	3	0	3	0	pre-B-ALL
(f,8y)	R-2(50)[d]	FC/BM	L1	91	88	94	0	94	87	–	49	0	88	93	93	0	1	1	0	0	0	pre-B-ALL
2717	D	FC/BM	L2	84	98	96	87	91	96	–	2	52	92	94	6	4	3	5	3	2	–	common ALL
(m,1y)	R-3(31)[d]	IF/BM	L2	91	97	97	–	3	74	–	3	79	59	84	0	–	2	1	1	1	–	null ALL
3559	D	IF/BM	L1	99[b]	82	90	–	83	77	–	6	25	69	82	34	1	4	3	1	1	–	pre-B-ALL
(f,10mo)	R-1(55)	IF/BM	L1	75	94	88	0	19	85	–	4	8	77	78	82	2	3	3	1	5	–	pre-B-ALL
3797	D	IF/BM	L1	76	92	56	2	84	82	68	62	66	73	75	3	3	5	4	0	1	12	common ALL
(m,2y)	R?-2(49)[d]	FC/BM	M5	83	0	36	1	3	11	–	5	2	83	7	0	–	12	14	5 (CDw65 66%)	25	66	ANLL
3837	D	FC/BM	L1	90	94	91	85	91	91	65	87	14	91	89	94	1	3	3	3	1	2	pre-B-ALL
(m,8y)	R-1(34)	IF/BM	L2	99	89	85	80	93	89	–	85	6	83	14	88	7	2	6	1	3	–	pre-B-ALL
	R-2(49)	IF/BM	L1	98	93	85	94	97	88	–	71	22	62	1	84	0	1	2	1	3	–	pre-B-ALL
3869	D	FC/BM	L2	58	70	81	76	20	80	58	64	12	66	84	2	4	5	14	8	0	–	common ALL
(m,9y)	R-1(38)	FC/BM	L1	92	97	94	93	88	91	–	36	3	91	95	0	0	0	0	0	3	–	common ALL
3963	D	FC/PB	L1	86[b]	58	90	71	5	96	–	4	7	77	8	23	0	5	6	6	6	0	pre-B-ALL
(m,9y)	D	FC/BM							87						38							
	R-1(9)	IF/BM	L1	73	73	80	11	4	34	–	3	2	62	4	0	0	1	5	11	–	–	null ALL
3995	D	IF/BM	L2	81	84	93	4	90	90	–	63	3	7	94	1	1	4	6	0	0	0	common ALL
(m,5y)	R-1(43)	FC/BM	L2	87	92	95	93	77	93	–	73	1	62	95	1	1	2	3	0	1	–	common ALL
	R-2(57)	FC/BM	L2	98	92	98	96	96	88	82	62	0	73	98	0	–	2	2	0	0	18	common ALL
4522	D	IP/BM	L1	89	89	95	5	94	81	42	–	0	60	–	23	0	5	6	–	–	0	pre-B-ALL
(f,13y)	R?-2(53)[d]	FC/BM	M4	39	2	69	14	6	45	0	14	8	7	10	7	–	–	–	0 (CD13 32%, MPO 56%)	10	0	ANLL
4553	D	FC/PB	L2	89	85	89	10	80	82	–	86	10	82	86	0	1	5	6	0	0	0	common ALL
(m,5y)	R-1(33)	IF/BM	L2	95	88	80	22	94	93	–	10	10	84	93	0	0	1	2	2	3	–	common ALL
4563	D	FC/BM	L1	80	82	88	5	42	59	77	68	4	29	83	0	0	5	5	0	19	0	common ALL
(m,7y)	R-1(18)	IF/BM	L1	42	55	51	0	43	0	0	1	2	29	56	0	0	18[c]	21[c]	2	12	–	common ALL
		per TdT					0	86	0		0	0	91	99								
4616	D	IF/BM	L1	48[b]	60	74	1	64	73	9	3	3	60	66	42	2	10	14	0	8	0	pre-B-ALL
(m,1y)	R-1(6)	IF/BM	L1	54[b]	36	94	2	87	94	–	93	2	87	88	81	1	1	2	1	1	3	pre-B-ALL

TABLE 1. Immunophenotype of precursor B-ALL with immunophenotypic shifts (continued)[a].

pat nr (sex, age)	D/R (time from diagnosis in months)	method/ cell sample	FAB	BL %	Non-lineage TdT	Non-lineage HLA DR	Non-lineage CD 34	B-lineage CD 10	B-lineage CD 19	B-lineage CD 22 Cy	B-lineage CD 22 Sm	B-lineage CD 20	B-lineage CD 9	B-lineage CD 24	B-lineage Cytgμ	B-lineage Smlgμ	T-lineage CD 3 Sm	T-lineage CD 7	Myeloid CD 14	Myeloid CD 15	Myeloid CD 33	Immuno-phenotype
4711	D	IF/BM	L2	88	81	96	2	95	95	93	90	92	36	97	0	0	3	5	2	1	2	common ALL
(f,15y)	R-1(7)	IF/BM	L2	88	0	99	5	99	97	–	91	48	95	99	0	0	0	0	0	0	0	common ALL
4714	D	FC/BM	L2	88	31	94	6	58	94	44	88	57	95	95	46	2	2	3	0	1	–	pre-B-ALL
(f,12y)	R-1(14)	FC/BM	L1	90	26	88	0	86	89	–	88	80	86	86	0	0	0	0	0	8	–	common ALL
4745	D	IF/BM	L1	97	95	82	30	97	97	92	97	98	93	96	99	0	3	3	0	0	0	pre-B-ALL
(f,6y)	R-1(6)	IF/BM	L2	93	96	87	9	94	94	–	92	92	70	93	96	1	1	2	2	3	3	pre-B-ALL
	R-2(11)	IF/BM	L1	90	94	76	20	93	90	–	88	92	73	94	96	3	3	6	3	3	–	pre-B-ALL
4790	D	IF/BM	L2	76	51	69	16	1	74	5	–	0	59	–	0	0	0	0	0	36	0	null ALL
(f,7mo)	R-1(8)	IF/BM	L2	75	66	93	1	3	94	79	56	1	32	9	0	0	0	2	1	5	–	null ALL
4797	D	IF/BM	L1	95[b]	93	94	69	91	94	–	92	90	83	90	0	0	5	5	0	1	0	common ALL
(m,4y)	R-1(38)	FC/BM	L1	92[b]	87	96	80	71	94	–	89	82	0	90	0	7	2	2	0	25	3	common ALL
4803	D	FC/BM	L1	86	90	94	10	94	96	79	96	77	94	51	62	0	4	5	3	3	3	pre-B-ALL
(m,3y)	R-1(17)	FC/BM	L1	22	30	29	2	25	25	–	28	23	23	28	1	0	45[c]	38[c]	4	14	–	common ALL
		per TdT					0	99	93		74	33	99	77								
4820	D	FC/BM	L1	77	76	85	64	79	86	–	–	21	–	–	0	–	10	14	1	1	2	common ALL
(m, 1y)		FC/PB			62						62		76	65								
	R-1(33)	FC/BM	L1	92[b]	86	94	83	93	94	–	93	86	95	94	0	–	4	6	1	1	0	common ALL
4870	D	FC/BM	L1	71	91	93	0	94	93	–	77	3	92	34	67	0	0	0	0	0	0	pre-B-ALL
(f,9y)	R-1(26)	FC/BM	L2	93	83	94	48	94	94	–	93	2	94	41	60	–	2	3	0	0	0	pre-B-ALL
4882	D	FC/BM	L1	90	98	97	60	98	98	–	97	95	97	97	0	1	3	3	78	6	2	common ALL
(f,11y)	R-1(21)	FC/BM	L1	72	80	87	4	83	86	–	87	3	67	88	1	–	3	3	2	4	4	common ALL
	R-2(29)	FC/BM	L1	83[b]	76	93	7	90	95	–	93	84	91	99	0	1	0	1	0	3	5	common ALL
5171	D	FC/BM	L1	65	68	79	4	61	69	36	45	54	27	74	10	27	15	21	0	6	3	common ALL
(m,10mo)	R-1(13)	FC/BM	L1	92	85	98	58	83	89	–	72	77	3	52	0	–	13	12	1	1	76	common ALL
5346	D	FC/BM	L1	81	69	81	42	73	81	–	53	8	65	81	3	8	12	15	0	7	6	common ALL
(m,2y)	R-1(6)	FC/BM	L1	93	90	92	80	91	89	81	85	5	34	90	2	–	5	6	3	3	62	common ALL

Symbols used: pat nr, DCLSG patient number; f, female; m, male; y, years; mo, months; D, diagnosis; R, relapse; R-1, first relapse; R-2, second relapse; R-3, third relapse; R?, relapse or secondary leukemia; FC, flow cytometer; IF, immunofluorescence microscopy; IP, immunoperoxidase; –, no immunophenotypic data were available with this McAb in this cell sample.

a. The expression of the used McAb is given in percentage positivity.

b. Percentage of blasts before Ficoll centrifugation; this implies that after cell separation the percentage of blast cells would be higher, due to enrichment.

c. Interpreted as normal T-lymphocytes.

d. First relapse in the bone marrow.

TABLE 2. Immunophenotype of precursor B-ALL without immunophenotypic shifts[a].

pat nr (sex, age)	D/R (time from diagnosis in months)	method/ cell sample	FAB	BL %	Non-lineage			B-lineage									T-lineage		Myeloid			Immuno-phenotype
					TdT	HLA DR	CD 34	CD 10	CD 19	CD 22 Cy	CD 22 Sm	CD 20	CD 9	CD 24	CyIgμ	SmIgμ	CD 3 Sm	CD 7	CD 14	CD 15	CD 33	
3968	D	IP/BM	L2	95	98	93	4	74	83	81	–	11	47	–	0	7	–	8	5	4	22	common ALL
(m,14y)	R-1(40)	IF/BM	L1	67	64	80	2	48	62	60	50	9	62	–	2	–	13	12	3	5	11	common ALL
		per TdT						67	80			10	73									
3997	D	IP/BM	L1	89	95	96	0	88	79	81	–	0	10	–	0	2	5	3	–	–	0	common ALL
(m,2y)	R-1(45)	IF/BM	L1	44	48	61	0	40	58	–	57	12	23	57	0	0	14	18	6	11	19	common ALL
		per TdT					1	98	82			0	1	96								
4511	D	IF/BM	L1	95[b]	94	88	72	92	94	20	39	2	5	86	0	2	7	6	3	1	21	common ALL
(m,4y)	R-1(39)	FC/BM	L1	35	47	62	23	60	61	–	59	10	19	10	4	0	8	9	3	1	24	common ALL
		per TdT						97	83			0	5	97								
4638	D	IP/BM	L1	77	77	77	61	58	43	63	–	10	77	–	1	2	23[c]	24[c]	–	–	0	common ALL
(f,2y)	R-1(36)	FC/BM	L2	79	86	55	44	47	56	–	56	8	42	51	1	–	25[c]	22[c]	0	22	0	common ALL
4675	D	IF/BM	L1	84	89	90	81	81	89	79	75	10	87	84	0	3	5	6	3	5	5	common ALL
(m,6y)	R-1(20)	FC/BM	L2	82[b]	72	78	70	67	76	–	68	3	74	72	4	5	7	11	4	7	–	common ALL
4777	D	IP/BM	L1	62	77	85	31	83	80	81	–	13	73	–	9	–	–	9	–	–	5	common ALL
(m,9y)	R-1(24)	FC/BM	L1	73	90	89	78	85	85	54	82	0	84	88	0	0	2	3	2	5	0	common ALL
4778	D	IP/BM	L2	66	72	62	6	65	64	65	–	63	72	–	2	4	15[c]	21[c]	–	12	2	common ALL
(m,1y)	R-2(29)[d]	FC/PB	L1	87	94	82	0	89	86	54	85	82	78	90	0	0	0	0	0	5	0	common ALL
4896	D	IP/BM	L1	86	91	93	71	60	54	57	–	0	84	–	0	3	8	13	–	–	5	common ALL
(m,13y)	R-1(22)	FC/BM	L1	91	95	91	90	88	84	–	88	0	95	91	0	8	0	0	10	7	18	common ALL
4980	D	IP/BM	L1	86	82	92	50	84	58	85	38	0	79	–	1	0	2	8	–	–	0	common ALL
(m,2y)	R-1(18)	FC/BM	L1	52	70	75	62	69	77	–	58	2	69	78	1	–	6	7	2	10	2	common ALL
		per TdT					70	99	90		55	1	99	99								

Symbols used: pat nr, DCLSG patient number; f, female; m, male; y, years; D, diagnosis; R, relapse; R-1, first relapse; R-2, second relapse; FC, flow cytometer; IF, immunofluorescence microscopy; IP, immunoperoxidase; – no immunophenotypic data were available with this McAb in this cell sample.

a. The expression of the used McAb is given in percentage positivity.

b. Percentage of blasts before Ficoll centrifugation; this implies that after cell separation the percentage of blast cells would be higher, due to enrichment.

c. Interpreted as normal T-lymphocytes.

d. First relapse in the bone marrow.

TABLE 3. Immunophenotype of T-ALL at diagnosis and relapse[a].

Pat nr (sex, age)	D/R (time from diagnosis in months)	method/ cell sample	FAB	BL %	Non-lineage TdT	Non-lineage HLA DR	Non-lineage CD 34	T-lineage CD 1	T-lineage CD 2	T-lineage CD 3 Cy	T-lineage CD 3 Sm	T-lineage TcR $\alpha\beta$	T-lineage TcR $\gamma\delta$	T-lineage CD 4	T-lineage CD 5	T-lineage CD 7	T-lineage CD 8	B-lineage CD 10	B-lineage CD 20	Myeloid CD 14	Myeloid CD 15	Myeloid CD 33	Immuno-phenotype
3729	D	IF/BM	L2	84[b]	78	18	0	0	95	88	84	0	49	1	88	96	0	0	1	0	2	3	TcR-$\gamma\delta$ T-ALL
(f,11y)	R-1(33)	IF/BM	L1	63[b]	72	10	2	1	96	92	92	4	75	0	77	97	0	0	2	3	4	–	TcR-$\gamma\delta$ T-ALL
3810	D	IF/BM	L1	63	51	1	3	77	83	85	15	–	–	83	90	86	35	38	1	0	0	0	CD3$^-$ T-ALL
(m,6y)	R-1(17)	FC/BM	L1	80	75	0	28	0	0	92	0	0	0	0	86	89	4	25	–	0	0	–	CD3$^-$ T-ALL
	R-2(25)	FC/BM	L1	81	38	1	52	2	2	95	2	2	1	3	–	98	3	60	2	2	1	–	CD3$^-$ T-ALL
3987	D	FC/BM	L1	97	85	1	1	44	98	97	89	69	5	57	98	98	57	1	0	–	–	0	TcR-$\alpha\beta$ T-ALL
(m,8y)	R-3(18)	FC/BM	L1	95	71	1	4	3	91	99	89	84	4	78	94	84	94	4	4	2	2	–	TcR-$\alpha\beta$ T-ALL
4529	D	FC/PB	L1	80	6	2	1	1	11	85	2	3	1	88	14	91	69	43	1	–	–	0	CD3$^-$ T-ALL
(m,13y)	R-2(45)	FC/PL	ND	95	0	0	–	0	99	73	0	–	–	99	95	99	61	64	–	–	–	9	CD3$^-$ T-ALL
4564	D	FC/BM	L1	59	58	18	2	1	72	60	20	12	0	25	63	65	53	21	11	5	0	0	CD3$^-$ T-ALL
(m,3y)	R-1(15)	FC/PB	L1	87	64	0	0	14	93	73	23	8	3	88	90	92	85	35	7	11	3	–	CD3$^-$ T-ALL
4623	D	FC/BM	L1	91	0	4	8	0	90	92	0	–	–	90	90	90	2	0	0	–	–	6	CD3$^-$ T-ALL
(m,9y)	R-1(12)	IF/BM	L1	79	76	6	58	0	6	68	6	9	1	18	78	5	3	9	1	3	7	–	CD3$^-$ T-ALL
	R-2(21)	IF/BM	L1	78	63	0	9	1	4	75	1	2	0	79	79	89	2	3	1	4	5	–	CD3$^-$ T-ALL
4727	D	FC/PB	L2	90	81	2	90	0	82	92	0	4	0	48	65	89	4	42	2	0	0	0	CD3$^-$ T-ALL
(m,7y)	R-1(12)	FC/BM	L2	88	70	3	62	0	0	60	0	0	0	30	20	92	4	89	1	0	0	77	CD3$^-$ T-ALL
5158	D	FC/BM	L2	95	95	0	27	4	33	99	91	0	+	80	98	97	76	94	0	0	1	0	TcR-$\gamma\delta$ T-ALL
(m,4y)	R-1(11)	FC/BM	L2	74	54	31	71	2	47	85	79	4	65	68	74	85	47	60	5	2	6	5	TcR-$\gamma\delta$ T-ALL

Symbols used: pat nr, DCLSG patient number; f, female; m, male; y, years; D, diagnosis; R, relapse; R-1, first relapse; R-2, second relapse; R-3, third relapse; FC, flow cytometer; IF, immunofluorescence microscopy; PL, pleural exudate; ND, not determined; +, positive expression of McAb; –, no immunophenotypic data were available with this McAb in this cell sample.

a. The expression of the used McAb is given in percentage positivity.

b. Percentage of blasts before Ficoll centrifugation; this implies that after cell separation the percentage of blast cells would be higher, due to enrichment.

CD14, CD15, and CD33 were found to change in expression. Examples of marker-shifts in CD10 and CD34 are given in Figure 2.

In four precursor B-ALL cases (13%), the shifts in marker expression lead to an intra-lineage shift: one common ALL at diagnosis changed to null ALL at relapse (patient 2717) and three pre-B-ALL at diagnosis changed to common ALL (patients 4714 and 4803) or to null ALL (patient 3963). Two precursor B-ALL cases (one common ALL and one pre-B-ALL) were diagnosed as ANLL at relapse (6%), both by morphology as well as by immunological marker analysis (Table 1).

TABLE 4. Total of immunophenotypic shifts per McAb in precursor B-ALL[a].

	null ALL (n=1)	common ALL (n=21)	pre-B-ALL (n=10)	Total (n=32)
FAB	0% (0/1)	33% (7/21)	40% (4/10)	34% (11/32)
non-lineage markers				
TdT	0% (0/1)	10% (2/21)	20% (2/10)	13% (4/32)
HLA-DR	0% (0/1)	0% (0/21)	0% (0/10)	0% (0/32)
CD34	0% (0/1)	20% (4/20)	33% (3/9)	23% (7/30)
B-lineage markers				
CD10	0% (0/1)	14% (3/21)	20% (2/10)	16% (5/32)
CD19	0% (0/1)	10% (2/21)	10% (1/10)	9% (3/32)
CD22	100% (1/1)	19% (4/21)	10% (1/10)	19% (6/32)
CD20	0% (0/1)	14% (3/21)	20% (2/10)	16% (5/32)
CD9	0% (0/1)	24% (5/21)	10% (1/10)	19% (6/32)
CD24	—	7% (1/15)	11% (1/9)	8% (2/24)
Cyl$g\mu$	0% (0/1)	0% (0/21)	50% (5/10)	16% (5/32)
Sml$g\mu$	0% (0/1)	0% (0/12)	0% (0/9)	0% (0/22)
T-lineage markers				
SmCD3	0% (0/1)	0% (0/19)	0% (0/9)	0% (0/29)
CD7	0% (0/1)	0% (0/21)	0% (0/9)	0% (0/31)
Cross-lineage myeloid markers				
CD14	0% (0/1)	7% (1/15)	0% (0/9)	4% (1/25)
CD15	100% (1/1)	13% (2/16)	0% (0/9)	12% (3/26)
CD33	—	20% (3/15)	0% (0/9)	13% (3/24)

Symbols used: —, no immunophenotypic data were available with this McAb.
a. Percentage of immunophenotypic changes within the tested precursor B-ALL cases.

Immunological marker analysis of T-ALL at diagnosis and relapse

Based on expression of SmCD3, the eight T-ALL were classified as SmCD3$^-$ T-ALL (n=5) and SmCD3$^+$ T-ALL (one TcR-$\alpha\beta^+$ and two TcR-$\gamma\delta^+$) (Table 3). In five of the eight T-ALL cases marker-shifts were found. Shifts were observed in all tested T-cell markers, except for the lineage specific CD3 and TcR markers, i.e. CyCD3, SmCD3, TcR-$\alpha\beta$ and TcR-$\gamma\delta$ expression remained stable (Tables 3 and 5). Examples for marker-shifts in CD2 and CD5 expression are given in Figure 3. Like in precursor B-ALL no clear shift in HLA-DR antigen expression was found at relapse. Only one case (patient 5158)

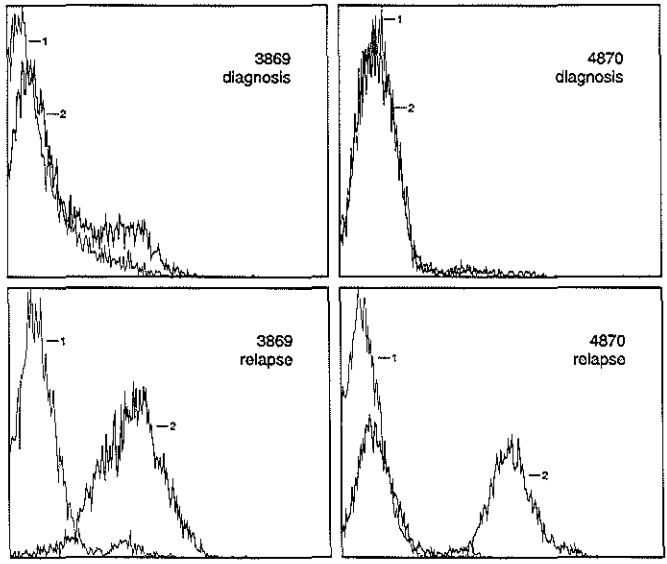

Figure 2. Histogram analysis of two precursor B-ALL patients at diagnosis and relapse. **Left part:** CD19 (VIL-A1 FITC) IF staining of BM cells at diagnosis (top) and at relapse (bottom) of patient 3869. **Right part:** CD34 (BI-3C5 FITC) IF staining of BM cells at diagnosis (top) and at relapse (bottom) of patient 4870. Symbols used: 1, IgG1 control; 2, McAb. Detailed information concerning the percentage positivity of expression of the immunological markers in both patients is shown in Table 1.

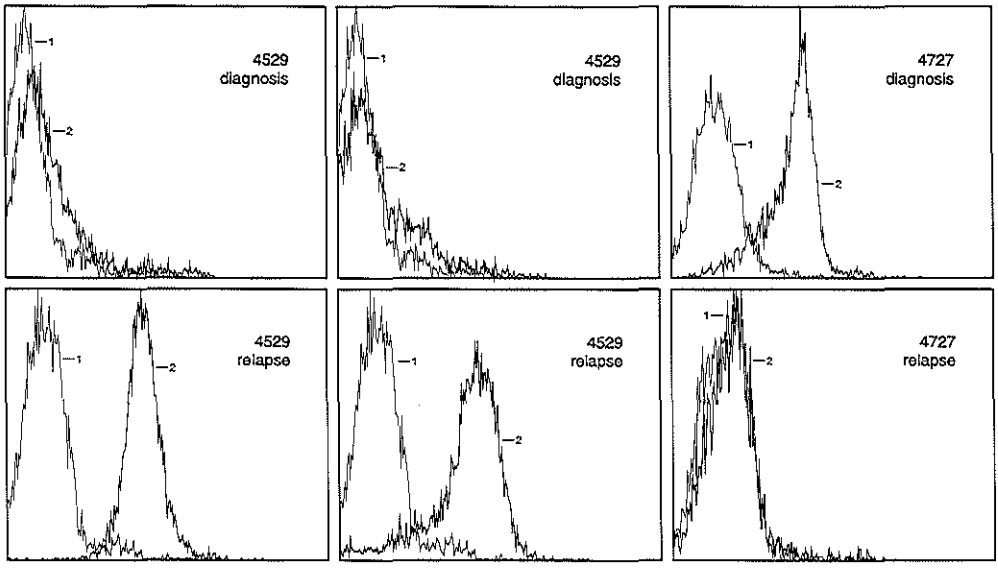

Figure 3. Histogram analysis of two T-ALL patients at diagnosis and relapse. **Left part:** CD2 (T11 FITC) IF staining and **middle part:** CD5 (Leu-1 FITC) IF staining of PB cells at diagnosis (top) and pleural exudate cells at relapse (bottom) of patient 4529. **Right part:** CD2 (T11 FITC) IF staining of PB cells at diagnosis (top) and BM cells at relapse (bottom) of patient 4727. Symbols used: 1, IgG1 control; 2, McAb. Detailed information concerning the percentage positivity of expression of the various immunological markers in both patients is shown in Table 3.

showed a small change from negative to positive HLA-DR expression, possibly due to contaminating non-malignant cells (Table 3). Despite the frequent marker-shifts (fre-

TABLE 5. Total of immunophenotypic shifts per McAb in T-ALL[a].

	CD3⁻ T-ALL (n=5)	TcR-αβ⁻ T-ALL (n=1)	TcR-γδ⁻ T-ALL (n=2)	Total (n=8)
FAB	0% (0/3)	0% (0/1)	50% (1/2)	14% (1/7)
Non-lineage markers				
TdT	20% (1/5)	0% (0/1)	0% (0/2)	13% (1/8)
HLA-DR	0% (0/5)	0% (0/1)	0% (0/2)	0% (0/8)
CD34	50% (2/4)	0% (0/1)	0% (0/2)	29% (2/7)
T-lineage markers				
CD1	20% (1/5)	100% (1/1)	0% (0/2)	25% (2/8)
CD2	80% (4/5)	0% (0/1)	0% (0/2)	50% (4/8)
CyCD3	0% (0/5)	0% (0/1)	0% (0/2)	0% (0/8)
SmCD3	0% (0/5)	0% (0/1)	0% (0/2)	0% (0/3)
TcR-αβ	0% (0/3)	0% (0/1)	0% (0/2)	0% (0/6)
TcR-γδ	0% (0/3)	0% (0/1)	0% (0/2)	0% (0/6)
CD4	60% (3/5)	0% (0/1)	0% (0/2)	38% (3/8)
CD5	20% (1/5)	0% (0/1)	0% (0/2)	13% (1/8)
CD7	20% (1/5)	0% (0/1)	0% (0/2)	13% (1/8)
CD8	20% (1/5)	0% (0/1)	0% (0/2)	13% (1/8)
B-lineage markers				
CD10	0% (0/5)	0% (0/1)	0% (0/2)	0% (0/8)
CD20	0% (0/3)	0% (0/1)	0% (0/2)	0% (0/6)
Cross-lineage myeloid markers				
CD14	0% (0/3)	—	0% (0/2)	0% (0/5)
CD15	0% (0/3)	—	0% (0/2)	0% (0/5)
CD33	50% (1/2)	—	0% (0/1)	33% (1/3)

Symbols used: —, no immunophenotypic data were available with this McAb.
a. Percentage of immunophenotypic changes within the tested T-ALL cases.

quently multiple marker-shifts per leukemia), no intra-lineage nor inter-lineage shifts were found in the 8 T-ALL cases.

Time of relapse and changes in marker expression

There seems to be neither a hierarchical order nor a relation with time for changes in marker expression between diagnosis and relapse except perhaps for CD10. Shifts in CD10 expression were only observed in relapses occuring 30 months or more after diagnosis. The two inter-lineage shifts, from ALL to ANLL were also found late, i.e. occuring 49 months (patient 3797) and 53 months (patient 4522) after diagnosis and off-therapy.

DISCUSSION

Comparative immunophenotyping studies of leukemias at diagnosis and relapse might be hampered by several problems such as: 1. the lack of adequate cell samples at both diagnosis and relapse; 2. changes in the applied immunophenotyping method during the time interval between diagnosis and relapse; 3. limited McAb panels at

diagnosis or relapse and changes in McAb panels with time; 4. interpretation difficulties due to low blast cell counts in the studied cell samples at relapse; and 5. unclear criteria for defining an immunophenotypic shift. In our study, most of these problems were neutralized. Originally cell samples at diagnosis were tested with immunoperoxidase stainings, but we repeated the analyses by immunofluorescence microscopy or flow cytometry in case a shift was found in order to be sure that the shift was not due to differences in the applied methods. Furthermore, nearly identical McAb panels were used at diagnosis and at relapse: expression of non-lineage specific markers (TdT, CD34, and HLA-DR), B-lineage markers (CD10, CD19, CD20, and CD22), T-lineage markers (CD1, CD2, CD3, CD4, CD5, CD7, and CD8), and cross-lineage myeloid markers (CD14, CD15, and CD33) were compared in all relevant cases (see Tables 1-3). The problem of low blast cell counts at relapse was met by use of double labeling for membrane markers and TdT in order to define the precise immunophenotype of the TdT^+ leukemic cells. Finally, the definition for marker-shifts was very strict, i.e. a change from positivity to negativity or vice versa (with a cutt-off point at 20% positivity) or a shift in positivity of $\geq$50%.

In our study, 40 ALL patients were analyzed at diagnosis and at relapse for cytomorphological and immunophenotypic shifts. Differences in cytomorphology between diagnosis and relapse were detected in 34% (11/32) of precursor B-ALL and 14% (1/7) of T-ALL (Tables 4 and 5). Differences in immunological marker analysis between diagnosis and relapse were found in 69% (22 cases) of precursor B-ALL and in 63% (5 cases) of T-ALL. Curiously, the morphological and immunophenotypic shifts were not correlated, which underlines the poor relationship between morphology (FAB type L1 and L2) and immunophenotype in ALL.

In precursor B-ALL, the phenotypic changes included four intra-lineage shifts and two inter-lineage shifts to ANLL. All four intra-lineage shifts concerned shifts to a more immature immunophenotypic classification. In T-ALL, no such shifts were observed, due to the stability in expression of the lineage specific markers CyCD3, SmCD3, and TcR. In precursor B-ALL, loss or acquisition of lineage specific markers like CD22 and/or Cyl$g\mu$ were found in ten patients and therefore precursor B-ALL seem to have a less stable phenotype. It should be noted that this is especially true for pre-B-ALL, because all ten pre-B-ALL showed at least one marker-shift at relapse.

Changes in immunophenotype between diagnosis and relapse have been described in some other reports (8-17). Early studies used very small panels of polyclonal antibodies (9,10,15) and found phenotypic shifts in common ALL antigen, HLA-DR, TdT or E-rosettes in 17-25% of cases. Later on, Pui et al. used a larger panel of McAb and reported changes in the expression of cell markers in ~25% of cases (11). This relatively low frequency of immunophenotypic shifts is probably due to the fact that only CD10 and HLA-DR were tested in all 68 relapses. Abshire et al. also reported shifts in expression of CD10 (14%), and HLA-DR (27%) (8). They found intra-lineage shifts in 15% of the 175 relapsed cases. This is comparable with the ~13% intra-lineage shifts in our series of precursor B-ALL. In contrast to other studies, we did not find a clear shift in HLA-DR expression except for a minor shift in percentage positivity in a T-ALL case (patient 5158).

Shifts in CD10 expression were found, but this appeared to occur only in late

relapses. The earliest CD10 shift in our study was found in a relapse occuring 31 months after diagnosis. This relationship between remission duration and marker-shift was not found for other markers, although there seems to be a greater chance for inter-lineage shifts in late "relapses" in our study. Pui et al. found a median time of 3 years in 13 cases with inter-lineage shifts (14). It should be emphasized that detection of an inter-lineage shift does not automatically imply the development of a secondary leukemia, since in patient 3991, shown in a previous study (21), with an inter-lineage shift from ALL to ANLL, seven rearranged Ig and TcR alleles were identical at relapse, excluding the development of a secondary leukemia (21). Apparently, in this case the leukemic clone at diagnosis had multi-lineage properties.

Reliable information about stability of marker expression and the occurrence of immunophenotypic shifts is essential when immunological marker analysis is used for detection of MRD. Combinations of T-cell markers and TdT are used for MRD detection in T-ALL (12,18,19,22,23). Campana et al. also proposed double marker analysis for myeloid markers (CD13 and/or CD33) and TdT, or Cyl$g\mu$ and TdT for MRD detection in precursor B-ALL (12). They stated that myeloid marker expression in B-lineage ALL remains stable and found loss of Cyl$g\mu$ expression in only one out of four pre-B-ALL in a total series of 10 precursor B-ALL. However it is clear from our data, that shifts can occur in any marker tested in precursor B-ALL, also myeloid markers and Cyl$g\mu$. Even CD34 expression might change at relapse in 20-25% of precursor B-ALL, although other scientists proposed to use CD34 as a marker in MRD studies (Table 4) (23). Although CD3 and TcR expression patterns appeared to be stable in our T-ALL series, shifts were frequently seen for other T-cell markers. Even loss of TdT expression was found in one T-ALL. Conversion to TdT negativity was also observed in three precursor B-ALL cases. This loss of TdT expression in ~12% of ALL cases at relapse is in line with other studies (12,16,18).

In 24 of the 40 patients here described, analysis of Ig and TcR receptor genes at diagnosis and subsequent relapse was performed (21). The presence of two or more subclones at diagnosis was found in the null ALL and three of seven pre-B-ALL studied, and was always accompanied by changes in marker expression at relapse. In ten common ALL and six T-ALL examined no subclones were found at diagnosis. No clear correlation was found between the immunophenotypic changes and the immunogenotypic changes at relapse: in contrast to the data of Bunin et al., new Ig and TcR gene rearrangement patterns at relapse were also found in patients without immunophenotypic shifts and vice versa (17,21).

In conclusion, marker-shifts in leukemia at relapse are frequently found in precursor B-ALL and T-ALL. In a small fraction this will lead to an intra-lineage shift (10%) or inter-lineage shift (5%). Therefore, monitoring for MRD by immunological marker analysis should not be restricted to one or two markers or marker combinations. If possible, several marker combinations should be used for MRD detection, preferentially in combination with PCR analysis of rearranged Ig and/or TcR genes or chromosome aberrations (21,22).

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CHAPTER 4.3

ANALYSIS OF IMMUNOGLOBULIN AND T-CELL RECEPTOR GENES IN 40 CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIAS AT DIAGNOSIS AND SUBSEQUENT RELAPSE: Implications for the detection of minimal residual disease by polymerase chain reaction analysis*

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ABSTRACT

The rearrangement patterns of immunoglobulin (Ig) and T-cell receptor (TcR) genes were studied by Southern blot analysis in 30 precursor B acute lymphoblastic leukemias (ALL) and 10 T-ALL at diagnosis and subsequent relapse. Eight precursor B-ALL appeared to contain bi-/oligoclonal Ig heavy-chain (IgH) gene rearrangements at diagnosis. Differences in rearrangement patterns between diagnosis and relapse were found in 67% (20 cases) of precursor B-ALL (including all eight bi-/oligoclonal cases) and 50% (five cases) of T-ALL. In precursor B-ALL, especially changes in IgH and/or TcR-δ gene rearrangements were found (17 cases), but also changes in TcR-β, TcR-γ, Igκ and/or Igλ genes (11 cases) occurred. The changes in T-ALL concerned the TcR-β, TcR-γ, TcR-δ and/or IgH genes. Two precursor B-ALL showed completely different Ig and TcR gene rearrangement patterns at relapse, suggesting the absence of a clonal relation between the leukemic cells at diagnosis and relapse and the development of a secondary leukemia. The clonal evolution in the other 23 ALL patients was based on continuing rearrangement processes and selection of subclones. The development of changes in Ig and TcR gene rearrangement patterns was related to remission duration, suggesting an increasing chance of continuing rearrangement processes with time. These immunogenotypic changes at relapse occurred in a hierarchical order with changes in IgH and TcR-δ genes occurring after only 6 months of remission duration, whereas changes in other Ig and TcR genes were generally detectable after 1 to 2 years of remission duration.

The heterogeneity reported here in Ig and/or TcR gene rearrangement patterns at diagnosis and relapse might hamper polymerase chain reaction (PCR)-mediated detection

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of minimal residual disease (MRD) using junctional regions of rearranged Ig or TcR genes as PCR targets. However, our data also indicate that in 75-90% of ALL, at least one major rearranged IgH, TcR-γ, or TcR-δ band (allele) remained stable at relapse. We conclude that two or more junctional regions of different genes (IgH, TcR-γ, and/or TcR-δ) should be monitored during follow-up of ALL patients for MRD detection by use of PCR techniques. Especially in bi-/oligoclonal precursor B-ALL cases, the monitoring should not be restricted to rearranged IgH genes, but TcR-γ and/or TcR-δ genes should be monitored as well, because of the extensive changes in IgH gene rearrangement patterns in this ALL subgroup.

INTRODUCTION

Despite major improvements in the treatment of ALL patients during the last two decades, 20-30% of children with ALL still relapse (1-3). The pathogenesis of leukemia relapse is still poorly understood (4). To obtain insight into the mechanisms that determine development of relapse in ALL, several studies have been performed: detection of morphological and immunophenotypic shifts at relapse (5-10), detection of shifts in leukemic cell karyotype at relapse (9,11), comparative glucose-6-phosphate dehydrogenase studies (12) and analysis of Ig and TcR genes at diagnosis and relapse (13-18).

Ig and TcR gene rearrangement patterns in leukemias and malignant lymphomas have been used as markers for clonality, which are unique for each malignancy (19). This especially concerns the junctional regions of rearranged Ig and TcR genes, because they are regarded as "tumor-specific" markers (20-22). Based on this assumption, several investigators have used the PCR for amplification of the "tumor-specific" junctional regions of rearranged IgH, TcR-γ and TcR-δ genes to detect MRD during follow-up of ALL patients (23-27). However, it should be noted that multiple rearranged IgH genes occur in 30-40% of precursor B-ALL (16,28-30). When excluding hyperdiploidy of chromosome 14, the multiple IgH gene rearrangements are most probably due to subclone formation (16,28-30), which is suggested to be related to poor prognosis in childhood ALL (28,30). This subclone formation can be explained by continuing rearrangement processes such as VH to D-JH joining or VH replacements (29-36).

Detailed analysis of the leukemic cells at diagnosis and relapse by use of Ig and TcR gene rearrangement patterns may give insight into the heterogeneity at diagnosis (process of subclone formation) and at relapse (selection of subclones, possibly related to development of therapy resistance), and into the implications of this heterogeneity for MRD detection by PCR-mediated amplification of junctional regions. So far, only a few studies on limited numbers of patients have been published (13-18). These studies were restricted primarily to IgH, Igκ and/or TcR-β genes and demonstrated changes in rearrangement patterns in 15-40% of ALL (13-18). Therefore, we performed detailed comparative Southern blot analyses of IgH, Igκ, Igλ, TcR-β, TcR-γ and TcR-δ gene rearrangement patterns in 30 precursor B-ALL and 10 T-ALL at diagnosis and subsequent relapse.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) and/or bone marrow (BM) samples from children with ALL (30 precursor B-ALL and 10 T-ALL) were obtained at initial diagnosis and at first relapse in 32 cases and at first and at second relapse in eight additional cases (six precursor B-ALL and two T-ALL). In one T-ALL, we received bilateral testis biopsies at relapse as well. Twenty-one children were treated at the Sophia Children's Hospital, Rotterdam. Their diagnosis and relapse cell samples were collected by the Department of Immunology, Erasmus University, Rotterdam. The other 19 children were treated at different hospitals in The Netherlands and their diagnosis and relapse cell samples were collected by the Dutch Childhood Leukemia Study Group (DCLSG), The Hague.

The diagnosis of acute leukemia was made according to the French-American-British (FAB) classification (37), based on cytomorphology of BM smears stained with May-Grünwald Giemsa and cytochemistry (periodic acid-Schiff, acid phosphatase, Sudan black B, myeloperoxidase and α-naphtylesterase). Each diagnosis and relapse was independently confirmed by the laboratory of the DCLSG.

Mononuclear cells (MNC) were isolated from PB and/or BM samples by Ficoll-Pague centrifugation (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). The freshly obtained MNC samples were subjected to a detailed immunological marker analysis according to standard protocols (30,38). Remaining MNC were stored in liquid nitrogen. A leukemia was considered to be a precursor B-ALL if the malignant cells were positive for terminal deoxynucleotidyl transferase (TdT), CD19 and HLA-DR (null ALL), or for TdT, CD10, CD19 and HLA-DR (common ALL), or for TdT, CD10, CD19, HLA-DR, and cytoplasmic Ig heavy-chain μ (pre-B-ALL) (39,40). A leukemia was considered to be a CD3$^-$ T-ALL if the malignant cells were positive for TdT and cytoplasmic CD3, as well as for CD2, CD5 and/or CD7. A CD3$^+$ T-ALL was diagnosed, if the malignant cells were positive for TdT and membrane TcR-CD3 complex expression as well as for CD2, CD5, CD7 and CD4 or CD8 (39,40). A leukemia was considered to be an acute myeloid leukemia (AML) if the malignant cells were positive for CD13, CD33, CDw65 and/or myeloperoxidase (MPO), as well as for HLA-DR, CD34, CD14, CD15 and/or CD36 (39).

Southern blot analysis

DNA was isolated from frozen MNC as described previously (41,42). Control DNA was obtained from granulocytes or other cell samples with germline Ig and TcR genes. Fifteen micrograms of DNA was digested with the appropriate restriction enzymes (Pharmacia). Completeness of digestion was checked with λ-phage DNA and plasmid DNA in parallel digests as described previously (42). The restriction fragments were size-separated in 0.7% agarose gels and transferred onto Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) as described previously (42). To take care that the diagnosis and relapse lanes of each patient during the gel electrophoresis contained comparable amounts of DNA, the DNA contents of the digested samples were checked (and adapted, if necessary) before loading into the agarose gel. This was further verified by staining of the agarose gel with ethidium bromide (42).

IgH gene rearrangements were detected with a ^{32}P-random oligonucleotide labeled joining (J)H probe. The JH probe (IGHJ6) was a 1.0-kb probe, which recognizes sequences just 3′ of the JH gene segments (43). The configuration of the Igκ genes was analyzed with a ^{32}P-labeled Jκ probe, a ^{32}P-labeled constant (C)κ probe, and a ^{32}P-labeled κ deleting element (Kde) probe. The Jκ probe was a 0.55-kb *Hae*III fragment, which recognizes sequences just 3′ of the Jκ gene segments (42), the Cκ probe was a 2.5-kb *Eco*RI fragment (42), and the Kde probe (IGKDE) was a 0.5-kb probe (see chapter 2.3). All diagnosis and relapse DNA samples were analyzed by use of the JH, Jκ, and Cκ probes in *Bgl*II digests and *Bam*HI-*Hin*dIII double digests and by use of Kde probe in *Bgl*II and *Hin*dIII digests. If necessary for confirmation, *Eco*RI and/or *Hin*dIII digests were also used. The configuration of the Igλ genes was analyzed with the ^{32}P-labeled Cλ3 probe (a 0.8-kb *Bgl*II-*Eco*RI fragment) in *Eco*RI-*Hin*dIII double digests and/or in *Eco*RI digests (42).

TcR-β gene rearrangements were detected with ^{32}P-labeled Jβ1, Jβ2, and Cβ probes in *Eco*RI, and *Hin*dIII digests, and for confirmation in *Bgl*II digests (42). The configuration of the TcR-γ genes was analyzed by use of the ^{32}P-labeled Jγ1.2, Jγ1.3 and Jγ2.1 probes in *Eco*RI digests, and for confirmation in *Kpn*I and/or *Bgl*II digests (42). The configuration of the TcR-δ genes was analyzed by use of ^{32}P-labeled Jδ1, Jδ2, and Cδ probes in *Eco*RI, *Hin*dIII, and *Bgl*II digests (42). In 24 cases, the TcR-δ gene rearrangements were further

analyzed with ^{32}P-labeled variable (V)δ1, Vδ2, Vδ3, δREC, diversity (D)δ1, and ψJα probes in *Eco*RI, *Hin*dIII, and *Bgl*II digests (44).

RESULTS

Immunological marker analysis

Immunological marker analysis of the 30 precursor B-ALL revealed that two were null ALL, 17 were common ALL, and 11 were pre-B-ALL. Changes in immunophenotypic classification occurred in seven precursor B-ALL. In two of them, an inter-lineage shift occurred to AML (Tables 1 and 2).

The 10 T-ALL could be divided in six CD3$^-$ T-ALL and four CD3$^+$ T-ALL (three TcR-αβ$^+$ T-ALL and one TcR-γδ$^+$ T-ALL). No changes in immunophenotypic classification were found in the T-ALL (Table 3).

Southern blot analysis of 30 precursor B-ALL at diagnosis

DNA samples from 30 precursor B-ALL at diagnosis were examined for the presence of rearranged Ig and TcR genes. At diagnosis, rearrangements and/or deletions of the IgH, Igκ, and Igλ genes were found in 100% (all 30 cases), 57% (17 cases), and 17% (five cases) of precursor B-ALL, respectively (Tables 1, 2 and Figure 1). In 10 cases, multiple rearranged IgH gene bands, generally differing in density, were found at diagnosis (30). In eight patients we could exclude hyperdiploidy of chromosome 14 as a cause of these multiple rearranged bands. In the other two patients (2665 and 4553), no cytogenetic data were available, but the density of the three rearranged IgH gene bands were comparable, suggesting trisomy 14. In patient 2665, this was further supported by the presence of one germline and two rearranged TcR-δ gene bands of comparable density (Table 2). Therefore, we have concluded that in eight precursor B-ALL the presence of multiple rearranged bands was due to the presence of two or more subclones (bi-/oligoclonality) with differently rearranged IgH genes (Tables 1, 2 and Figure 1) (30).

Rearrangements and/or deletions of the TcR-β, TcR-γ, and TcR-δ genes at diagnosis were found in 33% (10 cases), 53% (16 cases), and 83% (25 cases) of precursor B-ALL, respectively (Tables 1, 2 and Figure 2).

Southern blot analysis of 10 T-ALL at diagnosis

DNA samples from 10 T-ALL at diagnosis were examined for the presence of rearranged Ig and TcR genes. Rearrangements of the IgH genes at diagnosis were found in 40% (four cases) of T-ALL (Table 3). No rearrangements and/or deletions of the Ig light-chain (IgL) genes were found (Table 3). Rearrangements and/or deletions of the TcR-β, TcR-γ, and TcR-δ genes were found in all 10 T-ALL at diagnosis (Table 3 and Figure 2). In one T-ALL (patient 3288), three rearranged TcR-β2 gene bands with different density were found at diagnosis, suggesting subclone formation at the TcR-β gene level (cytogenetic analysis showed diploidy of chromosome 7).

Changes in Ig and TcR gene rearrangement patterns in precursor B-ALL at relapse

Comparative analysis of Ig and TcR gene rearrangement patterns at diagnosis and relapse

TABLE 1. Southern blot analysis of 10 precursor B-ALL patients without differences in immunogenotype between diagnosis and subsequent relapse(s).

DCLSG number (sex/age)	disease stage (time from diagnosis)[a]	immuno-phenotypic classification	sample	leukemic cells (%)[b]	Ig genes[c]			TcR genes[c]			
					IgH	Igκ	Igλ	TcR-β		TcR-γ	TcR-δ
								Jβ1	Jβ2		
0128	D	common ALL	BM	77	R,R	G	G	G	G	R,G	R,G
(M/2 y)	R1 (11)	common ALL	BM	41	R,R,g	G	G	G	G	R,G	R,G
	R2 (45)[d]	common ALL	BM	89	R,R	G	G	G	G	R,G	R,G
3697	D	pre-B-ALL	BM	93	R,R	G	G	G	G	G	G
(M/9 y)	R (15)	pre-B-ALL	BM	76	R,R	G	G	G	G	G	G
3837	D	pre-B-ALL	BM	98	R,R	G	G	G	R,G	R,R	D,D
(M/8 y)	R1 (35)	pre-B-ALL	BM	97	R,R	G	G	G	R,G	R,R	D,D
	R2 (50)	pre-B-ALL	BM	98	R,R	G	G	G	R,G	R,R	D,D
3995	D	common ALL	BM	90	D,R	G	G	D,D	R,R	R,R	D,D
(M/5 y)	R1 (43)	common ALL	BM	94	D,R	G	G	D,D	R,R	R,R	D,D
	R2 (57)	common ALL	BM	93	D,R	G	G	D,D	R,R	R,R	D,D
4535	D	pre-B-ALL	BM	85	R,R	G	G	G	G	R,R	D,R
(M/12 y)	R (7)	pre-B-ALL	BM	81	R,R	G	G	G	G	R,R	D,R
4553	D	common ALL	PB	85	R,R,R	G	G	G	G	G	R,R
(M/5 y)	R (32)	common ALL	BM	88	R,R,R	G	G	G	G	G	R,R
4711	D	common ALL	BM	95	R,R	D,R	R,R	G	R,R	G	G
(F/15 y)	R (7)	common ALL	BM	99	R,R	D,R	R,R	G	R,R	G	G
4714	D	pre-B-ALL	BM	90	R,R	G	G	G	G	G	R,G
(F/12 y)	R (14)	common ALL	BM	40	R,R,g	G	G	G	G	G	R,G
4745	D	pre-B-ALL	BM	95	R,R	D,D	G	G	R,G	R,R	D,D
(F/6 y)	R1 (6)	pre-B-ALL	BM	93	R,R	D,D	G	G	R,G	R,R	D,D
	R2 (11)	pre-B-ALL	BM	96	R,R	D,D	G	G	R,G	R,R	D,D
4882	D	common ALL	BM	98	R,R^w	D,G	G	G	G	R,R	D,D
(F/11 y)	R (22)	common ALL	BM	88	R,R^w	D,G	G	G	G	R,R	D,D

Abbreviations: M, male; F, female; y, years; D, diagnosis; R, relapse; BM, bone marrow; PB, peripheral blood.

a. The time from diagnosis (in months) is time between diagnosis and (first or second) relapse.

b. The leukemic cell load was determined by the percentages of TdT^+, $CD10^+$, and/or $CD19^+$ cells.

c. Configuration of Ig and TcR genes: (G) allele in germline configuration; (g) germline band due to the presence of non-leukemic cells (≥ 25%); (R) rearranged band; (R^w) weak rearranged band; (D) deletion of the involved gene (segment).

d. Second relapse after bone marrow transplantation.

were performed in all 30 precursor B-ALL (Tables 1 and 2). Differences in IgH, Igκ, and Igλ gene rearrangement patterns between diagnosis and relapse were found in 40%, 28%, and 43% of precursor B-ALL (with rearrangements or deletions of these genes), respectively (Table 4). The precursor B-ALL with these immunogenotypic shifts included all precursor B-ALL with bi-/oligoclonal IgH gene rearrangements. Differences in TcR-β, TcR-γ, and TcR-δ gene rearrangement patterns were found in 45%, 33%, and 44% of precursor B-ALL (with rearrangements or deletions of these genes) respectively (Table 4).

In 10 precursor B-ALL, no changes in immunogenotype at relapse were found (Table 1). The other 20 precursor B-ALL showed major or minor changes at relapse (Table 2). Two monoclonal precursor B-ALL (patients 3779 and 3797) had completely different Ig and TcR gene rearrangement patterns at relapse. In patient 3779, each rearranged

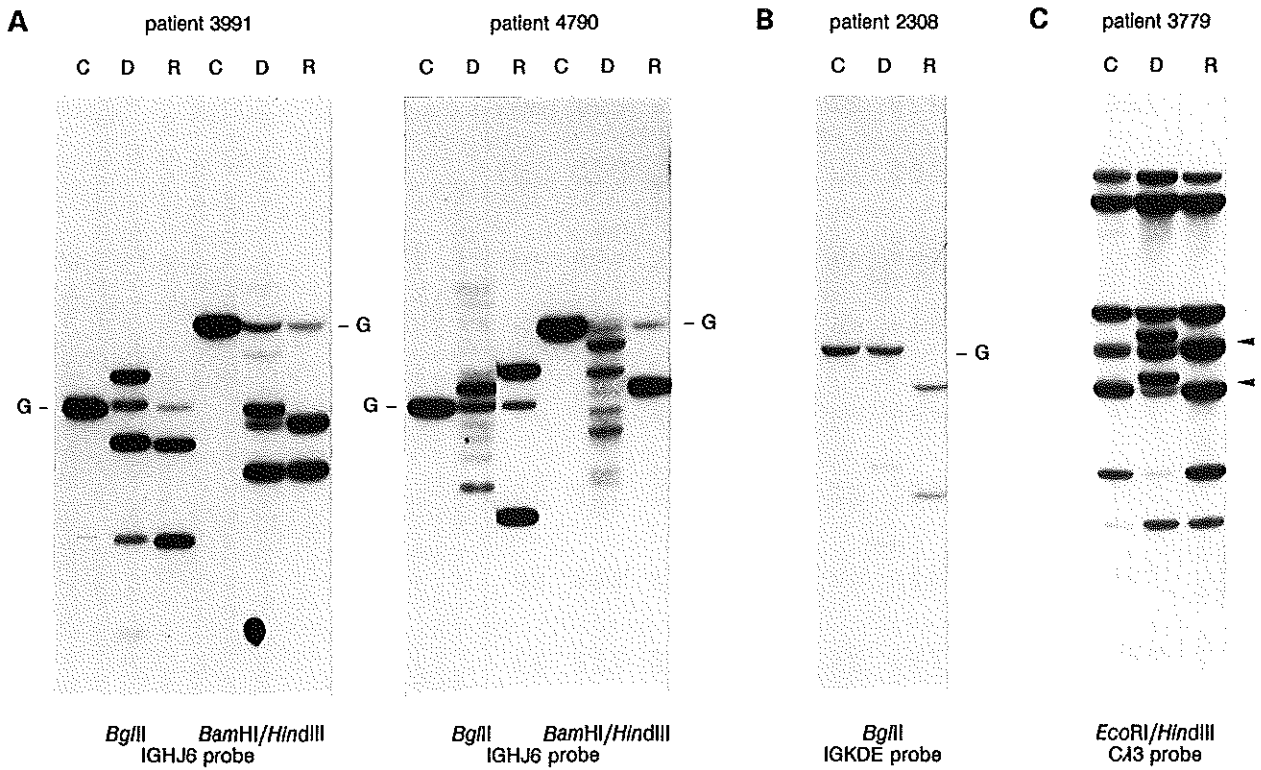

Figure 1. Southern blot analysis of the Ig genes in several precursor B-ALL patients at diagnosis (D) and relapse (R). Control DNA (C) and DNA from the precursor B-ALL patients were digested with the appropriate restriction enzymes, site-separated, and blotted onto nylon membrane filters, which were hybridized with ^{32}P-labeled probes. **A,** IgH gene analyses using *Bgl*II and a combination of *Bam*HI and *Hind*III with the IGHJ6 probe. **B,** Ig*κ* gene analyses using *Bgl*II with the IGKDE probe. **C,** Igλ gene analyses using a combination of *Eco*RI and *Hind*III with the Cλ3 probe. The germline bands (G) are indicated (A,B). The rearranged Igλ gene bands are indicated with arrows (C). Detailed information concerning the configuration of the different Ig genes is given in Table 2.

TABLE 2. Southern blot analysis of 20 precursor B-ALL patients with differences in immunogenotype between diagnosis and subsequent relapse(s).

DCLSG number (sex/age)	disease stage (time from diagnosis)[a]	immuno-phenotypic classification	sample	leukemic cells (%)[b]	Ig genes[c]			TcR genes[c]			
					IgH	Igκ	Igλ	TcR-β		TcR-γ	TcR-δ
								Jβ1	Jβ2		
IgH monoclonal cases at diagnosis											
2665	D	common ALL	BM	94	R,R,R	D,G	G	G	G	G	R,R,G
(M/5 y)	R1 (75)	common ALL	BM	85	D,R,R	D,G	G	G	G	R,R	D,D,D
	R2 (100)	common ALL	BM	89	D,R,R	D,G	G	G	G	R,R	D,D,D
2717	D	common ALL	BM	90	R,R	D,D	G	G	R,G	R,R	D,R
(M/1 y)	R (32)	null ALL	BM	97	R,R	D,D	G	D,D	Rn,Rn	R,Rn	D,D
3779	D	common ALL	PB	58	R,R,g	D,R,g	R,R,g	G	R,R,g	G	G
(F/3 y)	R (48)	common ALL	BM	92	Rn,Rn	D,Rn	G	G	G	R,R	D,D
3797	D	common ALL	BM	92	R,R	D,G	G	G	G	R,R	D,D
(M/2 y)	R (49)	AML	BM	75	Rn,G	G	G	G	G	G	G
3869	D	common ALL	BM	74	D,R,g	D,R,g	R,G	G	R,G	G	R^w,G
(M/9 y)	R (38)	common ALL	PB	95	D,R	D,R	R,G	G	R,G	G	G
3968	D	common ALL	BM	95	R,R	G	G	G	G	G	D,D
(M/14 y)	R (40)	common ALL	BM	67	R,R,g	G	G	D,G	R,G	G	D,D,g
4501	D	common ALL	PB	54	R,R,g	D,G	G	G	G	R,R,g	D,D,g
(F/5 y)	R (22)	pre-B-ALL	BM	93	R,Rn	D,G	G	G	G	R,R	D,D
4511	D	common ALL	BM	94	D,D	D,D	G	G	G	R,R	R,R
(M/4 y)	R (39)	common ALL	BM	50	D,D,g	D,D,g	R,G	G	G	R,R,g	D,R,g
4515	D	common ALL	PB	50	R,R,g	G	G	G	G	G	G
(F/12 y)	R (18)	common ALL	BM	70	R,R,g	G	G	G	G	G	R,G
4616	D	pre-B-ALL	BM	60	R,R,g	R,R,g	G	G	G	G	D,D,g
(M/1 y)	R (7)	pre-B-ALL	BM	93	R,R	R,R	R,G	G	G	G	D,D
4675	D	common ALL	BM	89	R,R	R,R	G	G	G	R,R	R,R
(M/6 y)	R (21)	common ALL	BM	72	R,R,g	R,Rn,g	G	G	G	R,R,g	R,Rn,g
4778	D	common ALL	PB	65	R,R,g	D,G	G	G	R,G	R,R,g	D,D,g
(M/1 y)	R (29)	common ALL	PB	87	R,R	D,G	G	G	R,Rn	R,R	D,D
IgH bi-/oligoclonal cases at diagnosis											
2308	D	pre-B-ALL	PB	80	3R,R^w	R^w,G	G	G	G	R,G	R^w,R
(F/8 y)	R (50)	pre-B-ALL	BM	93	Rn,R^w	D,D	G	G	G	G	R^w,G
2678	D	common ALL	BM	73	2R,2R^w,g	R,R,g	G	G	G	G	R,R,g
(M/1 y)	R1 (39)	common ALL	BM	85	2R,2Rn^w	R,R	G	G	G	G	R,R
	R2 (66)[d]	pre-B-ALL	BM	45	R^w,Rn^w,g	R^w,R^w,g	G	G	G	G	R^w,R^w,g
3510	D	pre-B-ALL	BM	82	R,R,R	G	R,G	G	G	G	D,R
(M/2 y)	R (8)	pre-B-ALL	BM	85	R,R,G	G	R,G	G	G	G	D,G
3559	D	pre-B-ALL	BM	82	R,5R^w,G	G	G	D,G	R,R,G	R,R	D,R^w,G
(F/11 mo)	R (52)	pre-B-ALL	BM	91	Rn,4Rn^w	D,G	G	D,G	R,G	Rn,G	D,R
3829	D	null ALL	PB	77	R,4R^w,G	G	G	G	G	G	D,R
(M/2 mo)	R (6)	null ALL	BM	95	2R^w,Rn^w,G		G	G	G	G	GD,R
3963	D	pre-B-ALL	BM	90	2R,4R^w	G	G	G	G	G	R^w,G
(M/9 y)	R (9)	null ALL	BM	80	Rn,Rn	G	G	G	G	G	G
3991	D	pre-B-ALL	BM	73	2R,2R^w,g	R,G	R,G	D,D,g	R,R,g	R,G	D,D,g
(F/5 y)	R (17)	AML	BM	62	2R,g	R,G	R,G	D,D,g	R,R,g	R,G	D,D,g
4790	D	null ALL	BM	74	2R,7R^w,g	G	G	G	G	R,R,g	G
(F/7 mo)	R (9)	null ALL	BM	94	Rn,Rn	G	G	G	G	R,R	G

Abbreviations: M, male; F, female; mo, months; y, years; D, diagnosis; R, relapse; BM, bone marrow; PB, peripheral blood.

a. The time from diagnosis (in months) is time between diagnosis and (first or second) relapse.

b. The leukemic cell load was determined by the percentages of TdT^+, $CD10^+$, and/or $CD19^+$ cells. In case of AML the leukemic cell load was determined by the percentages of $CD13^+$, $CD33^+$, $CDw65^+$ and/or MPO^+ cells.

c. Configuration of Ig and TcR genes: (G) allele in germline configuration; (g) germline band due to the presence of non-leukemic cells (≥ 25%); (R) rearranged band; (R^w) weak rearranged band; (Rn) new rearranged band, not found at diagnosis; (Rn^w) weak new rearranged band; (D) deletion of the involved gene (segment).

d. Second relapse after BM transplantation.

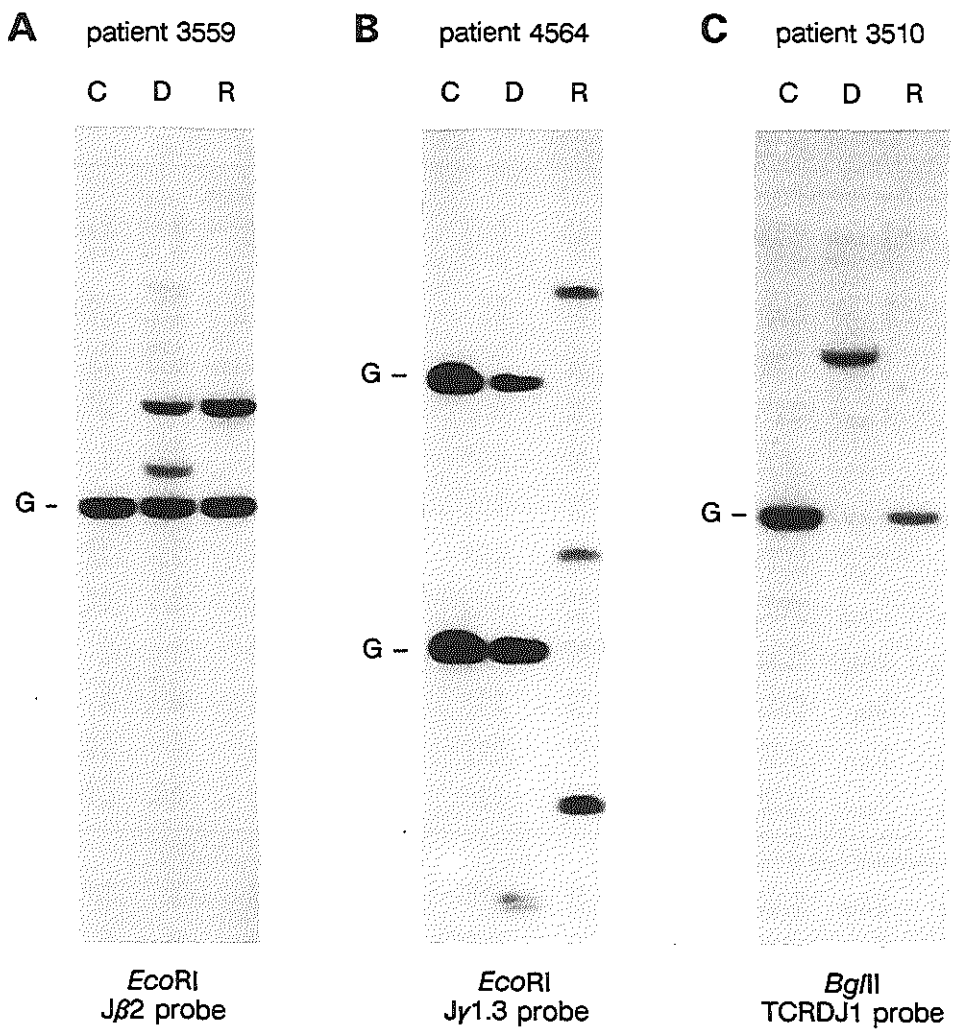

Figure 2. Southern blot analysis of TcR genes in several ALL patients at diagnosis (D) and relapse (R). Control DNA (C) and DNA from ALL patients were digested with the appropriate restriction enzymes, size separated, and blotted onto nylon membrane filters, which were hybridized with ^{32}P-labeled probes. **A,** TcR-β gene analyses using *Eco*RI with the Jβ2 probe. **B,** TcR-γ gene analyses using *Eco*RI with the Jγ1.3 probe. **C,** TcR-δ gene analyses using *Bgl*II with the Jδ1 probe. The germline bands (G) are indicated. Detailed information concerning the configuration of the different TcR genes is given in Tables 2 and 3.

Ig and TcR gene changed at relapse, while in patient 3797 a completely different IgH gene rearrangement pattern was found at relapse with IgL and TcR genes in germline configuration (Table 2). In the other 18 precursor B-ALL with immunogenotypic changes at relapse, a clonal relation could be established, based on the presence of at least one identically rearranged Ig and/or TcR band (allele) (Table 2).

In two oligoclonal precursor B-ALL cases (patients 2308 and 3829), the clonal relation

could only be proven by weak rearranged IgH gene bands (one minor subclone) at diagnosis, which remained stable at relapse. In patient 2308, this subclone probably also had a weak rearranged TcR-δ gene band (Vδ2-Dδ3), which remained stable at relapse. This could be proven by a mono-allelic *Bam*HI restriction site polymorphism in the Vδ2 gene segment used in this rearrangement (44,45).

In four precursor B-ALL (patients 2665, 3510, 3869, and 3963) loss of one rearranged IgH gene band (patients 2665, 3510, and 3963) and/or one rearranged TcR-δ gene band (all four cases) was found at relapse, indicating loss of one subclone or loss of one copy of chromosome 14. In two monoclonal precursor B-ALL (patients 4511 and 4616) the Igλ genes changed from germline at diagnosis to one rearranged allele at relapse, with no changes in the other Ig genes. In five precursor B-ALL (patients 2717, 3869, 3968, 4515, and 4778), changes in TcR-β, TcR-γ, and/or TcR-δ genes were found on one or both alleles, while the configuration of the IgH and IgL genes remained stable. Several examples of changes in Ig and TcR gene rearrangement patterns in precursor B-ALL at relapse are shown in Figures 1 and 2.

Changes in Ig and TcR gene rearrangement patterns in T-ALL at relapse

Comparative analyses of Ig and TcR gene rearrangement patterns at diagnosis and relapse were performed in all 10 T-ALL (Table 3). Differences in IgH gene rearrangement patterns between diagnosis and relapse were found in two of the four T-ALL with IgH gene rearrangements at diagnosis (Tables 3 and 4). Differences in TcR-β, TcR-γ, and TcR-δ gene rearrangement patterns were found in 30%, 20% and 10% of T-ALL respectively (Tables 3 and 4). This concerned five patients, including patient 3288 with subclone formation at the TcR-β2 gene level at diagnosis. In all five T-ALL, a clonal relation between diagnosis and relapse could be proven based on the presence of at least three identically rearranged Ig and/or TcR alleles (Table 3). In two CD3$^+$ T-ALL (patients 3288 and 3977), the changes in the TcR-β gene configuration did not affect their TcR-$\alpha\beta^+$ immunophenotype. Apparently, the changes in these patients concerned the non-functionally rearranged TcR-β alleles, leaving the functionally rearranged alleles unchanged. The change in TcR-γ gene rearrangement pattern in patient 4564 at relapse is shown in Figure 2.

Immunophenotypic shifts are related to immunogenotypic shifts at relapse in precursor B-ALL

In six out of seven precursor B-ALL, the changes in immunophenotypic classification of the leukemic cells could be correlated with changes at the Ig and/or TcR gene level (Table 2). In four cases, an intra-lineage immunophenotypic shift occurred: in two cases (patients 2717 and 3963) to an "immature" phenotype and in two cases (patients 2678 and 4501) to a more "mature" phenotype. In the two other precursor B-ALL (patients 3797 and 3991), an inter-lineage immunophenotypic shift to AML was observed (Table 2). In one patient (4714) an immunophenotypic shift from pre-B-ALL to common ALL occurred, while no immunogenotypic changes at relapse were found (Table 1).

In patients 3779 and 3797, no clonal relation in immunogenotype between diagnosis and relapse could be established. In patient 3797, this immunogenotypic shift correlated with the inter-lineage shift from common ALL at diagnosis to AML at relapse, while patient 3779 with common ALL also had a common ALL at relapse (Table 2 and Figures 1 and 2).

TABLE 3. Southern blot analysis of 10 T-ALL patients at diagnosis and subsequent relapse(s).

DCLSG number (sex/age)	disease stage (time from diagnosis)[a]	immuno-phenotypic classification	sample	leukemic cells (%)[b]	Ig genes[c]			TcR genes[c]			
					IgH	Igκ	Igλ	TcR-β		TcR-γ	TcR-δ
								Jβ1	Jβ2		
Patients without differences between diagnosis and relapse											
3052	D	CD3⁻ T-ALL	BM	98	G	G	G	D,D	R,R	R,R	R,R
(F/8 y)	R (6)	CD3⁻ T-ALL	BM	82	G	G	G	D,D	R,R	R,R	R,R
3729	D	TcR-γδ⁺ T-ALL	PB	69	G	G	G	R,G	G	R,R	R,R
(F/11 y)	R (33)	TcR-γδ⁺ T-ALL	PB	75	G	G	G	R,G	G	R,R	R,R
3987	D	TcR-αβ⁺ T-ALL	PB	95	G	G	G	R,G	R,G	R,R	D,D
(M/7 y)	R (31)	TcR-αβ⁺ T-ALL	BM	91	G	G	G	R,G	R,G	R,R	D,D
4623	D	CD3⁻ T-ALL	BM	83	R,R	G	G	D,R	R,R	R,R	D,R
(M/9 y)	R1 (12)	CD3⁻ T-ALL	BM	76	R,R	G	G	D,R	R,R	R,R	D,R
	R2 (21)	CD3⁻ T-ALL	BM	78	R,R	G	G	D,R	R,R	R,R	D,R
4727	D	CD3⁻ T-ALL	PB	96	G	G	G	R,R	G	R,R	R,R
(M/7 y)	R (12)	CD3⁻ T-ALL	BM	95	G	G	G	R,R	G	R,R	R,R
	R (12)	NT	testes	NT	G	G	G	R,R	G	R,R	R,R
Patients with differences between diagnosis and relapse											
3288	D	TcR-αβ⁺ T-ALL	PB	80	G	G	G	D,R	R,2R^{W}	R,R	D,D
(F/5 y)	R (13)	TcR-αβ⁺ T-ALL	PB	78	G	G	G	D,R	R,Rn	R,R	D,D
3810	D	CD3⁻ T-ALL	BM	90	R,R	G	G	D,G	R,R	R,R	R,R
(M/6 y)	R1 (17)	CD3⁻ T-ALL	BM	94	R,G	G	G	D,G	Rn,Rn	R,R	D,R
	R2 (26)[d]	CD3⁻ T-ALL	PB	96	R,G	G	G	D,G	Rn,Rn	R,R	D,R
3977	D	TcR-αβ⁺ T-ALL	BM	85	R,G	G	G	D,G	R,G	R,R	D,R
(M/8 y)	R (15)	TcR-αβ⁺ T-ALL	PB	90	R,G	G	G	D,Rn	R,Rn	R,R	D,R
4564	D	CD3⁻ T-ALL	PB	81	G	G	G	R,R	R,G	R,R	D,D
(M/3 y)	R (16)	CD3⁻ T-ALL	PB	90	G	G	G	R,R	R,G	Rn,Rn,Rn	D,D
4643	D	CD3⁻ T-ALL	PB	66	R,R^{W},G	G	G	D,D,g	R,R,g	R,R,g	R,R^{W},G
(M/10 y)	R (14)	CD3⁻ T-ALL	BM	93	R,G	G	G	D,D	R,R	R,Rn	R,R

Abbreviations: M, male; F, female; y, years; D, diagnosis; R, relapse; BM, bone marrow; PB, peripheral blood.
a. The time from diagnosis (months) is time between diagnosis and (first or second) relapse.
b. The leukemic cell load was determined by the percentage of TdT^{+}, CyCD3^{+} or TcR-CD3^{+} cells.
c. Configuration of Ig and TcR genes: (G) allele in germline configuration; (g) germline band due to the presence of non-leukemic cells (≥ 25%); (R) rearranged band; (R^{W}) weak rearranged band; (Rn) new rearranged band, not found at diagnosis; (D) deletion of the involved gene (segment).
d. Second relapse after bone marrow transplantation.

Although patient 3991 (pre-B-ALL) relapsed with AML with changes on two IgH alleles, the clonal relation was based on seven identically rearranged Ig/TcR alleles at diagnosis and relapse (Table 2 and Figure 1).

The changes at the Ig and TcR gene level in T-ALL were not associated with changes in immunophenotypic classification.

Changes in Ig and TcR gene rearrangement patterns are related to remission duration

In the 22 monoclonal precursor B-ALL, changes at the IgH and TcR-δ gene level were found after remission duration of at least 18 months (Tables 1 and 2). The only exception was

patient 4616 with an Igλ gene rearrangement as single change at relapse (Table 2). Changes at the TcR-β and TcR-γ gene level were found in cases with a remission duration of at least 30 months. Changes in rearrangement patterns on at least four Ig or TcR alleles were also only found after a remission duration of 30 months.

In the eight IgH bi-/oligoclonal precursor B-ALL, changes at the Ig and TcR gene level were found in cases with a remission duration of at least 6 months. These changes occurred only at the IgH and TcR-δ gene level. Changes in Igκ, TcR-β, and TcR-γ gene rearrangement patterns were found after remission duration of at least 50 months (Table 2).

In the series of 10 T-ALL, changes in IgH and/or TcR gene rearrangement patterns occurred in five patients with a remission duration of at least 1 year (Table 3).

DISCUSSION

Forty ALL patients were analyzed at diagnosis and relapse for the configuration of their Ig and TcR genes by use of Southern blot analysis. Differences in Ig and/or TcR gene rearrangement patterns between diagnosis and relapse were detected in 67% (20 cases) of precursor B-ALL (including all eight bi-/oligoclonal cases) and 50% (five cases) of T-ALL (Table 4). In precursor B-ALL especially, changes in IgH and/or TcR-δ gene rearrangements were found (17 cases), but also changes in Igκ, Igλ, TcR-β, and TcR-γ genes (11 cases) occurred. The changes in T-ALL concerned the TcR-β, TcR-γ, TcR-δ and/or IgH genes.

In 15 ALL patients (10 precursor B-ALL and five T-ALL) no differences in Ig and TcR gene rearrangement patterns between diagnosis and relapse were found, i.e., the original clone caused the relapse (Figure 3). In nine ALL patients with subclone formation at diagnosis (eight precursor B-ALL and one T-ALL), three different types of immunogenotypic changes occurred. First, in four patients, loss of (faint) rearranged Ig and/or TcR gene bands at relapse was observed, suggesting selection of a major subclone. Second, in two patients, a nearly complete change in immunogenotype at relapse occurred, except for stable rearranged IgH and/or TcR-δ gene bands, which were faint at diagnosis, suggesting selection of a minor subclone (Table 2 and Figure 3). In the remaining three bi-/oligoclonal

TABLE 4. Changes in Ig and TcR gene rearrangement patterns between diagnosis and relapse in childhood ALL.

	Ig genes			TcR genes			
	IgH	Igκ	Igλ	TcR-β	TcR-γ	TcR-δ	TOTAL
precursor B-ALL (n = 30)	40% (12/30)	28% (5/18)	43% (3/7)	45% (5/11)	33% (6/18)	44% (12/27)	67% (20/30)
T-ALL (n = 10)	50% (2/4)	– (0/0)	– (0/0)	30% (3/10)	20% (2/10)	10% (1/10)	50% (5/10)

The frequencies only concern ALL with Ig and/or TcR gene rearrangements. If no Ig or TcR gene rearrangement was found at diagnosis and/or relapse, the ALL was excluded from the calculations.

cases, as well as in 14 of the 16 monoclonal ALL patients, the differences at the Ig and/or TcR gene level between diagnosis and relapse were probably due to continuing rearrangement processes in the original clone (Figure 3) (29-36). However, in the latter ALL we cannot exclude the expansion of a minor subclone, which was not detectable at diagnosis due to the Southern blot detection limit of approximately 5% (Figure 3). It should be noted that especially in ALL with subclone formation at diagnosis, combinations of the mentioned processes of clonal evolution might occur. Only two of 25 ALL patients with immunogenotypic changes showed completely different Ig and TcR gene rearrangement patterns at relapse, suggesting the absence of a clonal relation between the leukemic cell populations at diagnosis and at relapse and the development of a secondary leukemia (Figure 3).

The changes in immunogenotype could be correlated with changes in immunophenotypic classification of the leukemic cells in six precursor B-ALL cases. In two precursor B-ALL (patients 3797 and 3991), an inter-lineage shift to AML occurred, suggesting the development of a secondary leukemia. In one of them (patient 3797), indeed a complete change in immunogenotype at relapse was found, supporting the diagnosis of secondary leukemia. However, in the other case (patient 3991), seven identically rearranged IgH/TcR alleles were still present at relapse, indicating that the AML was related to the precursor B-ALL, and thereby excluding the development of a secondary leukemia. In a third precursor B-ALL (patient 3779), the immunophenotype at diagnosis and relapse was identical, but a completely different immunogenotype at relapse was found. Based on the long remission

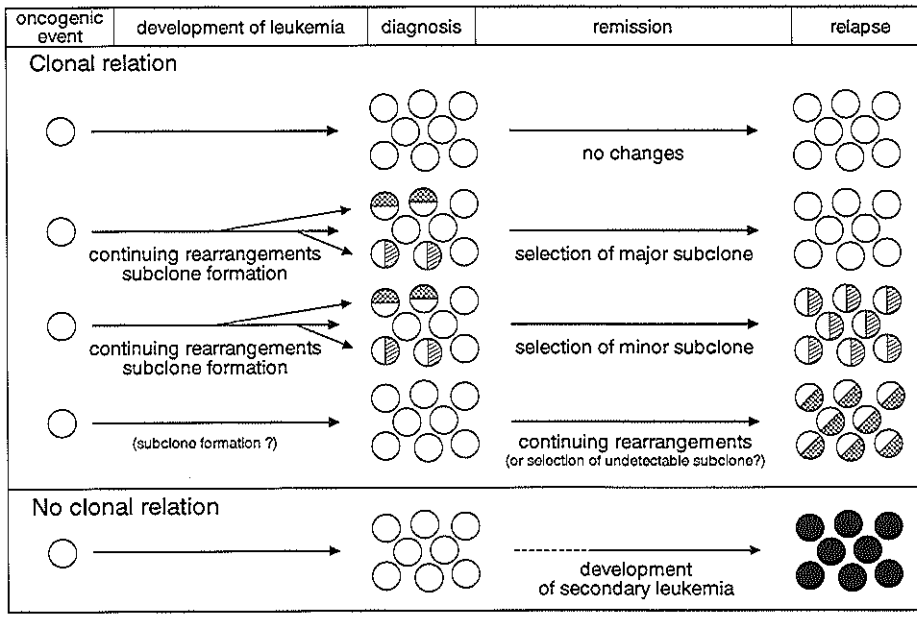

Figure 3. Hypothetical diagram of clonal evolution in ALL, based on changes in Ig and TcR gene rearrangement patterns. Note that combinations of the mentioned processes of clonal evolution might occur, especially in the leukemias with subclone formation at diagnosis. In these cases selection of a minor or major subclone at relapse may be accompanied by further continuing rearrangements, leading to the emergence of new subclones.

duration (4 years) and the extensive immunogenotypic shifts (on at least 10 alleles), a secondary precursor B-ALL was suggested. These three cases emphasize the difficulties in defining a secondary leukemia and the importance of cytogenetic analysis of malignant cell samples to determine the clonal relation or clonal evolution between diagnosis and relapse. Unfortunately, cytogenetic data in ALL patients are not always available, as was the case in patients 3779 and 3991.

Interestingly, the development of changes in Ig and TcR gene rearrangement patterns was related to remission duration, suggesting an increasing chance of continuing Ig and TcR gene rearrangement processes with time. This phenomenon has also been reported by Wasserman et al., who analyzed IgH gene junctional regions in 12 leukemias at diagnosis and relapse (34). A second phenomenon was the finding that these changes in Ig and/or TcR gene rearrangement patterns occurred in a hierarchical order, i.e. changes in IgH and TcR-δ genes were already detectable after 6 months of remission duration, whereas changes in other Ig and TcR genes were generally found after 1 to 2 years of remission duration. This hierarchical order might be related to the hierarchical order in Ig and TcR gene rearrangements during B- and T-cell differentiation.

As indicated earlier, the PCR-technique can be used for the amplification of "tumor-specific" junctional regions of rearranged IgH, TcR-γ and/or TcR-δ genes to detect MRD in ALL (20-27).

Recently, the significance and prognostic implications of MRD detection by PCR in childhood ALL at different time points during therapy has been determined (46-48). Rovera et al. concluded that PCR detection of high levels of residual disease at the end of induction therapy identifies patients at increased risk for relapse during therapy (46,47). Furthermore, they concluded that absence of detectable MRD at the end of chemotherapy is not sufficient to assure that the patient is cured, indicating that after treatment frequent serial monitoring is required for the early prediction of relapse (46,47). Potter et al. subscribe to these findings and conclude that detectable MRD at the end of treatment would predict future relapse (48).

It should be noted that the extensive changes reported here in Ig and/or TcR gene rearrangement patterns between diagnosis and relapse (clonal evolution) might hamper the detection of MRD by PCR-mediated amplification of "leukemia-specific" Ig and/or TcR gene junctional regions, especially in case of bi-/oligoclonal precursor B-ALL (27,49). This might lead to false-negative MRD results (27,49). It has been argued that changes in Ig and/or TcR gene rearrangement patterns as detected by Southern blotting do not necessarily lead to false-negative PCR results, because a change in Southern blot band pattern does not automatically imply that the complete sequence of the junctional region of a rearranged Ig or TcR gene has changed (50). For example, VH replacements in completely rearranged IgH genes generally do not affect the original V-D-JH junctional regions, and also the D-JH junctional region will generally remain stable in subclones, which rearranged different VH gene segments to an identical D-JH precursor.

Recent studies on IgH junctional regions in 13 bi-/oligoclonal precursor-B-ALL at diagnosis (34-36) showed that, indeed, most multiple IgH gene rearrangements can be explained by VH replacements (seven cases), or rearrangement of different VH gene segments to a preexisting D-JH rearrangement (four cases). In two cases, unrelated junctional regions were found, suggesting *de novo* IgH gene rearrangements. Also,

comparison of junctional regions at diagnosis and subsequent relapse showed that in IgH monoclonal precursor B-ALL, the instability of V-D-JH joinings was partly generated via VH replacements with a conserved junctional region, partly via rearrangements of different VH gene segments to an identical D-JH precursor, and partly due to minor changes in the junctional regions (34,51). However, in the two published bi-/oligoclonal precursor B-ALL cases, extensive changes in V-D-JH junctional regions were found at relapse, which could be explained by outgrowth of a minor subclone, *de novo* IgH gene rearrangements, and loss of a subclone at relapse (35,36). Changes in TcR junctional regions between diagnosis and subsequent relapse were reported in two cases (52,53). One case could be explained by the occurrence of continuing rearrangements at the TcR-γ gene level, and, in the other case, the major subclone disappeared as deduced from TcR-δ gene analysis (52,53).

Although in several cases, the V-D-JH or D-JH junctional regions remained unchanged, it should be noted that differences in VH gene segments might influence the efficiency of the PCR due to changes in VH primer annealing, dependent on the sequence of the newly rearranged VH gene segment. This pitfall might partly be overcome by using a panel of seven VH FR1 family-specific primers for PCR-mediated MRD detection during follow-up, as suggested by Deane et al. (54).

In Table 5, we have summarized our data concerning the changes and stability of IgH, TcR-γ, and TcR-δ gene rearrangement patterns at relapse, because the junctional regions of these genes are generally used as PCR targets for the detection of MRD. Table 5 shows that changes in IgH gene rearrangement patterns at relapse occur at high frequency in precursor B-ALL, especially in bi-/oligoclonal cases. Changes in TcR-γ and TcR-δ gene rearrangements at relapse are found in both precursor B-ALL and T-ALL, but generally concern only one allele. Despite the high frequency of immunogenotypic changes, at least one major IgH, TcR-γ and/or TcR-δ rearranged band (allele) remained stable in 75-90% of precursor

TABLE 5. Changes in IgH, TcR-γ and TcR-δ gene rearrangement patterns in 40 childhood ALL at relapse.

	Changes in rearrangement patterns at relapse			Stability of at least one major rearranged band (allele)		
	IgH	TcR-γ	TcR-δ	IgH	TcR-γ and/or TcR-δ	IgH, TcR-γ and/or TcR-δ
precursor B-ALL with monoclonal IgH genes at diagnosis (n = 22)	18% (4/22)	29% (4/14)	40% (8/20)	90% (19/21)[a]	72% (13/18)[b]	91% (20/22)
precursor B-ALL with bi/oligoclonal IgH genes at diagnosis (n = 8)	100% (8/8)	50% (2/4)	57% (4/7)	38% (3/8)[c]	63% (5/8)	75% (6/8)
T-ALL (n = 10)	50% (2/4)	20% (2/10)	10% (1/10)	100% (4/4)	90% (9/10)	90% (9/10)

The frequencies only concern ALL with IgH, TcR-γ and/or TcR-δ gene rearrangements. If no IgH, TcR-γ or TcR-δ gene rearrangement was found at diagnosis and/or relapse, the ALL was excluded from the calculations.

a. Patient 4511 was excluded from the calculations because both IgH alleles were deleted at diagnosis and at relapse.

b. Patients 3968 and 4616 were excluded from the calculations because of germline TcR-γ genes and biallelic TcR-δ gene deletions.

c. In two additional bi-/oligoclonal cases (patients 2308 and 3829), a rearranged band at relapse was identical to a weak rearranged band at diagnosis, suggesting selection of a minor subclone.

B-ALL and 90% of T-ALL. The optimal targets for MRD detection in monoclonal precursor B-ALL are IgH genes, but it might be valuable to monitor the TcR-γ gene rearrangements as well, especially in cases with germline or deleted IgH genes on one or both alleles. In T-ALL, the TcR-γ and TcR-δ genes represent optimal MRD-PCR targets. However, in bi-/oligoclonal precursor B-ALL (30-40% of the total group of precursor B-ALL), it will be difficult to estimate which minor or major IgH gene band (allele) will remain stable, especially in cases with more than two subclones. This implies that in these leukemias the MRD-PCR monitoring should not be restricted to IgH genes, but that TcR-γ and TcR-δ gene rearrangements should be monitored as well. Therefore, our data indicate that MRD detection in ALL patients by PCR techniques needs monitoring of two or more junctional regions of IgH, TcR-γ, and/or TcR-δ genes in order to prevent false-negative results.

Our study indicates that changes in Ig and TcR gene rearrangement patterns between diagnosis and relapse occur at a high frequency (67% in precursor B-ALL and 50% in T-ALL) and are caused by different processes of clonal evolution. These changes in immunogenotype appear to be related to remission duration and seem to occur in a hierarchical order. The heterogeneity reported here in Ig and/or TcR gene rearrangement patterns at diagnosis and relapse has implications for the detection of MRD using PCR-mediated amplification of "tumor-specific" Ig and TcR gene junctional regions, because it can be foreseen that subclone formation at diagnosis and clonal evolution at relapse might lead to false-negative results. Therefore, it is important to define optimal strategies in exploiting IgH, TcR-γ, and/or TcR-δ gene rearrangements for the detection of MRD by use of PCR techniques.

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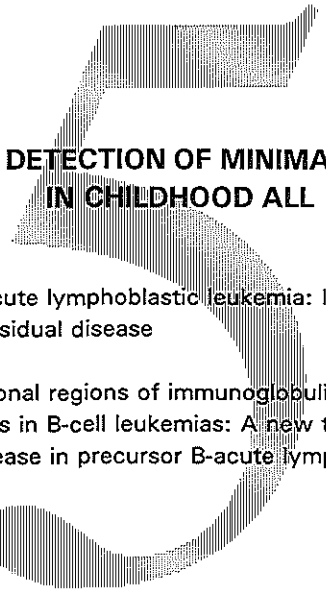

POSSIBILITIES FOR DETECTION OF MINIMAL RESIDUAL DISEASE IN CHILDHOOD ALL

CHAPTER 5.1

MOLECULAR BIOLOGY OF ACUTE LYMPHOBLASTIC LEUKEMIA: Implications for detection of minimal residual disease*

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ABSTRACT

Acute lymphoblastic leukemias (ALL) are characterized by high frequencies of clonal chromosome aberrations (ploidy aberrations and translocations) as well as by clonal rearrangements of immunoglobulin (Ig) and T-cell receptor (TcR) genes. These two types of clonal molecular characteristics can be used as patient-specific markers for detection of minimal residual disease (MRD) by use of polymerase chain reaction (PCR) technology.

In case of chromosome aberrations, this concerns translocations which result in fusion genes and fusion transcripts, such as in t(9;22), t(1;19), and t(4;11) in precursor B-ALL, or aberrations with site-specific breakpoints such as *TAL1* deletions in T-ALL. In fact, any precisely identifiable breakpoint fusion region of a chromosome aberration can be used as PCR target for MRD detection during follow-up of leukemia patients. So far such breakpoint fusion region can be identified in 15-20% of childhood ALL and 25-30% of adult ALL.

Junctional regions of rearranged Ig and TcR genes represent the second type of MRD-PCR target, which can be precisely identified in ~80% of precursor B-ALL and in >90% of T-ALL. This especially concerns the junctional regions of rearranged Ig heavy-chain (IgH), TcR-γ, and TcR-δ genes. In contrast to chromosome aberrations, the junctional regions of Ig and TcR genes might not remain stable during the disease course, because of continuing rearrangement processes and subsequent subclone formation. These continuing rearrangements are extensive in IgH genes, resulting in the presence of subclones in 30-40% of precursor B-ALL at diagnosis and changes in rearrangement patterns at relapse in 40% of cases. Continuing rearrangement processes also cause changes in TcR-γ and TcR-δ gene rearrangement patterns at relapse in 10-20% of T-ALL and 35-45% of precursor B-ALL. This heterogeneity in Ig/TcR gene rearrangement patterns at diagnosis and relapse might hamper PCR-mediated MRD detection. However in 75-90% of ALL cases, at least one IgH, TcR-γ, or TcR-δ allele remains stable at relapse. Therefore, two or more junctional

* Haematol Blood Transfus, in press.

regions of different Ig/TcR genes should be monitored for optimal MRD detection during follow-up of ALL patients.

Well-designed prospective studies on large series of ALL patients have to demonstrate the clinical impact of MRD detection.

INTRODUCTION

Approximately 80-85% of childhood leukemias and approximately 7% of adult leukemias represent ALL with an incidence of 3-4 per 100,000 children and 1-2 per 100,000 adults, respectively. Despite major improvements in ALL treatment during the last two decades, 20-30% of children with ALL and 60-75% of adult ALL patients relapse (1-7). Apparently, the current treatment protocols are not capable of killing all leukemic cells in these patients, although the far majority reach complete remission according to cytomorphological criteria. Since the detection limit of cytomorphological techniques is not lower than 1-5% leukemic cells, it is obvious that such techniques can only provide superficial information about the effectiveness of leukemia treatment. Techniques with a higher sensitivity to detect MRD are needed to obtain better insight in the reduction of tumor mass during induction treatment and further eradication of the leukemic cells during maintenance treatment (Figure 1).

During the last decade, several methods for detection of MRD have been developed and evaluated, such as cytogenetics, cell culture systems, immunological marker analysis,

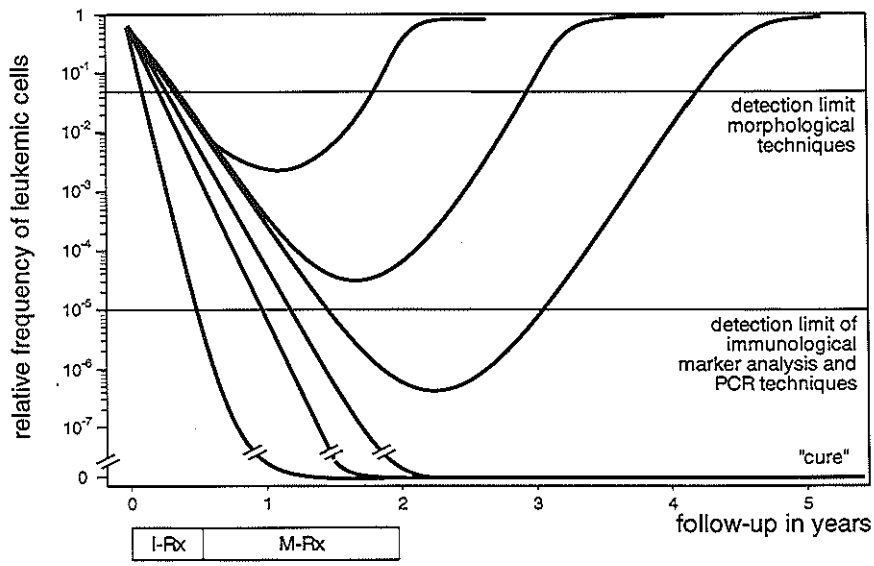

Figure 1. Diagram of putative relative frequencies of ALL cells in peripheral blood and bone marrow during treatment and development of relapse. The detection limit of cytomorphological techniques as well as the detection limit of immunological marker analysis and PCR techniques are indicated. I-Rx = induction treatment; M-Rx = maintenance treatment.

fluorescence in situ hybridization, and molecular-biological techniques (8-18). In most studies, the detection limits of these techniques are 1-5% malignant cells. However, depending on the immunophenotype and the genotype of the leukemia, immunological marker analysis and the PCR technique are able to detect lower frequencies of leukemic cells, as low as 10^{-4} to 10^{-5} (10 to 1 leukemic cells per 100,000 normal cells) (Figure 1) (9,10,15,19-21).

In the PCR technique, patient-specific nucleotide sequences are used as targets for MRD detection, such as junctional regions of rearranged Ig and TcR genes as well as breakpoint fusion regions of chromosome aberrations.

Ig and TcR gene rearrangement processes

Ig and TcR gene complexes consist of multiple variable (V), diversity (D), and joining (J) gene segments, combinations which code for the variable protein domains of the antigen-specific receptors (16).

The rearrangement processes in Ig and TcR genes during early lymphoid differentiation result in specific combinations of V, (D,) and J gene segments, thereby deleting all intervening sequences. These gene rearrangements are mediated via recombination signal sequences (RSS), generally heptamer-nonamer sequences, which flank the V, (D,) and J gene segments (16,22,23).

The many possible different combinations of V, (D,) and J gene segments form the so-called combinatorial diversity. This diversity is drastically increased by random deletion and insertion of nucleotides at the junctions of the rearranging gene segments, which together form the junctional diversity. The junctional regions are different in each lymphocyte, even in unrelated lymphocytes which have rearranged the same V, (D,) and J gene segments (16,22,23). Therefore, junctional regions are "fingerprint-like" sequences, which can be used for identification of lymphocytes (20). The combined combinatorial and junctional diversity not only guarantees the enormous diversity of antigen-specific receptors of normal lymphocytes, but also represents an unique diagnostic identification system for "immature" and "mature" clonal lymphoproliferative diseases, such as ALL and other lymphoid leukemias and lymphomas (24).

The far majority of both B-lineage ALL (i.e. precursor B-ALL) and T-lineage ALL (T-ALL) indeed have rearranged Ig and TcR genes, respectively (24-27). Also cross-lineage gene rearrangements occur in high frequencies. This especially concerns TcR gene rearrangements in precursor B-ALL (Table 1) (24,25,27).

Southern blot and PCR analysis of Ig and TcR genes allow the detection of clonal Ig and TcR gene rearrangements. Application of the Southern blot technique is based on detection of clonal changes in length of restriction fragments due to clonal rearrangements of V, (D,) and J gene segments and therefore takes advantage of the combinatorial diversity. This technique has a detection limit of ~5%, i.e. ~5 clonal cells between 100 normal (polyclonal) cells (16). The PCR technique focusses on junctional region of rearranged Ig and TcR genes and is more sensitive than the Southern blot technique, especially if junctional region-specific probes are used (see below).

Chromosome aberrations

Like other malignancies, ALL can be regarded as an acquired genetic disease, which is

TABLE 1. Frequencies of Ig and TcR gene rearrangements and deletions in precursor B-ALL and T-ALL.

	IgH		Igκ		Igλ	TcR-β	TcR-γ	TcR-δ	
	R	D	R	D	R	R	R	R	D
Precursor B-ALL (n=108)	96%	3%	29%	50%	21%	36%	57%	50%	40%
T-ALL (n=138)	20%	0%	0%	0%	0%	90%	93%	70%	25%
Total ALL[a]	81%	2%	23%	40%	17%	47%	64%	54%	37%

Abbreviations: R, one or both alleles rearranged; D, both alleles deleted or one allele deleted with the other in germline configuration.
a. Estimated frequencies, based on the fact that in children precursor B-ALL and T-ALL represent 80-85% and 15-20% of ALL, respectively and that in adults precursor B-ALL and T-ALL represent 75-80% and 20-25% of ALL, respectively.

caused by alterations in the structure or expression of critical genes. In particular, derangements of genes which normally control growth and differentiation of early lymphoid cells are thought to play a role in the development of ALL, such as proto-oncogenes, tumor suppressor genes, transcription factors, and especially Ig and TcR genes. The latter is illustrated by the fact that chromosome breakpoints in ALL often involve chromosome bands, which contain Ig or TcR genes: 14q32 (IgH gene), 2p12 (Igκ gene), 22q11 (Igλ gene), 14q11 (TcR-α/δ gene complex), 7q35 (TcR-β gene), and 7p15 (TcR-γ gene). Several chromosome aberrations in ALL appear to be associated with particular subtypes (28-30).

So far, most chromosome aberrations have been detected by routine microscopic cytogenetics (G and R band staining patterns). Recently *in situ* hybridization and flow karyotyping have been introduced for detection of chromosome aberrations, but their application is dependent on the availability of suitable probes and the resolution of scatter/staining patterns of chromosomes, respectively. Futhermore, if the breakpoints of a particular chromosome aberration in different patients are well-defined and/or clustered in a small area, also Southern blotting and even the PCR technique can be used to detect these aberrations (see below).

Several studies indicate that RSS-like sequences probably play a role in aberrant gene rearrangements in lymphoid malignancies, especially in chromosome aberrations involving Ig and TcR genes. In T-ALL the TcR-α/δ locus in band 14q11 is frequently involved, such as in t(10;14)(q24;q11) and t(1;14)(p34;q11) (31-33). In the latter aberration the *TAL1* gene on chromosome 1 is translocated to the TcR-δ gene. Studies on this translocation have lead to the discovery of site-specific (sub-microscopic) deletions of ~90 kb in the *SIL* gene/*TAL1* gene region on chromosome 1 (31,32). These so-called *TAL1* gene deletions are reported to occur in 10-30% of T-ALL (32,34-42). So far five types of *TAL1* deletions have been described, all of which represent rearrangements occurring via RSS-like sequences (37,39,40). All five types of *TAL1* deletions appear to use the same 5′ heptamer RSS, located between the first and second *SIL* exons, but different 3′ heptamer-nonamer RSS, which are located in the 5′ part of the *TAL1* locus. This results in the deletion of all coding *SIL* exons and places the coding *TAL1* exons under direct control of the *SIL* gene regulatory elements. The fusion regions of the breakpoints in all five types of

TAL1 deletions show random deletion and insertion of nucleotides. Therefore, these fusion regions are different in each patient and resemble junctional regions of rearranged Ig and TcR genes (42-44).

DETECTION OF MRD BY USE OF THE PCR TECHNIQUE

Basic principles of PCR-mediated MRD detection

The PCR technique allows selective amplification of a particular DNA segment or messenger (m)RNA (after reverse transcription into copy (c)DNA) (45-47). If the target DNA or mRNA sequences are tumor-specific, it is possible to detect a few malignant cells in between many normal cells. Theoretically the detection limit of the PCR technique is approximately 10^{-6}, if a DNA segment is used as PCR target. This is based on the assumption that one cell contains ~10 pg DNA and that one PCR tube can contain maximally 10 μg DNA. This detection limit can indeed be reached, but generally varies between 10^{-4} and 10^{-6}, dependent on the type of tumor-specific PCR target (15,19,20,48,49). In the initial PCR studies on the detection of MRD, well-defined chromosome translocations were used as tumor-specific markers (48-51). However, it is also possible to detect MRD by use of PCR-mediated amplification of junctional regions of rearranged Ig and TcR genes (20,52-59). Because the PCR technique is highly sensitive, all possible precautionary measures should be taken to prevent cross-contamination of PCR products between patient samples in PCR-mediated MRD studies (47,60).

Chromosome aberrations as leukemia-specific PCR targets for MRD detection

In the initial MRD-PCR studies, t(14;18)(q32;q21) and t(9;22)(q34;q11) were used as PCR targets (48-51). For this purpose oligonucleotide primers were designed to recognize sequences at opposite sides of the breakpoint fusion region, so that the PCR product contained the tumor-specific fusion sequences. In routinely performed MRD-PCR analysis, the PCR products should not exceed ~2 kilobases (kb) (46,47). Therefore, PCR-mediated amplification of DNA sequences can only be used for chromosome aberrations in which the breakpoints of different patients cluster in a small area (total breakpoint area: <2 kb), such as in t(14;18) where the *BCL2* gene is juxtaposed to one of the J gene segments of the IgH genes (61,62). Other examples are the T-ALL-associated aberrations t(1;14)(p34;q11), t(10;14)(q24;q11) and the *TAL1* deletions (31-33).

In most translocations, the breakpoints are spread over much larger areas than 2 kb. This implies that the precise breakpoint recombination area has to be determined for each individual patient, which is a laborious and time-consuming effort (63). However, in several leukemias it has been found that, as a consequence of the translocation, a new leukemia-specific fusion gene has been created, which is transcribed into a leukemia-specific fusion mRNA. This fusion mRNA can be used as target for the MRD-PCR analysis after reverse transcription into cDNA (Table 2). Examples are: *bcr-abl* mRNA in case of t(9;22) (64-66), *E2A-PBX1* mRNA in most cases of t(1;19) (67-69), and the recently discovered *MLL/ALL1-AF4* mRNA in null ALL with t(4;11)(q21-q23) (Table 2) (70-78).

An advantage of using specific chromosome translocations as tumor-specific markers is their stability during the disease course. However, only 20-25% of childhood ALL and 30-35% of adult ALL, have a specific, microscopically detectable chromosome translocation

TABLE 2. PCR techniques for MRD detection in ALL patients.

Type of leukemia	PCR analysis of junctional regions of Ig or TcR genes[a]		PCR analysis of chromosome aberrations[b]		
	Ig or TcR gene	Frequency of applicability[c]	Aberration	Frequency of applicability[c]	Target (mRNA or DNA)
Precursor B-ALL	IgH: V-D-J	80%	t(9;22)(q34;q11)	adult: 30-35% childhood: 5-8%	*BCR-ABL* (mRNA)
	TcR-γ: Vγ-Jγ	55%	t(1;19)(q23;p13)	5-8%	*E2A-PBX1* (mRNA)
	TcR-δ: Vδ2-Dδ3 or Dδ2-Dδ3	40%	t(4;11)(q21;q23)	~3%	*ALL1-AF4* (mRNA)
T-ALL	IgH: V-D-J	15%?	deletion in *TAL1* gene	10-30%	del(*TAL1*) (DNA)
	TcR-γ: Vγ-Jγ	90%	t(1;14)(p34;q11)	1-3%	*TAL1*-TcR-δ (DNA)
	TcR-δ: Vδ-Jδ, Dδ-Jδ, or Vδ-Dδ	50%	t(10;14)(q24;q11)	1-3%	*TCL3*-TcR-δ (DNA)

a. The detection limit of PCR analysis of junctional regions of rearranged Ig and TcR genes varies from 10^{-3} to 10^{-6} and is dependent on "normal background" and the size of the junctional region.
b. The detection limit of PCR analysis of chromosome aberrations is 10^{-4} to 10^{-6}.
c. The indicated percentages represent frequencies within the precursor B-ALL and T-ALL groups.

and in a part of these aberrations the precise breakpoints are not (yet) known (30,36,79,80).

One should be aware that PCR products obtained via leukemia-specific fusion mRNA are not patient-specific. Therefore, false-positive results due to cross-contamination of PCR products between samples from different patients are difficult to recognize. This is in contrast to the PCR products obtained from breakpoint fusion regions of *TAL1* deletions, which can be identified by use of patient-specific oligonucleotide probes (42-44).

Junctional regions as patient-specific PCR targets for MRD detection

As indicated above, junctional regions of rearranged Ig and TcR genes are "fingerprint-like" sequences which are assumed to be different in each lymphocyte and therefore also in each ALL. Based on this assumption, it has been suggested that Ig and TcR gene junctional regions can be used as targets for MRD-PCR analysis, using V, (D,) and J gene-specific oligonucleotides as primers (Table 2) (52-54,56-59). The choice of primers is dependent on the type of Ig or TcR gene as well as the rearranged gene segments. It may be possible to design general primers, which recognize (virtually) all V or J gene segments of a particular Ig or TcR gene complex, or specific primers, which recognize individual V or J gene segments or families of V or J gene segments (52-54,81,82).

An advantage of using IgH, TcR-γ, and TcR-δ junctional regions as targets for the MRD-PCR technique is the fact that the IgH gene complex contains only six VH families and six JH gene segments (Figure 2) and the fact that the TcR-γ and TcR-δ genes contain only a few V, (D,) and J gene segments (16,83-89), whereas the junctional regions of most complete V-(D-)J rearrangements are large (57,58,82,88-91). This implies that only a restricted number of oligonucleotide primers is needed, while the junctional regions will differ extensively between the leukemias. In principle, also PCR analysis of junctional regions of rearranged Ig light-chain, TcR-α, and TcR-β genes may be applicable for detection

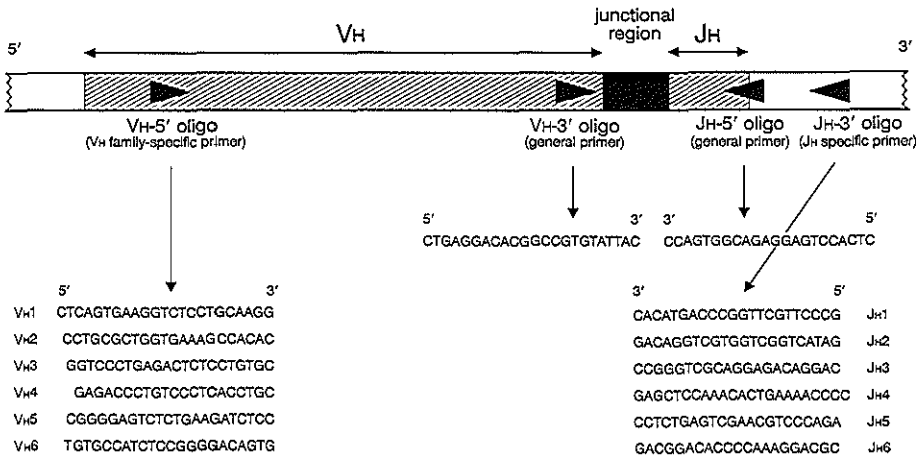

Figure 2. Schematic diagram of a VH gene segment joined to a JH gene segment via a fictitious junctional region, which consists of DH-derived nucleotides and randomly inserted nucleotides. The approximate position of the VH-family-specific primers, VH consensus primer, JH consensus primer, and JH specific primers are indicated with arrows. The presented oligonucleotides can be used as primers for the PCR-mediated amplification of the junctional regions of rearranged IgH genes (52,81,83).

of MRD. However, especially PCR analysis of TcR-α and TcR-β genes at the DNA level needs many different oligonucleotide primers.

In ~80% of precursor B-ALL, IgH gene rearrangements can be detected by PCR analysis with VH and JH primers (92), suggesting that in the remaining cases only incompletely rearranged (D-JH), germline, or deleted IgH genes occur (Table 2). In principle Vγ and Jγ primers detect each TcR-γ gene rearrangement, whereas ~80% of TcR-δ gene rearrangements in precursor B-ALL and ~70% of TcR-δ gene rearrangements in T-ALL can be identified by PCR analysis (Table 2) (27).

The obtained junctional region PCR products can be analysed in a dot blot or Southern blot by use of a patient-specific junctional region probe in order to discriminate between the leukemia-derived junctional regions and junctional regions from normal cells which have rearranged the same (or comparable) V and J gene segments as the leukemic cells. For each leukemia one should determine at diagnosis which junctional region(s) can be used as targets for the MRD-PCR analysis during follow-up and which primers are optimal for this purpose (Table 2). Also the patient-specific junctional region probes have to be designed for each individual patient at initial diagnosis (20,21,52-59).

PITFALLS OF MRD-PCR ANALYSIS OF JUNCTIONAL REGIONS

From the above described data, it can be concluded that PCR-mediated amplification of junctional regions for detection of MRD is a sensitive technique, which can be applied in the majority of ALL. However, one should realize that this technique has several pitfalls (Table 3).

TABLE 3. Limitations and pitfalls of MRD-PCR analysis of junctional regions.

1. Occurrence of oligoclonality at Ig or TcR gene level (e.g. IgH gene level in precursor B-ALL).
   Consequence: false-negative results.

2. Occurrence of clonal evolution at Ig or TcR gene level.
   Consequence: false-negative results.

3. Background of normal cells with the same rearranged gene segments as the leukemic cells. (e.g. Vδ1-Jδ1 rearrangements and Vγ1-Jγ2.3 rearrangements occur in 0.1-5% and a large part of normal blood T-lymphocytes, respectively).
   Consequence: lower sensitivity of the PCR technique and high dependence on the specificity of the junctional region probe for detection of patient-specific PCR products.

4. Type of rearrangement and size of junctional region (e.g. complete Vδ-Jδ rearrangements have large junctional regions, but Dδ-Dδ rearrangements have short junctional regions).
   Consequence: size of junctional region influences the sensitivity of the MRD-PCR technique.

5. Hybridization conditions, washing stringency and exposure time influence the specificity and sensitivity of the MRD-PCR technique.
   Consequence: careful evaluation of the conditions for each patient and use of positive and negative control samples in various dilutions is necessary.

Occurrence of oligoclonality

A necessary condition for the MRD-PCR technique is the stability of the patient-specific junctional region during follow-up. However, in 30-40% of precursor B-ALL at diagnosis multiple IgH gene rearrangements are found (Figure 3A) (26,93-96). These are probably caused by continuing rearrangement processes, which lead to subclone formation (bi-/oligoclonality) (26,94). Such subclone formation at diagnosis appeared to be rare at the TcR-γ and TcR-δ gene level in T-ALL and in cross-lineage TcR gene rearrangements in precursor B-ALL (24,27,97).

Occurrence of clonal evolution

Changes in Ig and TcR gene rearrangement patterns at relapse is also an important pitfall in PCR analysis of junctional regions for MRD detection. In 40 children with ALL, we compared the Ig and TcR gene rearrangement patterns at diagnosis and relapse. An example is shown in Figure 3B. In Table 4, we summarized the data concerning the changes and stability of IgH, TcR-γ, and TcR-δ gene rearrangements at relapse (97). This table shows that changes in IgH gene rearrangement patterns at relapse occur at high frequency in precursor B-ALL, especially when subclone formation is already present at diagnosis (97). Changes in TcR-γ and TcR-δ gene rearrangements at relapse are found in both precursor B-ALL and T-ALL, but generally concern only one allele (97). It should be emphasized that Table 4 focusses on changes and stability of gene rearrangements which already existed at diagnosis, i.e. "new" rearrangements at relapse in genes which were in germline configuration or deleted at diagnosis were not included in the calculations, because such rearrangements could not have been used for prospective MRD-PCR studies.

Figure 4 shows the changes and stability of rearranged Ig and TcR gene bands (alleles) and their relation to the remission duration in the 40 childhood ALL cases. Changes in at least one of the three major MRD-PCR targets (IgH, TcR-γ, and TcR-δ) were found in 40%

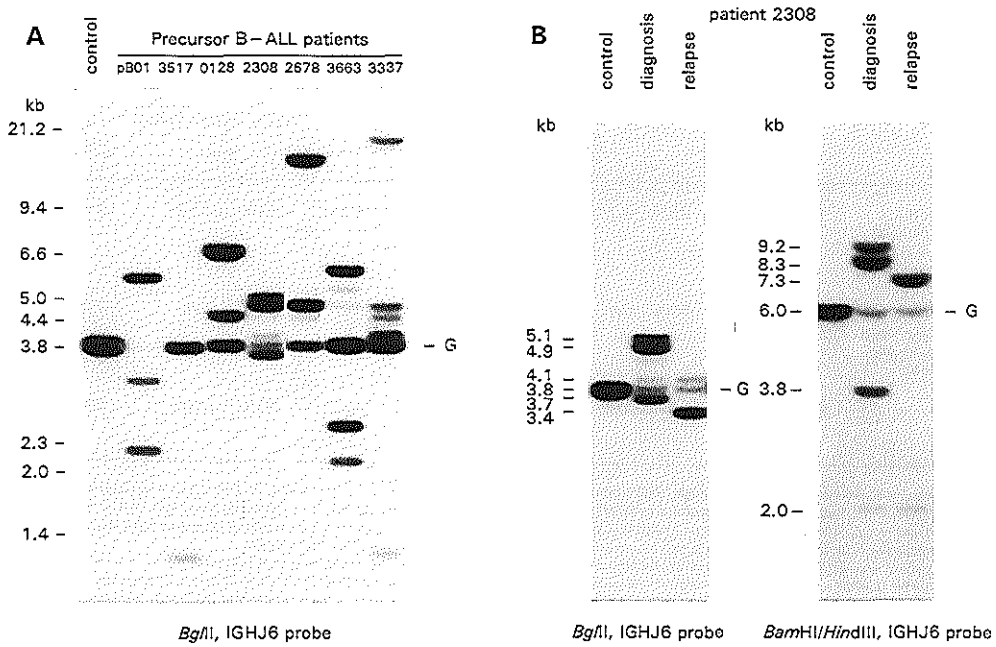

Figure 3. Southern blot analysis of IgH genes in several precursor B-ALL patients at diagnosis (**A**) and one precursor B-ALL patient (2308) at diagnosis and subsequent relapse (**B**). Control DNA and DNA from precursor B-ALL patients were digested with *Bgl*II and in case of patient 2308 also with *Bam*HI/*Hin*dIII, size-separated, and blotted onto nylon membrane filters, which were hybridized with the ^{32}P-labeled IGHJ6 probe. (**A**) In five of the here presented patients, more than two rearranged IgH gene bands were observed. The multiple IgH gene bands differed in density in most cases. With cytogenetic analysis we could exclude the presence of more than two chromosomes 14. (**B**) In patient 2308, three distinct and one weak rearranged band, were seen at diagnosis in both digests, whereas only one distinct and one weak rearranged band were detected at relapse. The weak band at diagnosis was probably identical to the weak band at relapse, but all other bands differed in size in both digests, indicating that clonal evolution had occurred in this precursor B-ALL patient at relapse.

(9/22) of the monoclonal precursor B-ALL after remission duration of at least 18 months (Figure 4A). All eight bi-/oligoclonal precursor B-ALL showed changes at relapse in IgH, TcR-γ, and/or TcR-δ genes, which were already found after a short remission duration of at least 6 months (Figure 4B). In 30% of T-ALL, changes in IgH, TcR-γ, and/or TcR-δ gene rearrangement patterns occurred after a remission duration of at least 14 months (Figure 4C). Despite the high frequency of immunogenotypic changes, at least one major IgH, TcR-γ, and/or TcR-δ rearranged band (allele) remained stable in 75-90% of precursor B-ALL and 90% of T-ALL (Figure 4 and Table 4).

Several reports indicate that changes in IgH gene rearrangements at relapse do not necessarily imply that the complete sequence of the junctional regions has changed (98,99). For example, VH replacements in completely rearranged IgH genes generally do not affect the original V-D-JH junctional regions, and also the D-JH junctional regions will generally remain stable in subclones, which rearranged different VH gene segments to an identical D-JH precursor (100).

TABLE 4. Changes in IgH, TcR-γ, and TcR-δ gene rearrangement patterns in 40 childhood ALL at relapse[a].

	Changes in rearrangement patterns at relapse			Stability of at least one major rearranged band (allele)		
	IgH	TcR-γ	TcR-δ	IgH	TcR-γ and/or TcR-δ	IgH, TcR-γ, and/or TcR-δ
precursor B-ALL with monoclonal IgH genes at diagnosis (n=22)	19% (4/21)[b]	29% (4/14)	40% (8/20)	90% (19/21)[b]	72% (13/18)[c]	91% (20/22)
precursor B-ALL with bi-/oligoclonal IgH genes at diagnosis (n=8)	100% (8/8)	50% (2/4)	57% (4/7)	38% (3/8)[d]	63% (5/8)	75% (6/8)
T-ALL (n=10)	50% (2/4)	20% (2/10)	10% (1/10)	100% (4/4)	90% (9/10)	90% (9/10)

a. The frequencies only concern ALL with IgH, TcR-γ, and/or TcR-δ gene rearrangements. If no IgH, TcR-γ, or TcR-δ gene rearrangement was found at diagnosis, the ALL was excluded from the calculations (all 40 ALL were extensively tested for the occurrence of IgH, TcR-γ, and TcR-δ gene rearrangements). "New" rearrangements at relapse in genes which were in germline configuration or deleted at diagnosis were also not included in the calculations, because such rearrangements could not have been used for prospective MRD-PCR studies.
b. One patient was excluded from the calculations because both IgH alleles were deleted at diagnosis and at relapse.
c. Two patients were excluded from the calculations because of germline TcR-γ genes and biallelic TcR-δ gene deletions at diagnosis.
d. In two additional bi-/oligoclonal cases, a rearranged band at relapse was identical to a weak rearranged band at diagnosis, suggesting selection of a minor subclone.

Our childhood ALL study indicates that IgH genes represent optimal targets for MRD detection in monoclonal precursor B-ALL, but it might be valuable to monitor the TcR-γ and/or TcR-δ gene rearrangements as well, especially in cases with germline or deleted IgH genes on one or both alleles. In T-ALL the TcR-γ and TcR-δ genes represent optimal MRD-PCR targets (97). However, in bi-/oligoclonal precursor B-ALL (30-40% of the total group of precursor B-ALL), it will be difficult to estimate which minor or major IgH gene band (allele) will remain stable, especially in cases with more than two subclones. This implies that in these leukemias the MRD-PCR monitoring should not be restricted to IgH genes, but that TcR-γ and TcR-δ gene rearrangements should be monitored as well. Two recently published PCR studies, describing changes and stability of IgH, TcR-γ, and TcR-δ gene rearrangements at relapse in ALL, subscribed to these findings (101,102). Therefore, we conclude that MRD detection in ALL patients by PCR techniques needs monitoring of two or more junctional regions of IgH, TcR-γ, and/or TcR-δ genes in order to prevent false-negative results (97).

The probability of changes in rearrangement patterns appears to increase with time (Figure 4). This implies that in case of early relapse generally no changes will be found. This might be important for the choice of PCR targets in MRD-PCR studies in adult ALL patients, because in adult ALL remission duration is essentially shorter than in childhood ALL.

Background of normal cells

The sensitivity of the PCR technique and the specificity of the junctional region probe for detection of leukemia-specific PCR products is highly dependent on the background of normal cells with the same rearranged gene segments as the leukemic cells. This concerns for instance Vδ1-Jδ1 rearrangements and Vγl-Jγ2.3 rearrangements which occur in 0.1-5%

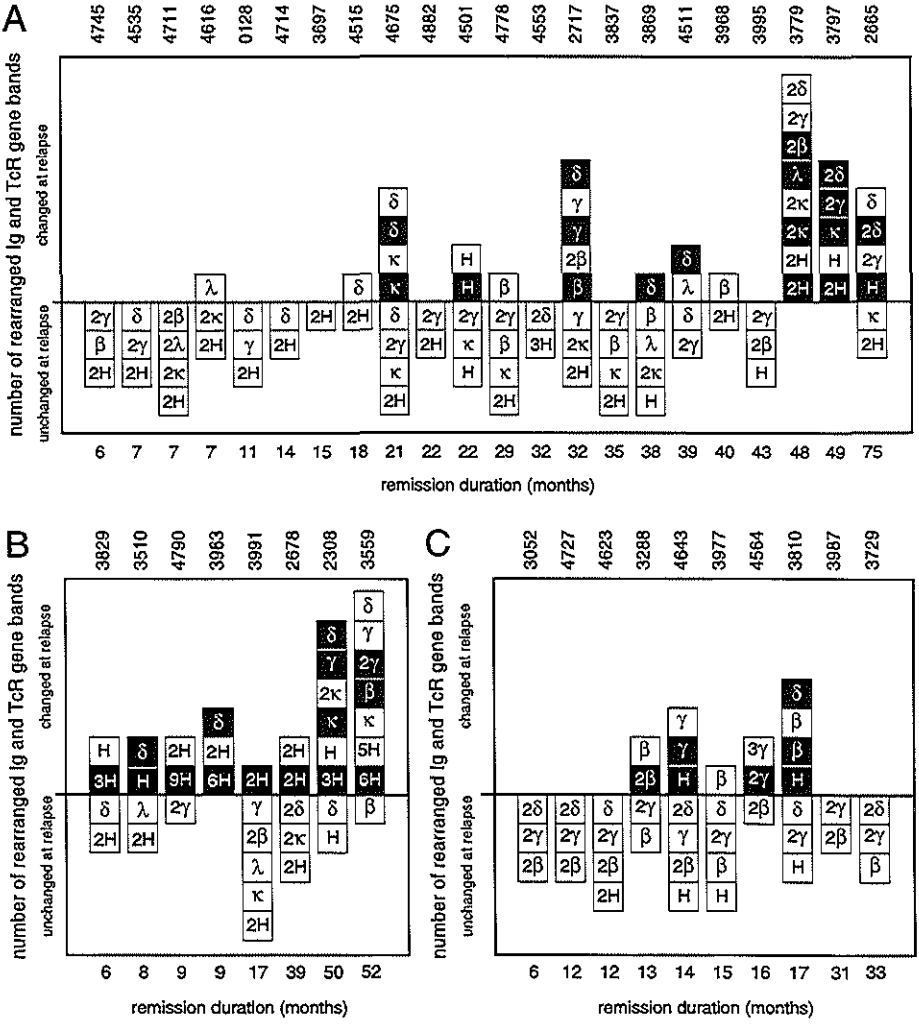

Figure 4. Changes in Ig and TcR gene rearrangement patterns at relapse and their relation with remission duration. Each bar summarizes the Ig and TcR gene configuration per patient, who are numbered according to the registry of the Dutch Childhood Leukemia Study Group. The letter codes in the boxes of each bar indicate the presence (open boxes) or loss (black boxes) of rearranged Ig and TcR gene bands at relapse (H, IgH; κ, Igκ; λ, Igλ; β, TcR-β; γ, TcR-γ; δ, TcR-δ); the numbers indicate the sum of rearranged bands. The changes at relapse are shown above the horizontal line (black boxes: loss of rearranged bands; open boxes: "new" rearranged bands), whereas the stable rearrangements are shown underneath. **A**, 22 monoclonal precursor B-ALL (remission duration 6-75 months), **B**, eight bi-/oligoclonal precursor B-ALL (remission duration 6-52 months), and **C**, 10 T-ALL (remission duration 6-53 months). Detailed information concerning the changes in Ig and TcR gene configuration at relapse are given in reference 97.

and in a large part of normal blood T-lymphocytes, respectively (82). This may result in lower sensitivity and specificity of the junctional region MRD-PCR technique.

Type of rearrangement and size of the junctional region

It should be emphasized that the detection limit of the MRD-PCR technique is related to the size of the junctional region. Junctional regions of complete Vδ-Jδ rearrangements are three to four times larger than Vγ-Jγ junctional regions (27,57,82), implying that TcR-δ junctional regions are more suitable targets for MRD-PCR analysis (27,82). However, TcR-δ gene rearrangements may be incomplete, such as Vδ-Dδ and Dδ-Dδ rearrangements, which have relatively short junctional regions (27,82,103). Incomplete TcR-δ gene rearrangements with short junctional regions are especially found in precursor B-ALL, e.g. Vδ2-Dδ3 and Dδ2-Dδ3 (27,90,103-106). This may result in detection limits which are 10^{-3} to 10^{-4}. In case of short junctional regions, it may theoretically happen that normal cells occur which have junctional regions that are identical to those in leukemic cells.

Technical influences on the MRD-PCR technique

Finally, it should be emphasized that the specificity and sensitivity of the junctional region MRD-PCR technique is influenced by the hybridization conditions, washing stringency and film exposure time of the junctional region specific probe. These should be carefully determined to obtain reproducible results.

PCR ANALYSIS FOR MRD DETECTION IN CHILDHOOD ALL

So far the MRD-PCR studies are restricted to retrospective studies or short-term prospective follow-up studies on limited numbers of patients. Most investigators use junctional regions of rearranged Ig and TcR genes as PCR targets (55,58,59,92,107-112). Analysis of cell samples from ALL patients who developed a relapse, indicate that relapse during treatment might be predicted by persisting PCR positivity, often at a high level (e.g. 10^{-2} or 10^{-3}), or by an increase of PCR positivity over a period up to 12 months before cytomorphological relapse (55,58,59,92,107,111). Several research groups concluded that PCR detection of high levels of residual disease at the end of induction therapy identifies patients at increased risk for relapse during therapy (108-111). Furthermore, they concluded that absence of detectable MRD at the end of chemotherapy is not sufficient to assure that the patient is cured, indicating that after treatment frequent serial monitoring is required for the early prediction of relapse (108-111). In a small prospective study of 20 children followed for 7 to 30 months, Cavé et al. found progressive decrease of the tumor load and no detectable blasts within 6 months. In three patients who developed BM relapse, slower kinetics of decrease were found (112). They concluded that the kinetics of blast-decrease in the first months of treatment may be of prognostic value (112).

CONCLUSION

PCR analysis of chromosomal aberrations and junctional regions of antigen specific receptors is valuable for the detection of MRD in ALL. However, one should realize that each MRD-PCR target has its own limitations and pitfalls.

MRD-PCR analysis using chromosome aberrations has the advantage that these

aberrations are most probably directly related to the oncogenic event and therefore represent stable tumor-specific markers. The first limitation of this technique is the fact that in only 15-20% of childhood ALL and in 25-30% of adult ALL, chromosome aberrations with well-defined breakpoints have been found so far. The second limitation is the fact that in many translocations the PCR target is a fusion mRNA, which is not patient-specific and therefore might cause false-positive results due to cross-contamination between patient samples.

MRD-PCR analysis using junctional regions of rearranged Ig and TcR genes seems to be a promising technique, which can be applied in the majority of ALL. Despite the relatively high frequency of changes in Ig and TcR gene rearrangement patterns, which might cause false-negative results (due to subclone formation and clonal evolution), at least one major IgH, TcR-γ, and/or TcR-δ rearranged band (allele) remained stable in the majority (75-90%) of ALL. Still, the size of the junctional region will influence the detection limit of the PCR technique. This especially concerns incomplete TcR-δ gene rearrangements, which represent the most frequent TcR-δ rearrangements in precursor B-ALL.

Prospective studies on large groups of ALL patients using several PCR targets in parallel are needed to evaluate which target is most efficient and reliable for each patient group. In the future, the MRD-PCR target of choice will probably depend on the presence of a chromosome aberration with well-defined breakpoints and the presence of a rearranged Ig and/or TcR gene, as well as on the probability of changes in Ig/TcR gene rearrangement patterns. The origin of the cell sample (bone marrow, peripheral blood, or cerebrospinal fluid), its volume, and its cellularity will influence the choice as well.

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CHAPTER 5.2

HETEROGENEITY IN JUNCTIONAL REGIONS OF IMMUNOGLOBULIN KAPPA DELETING ELEMENT REARRANGEMENTS IN B-CELL LEUKEMIAS: A new target for detection of minimal residual disease in precursor B-acute lymphoblastic leukemia*

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ABSTRACT

Virtually all immunoglobulin (Ig)κ gene deletions are mediated via rearrangements of the so-called kappa deleting element (Kde). Kde rearrangements occur either to Vκ gene segments or to the heptamer recombination signal sequence in the Jκ-Cκ intron (intron RSS). The junctional regions of Kde rearrangements in 121 B-lineage leukemias (53 precursor B-acute lymphoblastic leukemia (ALL) and 68 chronic B-cell leukemias) were analyzed by the polymerase chain reaction (PCR) and subsequent sequencing analysis to obtain a more detailed view about Kde rearrangements in B-cell differentiation.

Variable (V)κ gene family usage in Vκ-Kde rearrangements in B-lineage leukemias was comparable to Vκ gene family usage in functional Vκ-joining (J)κ rearrangements in normal and malignant mature B-cells, except for a higher frequency of VκII family usage in precursor B-ALL.

Junctional region sequencing of the Kde rearrangements in precursor B-ALL revealed an overall average insertion and deletion of 4.9 and 4.2 nucleotides, respectively, resulting in a large junctional diversity, whereas in chronic B-cell leukemias this N-region insertion ($\sim$1.6) and deletion ($\sim$2.8) was significantly lower.

These relatively large junctional regions as well as the limited number of primers for PCR analysis of Kde rearrangements make the Kde rearrangements a potential PCR target for minimal residual disease (MRD) detection in precursor B-ALL. The junctional diversity of the Kde rearrangements allowed us to design leukemia/patient-specific oligonucleotide probes and a detection limit of $\sim 10^{-4}$ was observed in a dilution experiment, proving with the MRD-PCR technique. Kde rearrangements occur in $\sim$50% of precursor B-ALL cases and remain stable during the disease course as was proven in five studied precursor B-ALL

* Submitted for publication.

patients at relapse. These findings indicate that Kde rearrangements in precursor B-ALL represent sensitive and stable patient-specific PCR targets for detection of MRD.

INTRODUCTION

The various types of B-lineage leukemias, i.e. precursor B-ALL and B-chronic lymphocytic leukemias (CLL) are regarded as malignant counterparts of immature and mature B-cells, respectively (1-3). Therefore, analysis of these maligancies will give more insight in Ig gene rearrangement processes during B-cell differentiation (1,4-8). Based on leukemia studies, it is found that during B-cell differentiation Ig gene rearrangements occur in an ordered manner. First, Ig heavy-chain (IgH) genes rearrange, followed by Igκ genes, which in turn precede Igλ gene rearrangements (9-12). In general, Igλ gene rearrangements occur after or coincide with Igκ gene deletions (11,12).

The Igκ gene locus on chromosome 2p12 contains approximately 76 Vκ gene segments (13,14), five Jκ gene segments, one constant (C)κ gene segment, and a Kde, which is located ~24 kb downstream of the Cκ gene segment (15-19). The Vκ gene segments are divided in seven families on the basis of sequence homology (13,14,20). Forty Vκ gene segments are clustered and located adjacent to the Jκ-Cκ region and are indicated as proximal. About 36 Vκ gene segments are thought to have been duplicated and inserted further upstream from the Jκ-Cκ region in an inverted orientation and are indicated as distal (21,22).

The far majority (>98%) of Igκ gene deletions are mediated via a site-specific rearrangement of the Kde (15-19,23). Kde rearranges via its heptamer-nonamer recombination signal sequence (RSS) either to a heptamer RSS located in the intron RSS or to heptamer-nonamer RSS at the 3′ side of a Vκ gene segment (Figure 1) (15-18). The incidence of Kde-mediated Igκ gene deletions (Kde rearrangements) is ~50% in precursor B-ALL, ~32% in Igκ^+ chronic B-cell leukemias, and 100% in Igλ^+ chronic B-cell leukemias (23).

The combinatorial diversity of the Ig molecules of B-lymphocytes is formed by the many possible different combinations of specific V, (D (diversity)), and J gene segments of each Ig gene (IgH and Ig light-chain (IgL)) (9,10,24-28). The junctional diversity, determined by the junctional region, is made up by D-gene derived nucleotides (in case of IgH genes) (10,27), deletion of nucleotides by trimming of the involved gene segments

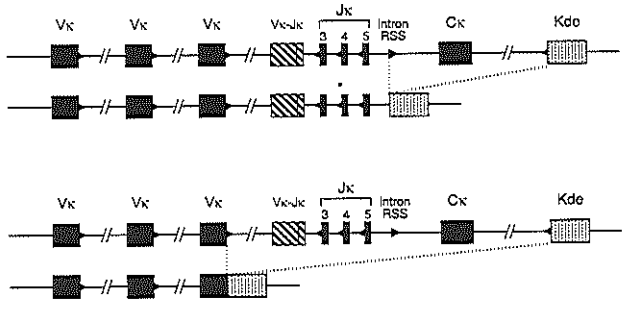


Figure 1. Schematic diagrams of Igκ gene deletions mediated by Kde rearrangements. Two types of Kde rearrangements can occur: (**upper diagram**) Kde rearrangement to the intron RSS; (**lower diagram**) Kde rearrangement to a Vκ gene segment.

(29), P-region nucleotides (30), and N-region nucleotides (28,29,31). D gene derived nucleotides and P-region nucleotides are template dependent, whereas N-nucleotides are randomly inserted at the junctions by the enzyme terminal deoxynucleotidyl transferase (TdT) (29,31). The combined combinatorial and junctional diversity provides the enormous diversity of antigen specific receptors of lymphocytes.

Junctional regions of Ig and TcR gene rearrangements are regarded as tumor-specific markers (32-35), because leukemias and malignant lymphomas represent clonal expansions from single transformed cells (5). Currently, it has been proven that IgH gene junctional regions as well as junctional regions of T-cell receptor (TcR)-γ and TcR-δ genes can be used as targets for MRD detection in ALL by use of the PCR technique (32-39). However, it should be noted that the applicability of these patient-specific sequences as targets for the MRD-PCR technique is dependent on the occurrence and stability of the involved Ig/TcR gene rearrangement (7,35,40,41). In principle, also PCR-mediated MRD detection of junctional regions of rearranged IgL or other TcR genes may be used. However, especially PCR analysis of TcR-α and TcR-β genes needs many different oligonucleotide primers and in case of IgL genes the junctional diversity is minimal (35,42).

The aim of our study was to obtain a more detailed view about Kde rearrangements during B-cell differentiation. Therefore, we examined the junctional regions formed by either Kde to Vκ rearrangements or Kde to the intron RSS rearrangements in a large group of 121 selected B-lineage leukemias, including 53 precursor B-ALL and 68 chronic B-cell leukemias. Furthermore, we analyzed the applicability of the Kde rearrangements as MRD-PCR target by studying the size, the composition, and the stability of the junctional regions in precursor B-ALL at diagnosis, and if available, also at relapse. Moreover the leukemia-specificity of this MRD-PCR target was evaluated and the detection limit was determined in a dilution experiment.

MATERIALS AND METHODS

Cell samples

A large group of 121 B-lineage leukemias at diagnosis and 7 precursor B-ALL also at relapse were selected based on the presence of Kde rearrangements as detected by Southern blotting in a previous study and the availability of DNA, necessary for the analyses (23,41). This group of leukemias comprised 53 precursor B-ALL (one null ALL, 38 common ALL, and 14 pre-B-ALL) and 68 chronic B-cell leukemias (60 CLL, four prolymphocytic leukemias (PLL), and four hairy cell leukemias (HCL)). Kde rearrangements to Vκ gene segments were found in ~69% of Kde rearrangements in precursor B-ALL, while these were found in 46% of Kde rearrangements in the chronic B-cell leukemia group (23).

Mononuclear cells (MNC) were obtained from peripheral blood (PB) or bone marrow (BM) samples of these 121 B-lineage leukemias or healthy adult volunteers by Ficoll-Paque centrifugation (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden). All MNC samples were frozen and stored in liquid nitrogen. All cell samples were obtained with the approval of the Medical Ethics Committee of the University Hospital Dijkzigt/Erasmus University, Rotterdam.

DNA was isolated from MNC samples as described previously (26,43).

PCR and direct sequencing analysis

PCR amplification was performed as described previously (44). An 1.0 μg sample of DNA, 12.5 pmol of the upstream and downstream oligonucleotide primers (Table 1) and one unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT, USA) were used in each PCR of 100μl.

TABLE 1. Oligonucleotide primers used in PCR, hybridization, and sequencing of Kde rearrangements.

Name	Code	Size	Position[a]	Sequence	Reference[b]
Kde rearrangement PCR primers					
5′ PCR primers				5′ 3′	
IGKVI5	Vκ-fr1	~379 bp	-221 bp	GTAGGAGACAGAGTCACCATCACT	13
IGKVII5	Vκ-fr1	~395 bp	-237 bp	GGAGAGCCGGCCTCCATCTCC	13
IGKVIII5	Vκ-fr1	~383 bp	-225 bp	AGGGGAAAGAGCCACTCTCTCC	13
IGKVIV5	Vκ-fr1	~397 bp	-239 bp	GGCGAGAGGGCCACCATCAAC	13
IGKVV5	Vκ-fr1	~376 bp	-218 bp	CCAGGAGACAAAGTCAACATCTCC	13
IGKVVI5	Vκ-fr1	~388 bp	-230 bp	CTGTGACTCCA(A/G)(A/G)GGAGAAAGTC	13
IGKVII5	Vκ-fr1	~391 bp	-233 bp	AGGACAGAGGGCCACCATCACC	13
IGKID5	intron-RSS-5	~457 bp	-299 bp	GTTATTCCCAAAAGCTTAATCTCAAAG	X67858
3′ PCR primer					
IGKDE3	Kde-3		+457 bp	TTCATAGACCCTTCAGGCACATGC	16
Kde rearrangement sequence primers					
5′ sequence primers					
IGKVI3	Vκ-fr3	~95 bp	-41 bp	TCAGCAGCCTG(C/G)AG(C/G)CTGAAGAT	13
IGKVII3	Vκ2-fr3	~88 bp	-34 bp	CAGCAGGGTGGAGGCTGAGGAT	13
IGKVV3	Vκ5-fr3	~126 bp	-72 bp	CAGCGGGTATGGAACAGATTTTAC	13
IGKI3	intron-RSS-3	~104 bp	-50 bp	CTATGCCGTGGCCACCCTGTGT	X67858
3′ sequence primer					
IGKDE3	Kde-5		+54 bp	ATGCAGCTGCAGACTCATGAGG	16
Junctional region patient-specific probes[c]					
patient 2308 (intron)				CCTGccctcgggGCCCTA	
patient 3264 (intron)				GCCAGCTTTataccgaCCCTA	
patient 3779 (intron)				GATGatggactCCTAGTGGCA	
patient 3869 (intron)				GCCGTAcccgggaCCAGG	
patient 4711 (intron)				CTGctggtagcgagGTGGC	
patient 5014 (intron)				CCTGATGgttgggCCTAGTG	
patient 3264 (Vκ II)				CTGGCCTCCtcgcaGAGCC	
patient 3797 (Vκ I)				ATCTCCCTCgggtcgaaGGA	
patient 4686 (Vκ I)				TACCgtgaacgGCCCAGGG	
patient 4711 (Vκ II)				ACACTGGCCaccGAGCCCT	
patient 4865 (Vκ III)				tccggggacggctaaccaG	
patient 5105 (Vκ I)				CTCCgtcggaCCCTAGTGG	

a. The position of the 3′ end of the oligonucleotide primers is indicated downstream (+) or upstream (-) of the closest joining site (=start of RSS). In case of the IGKDI5 and IGKDI3 primers, it concerns the heptamer RSS in the Jκ-Cκ intron.

b. Sequence information used to design the oligonucleotide primers was derived from the indicated literature references (13,16,45; Whitehurst et al. EMBL databank accession no. X67858).

c. Small characters in the sequence represent the nucleotides inserted at the junctional region of the Kde rearrangements in the precursor B-ALL patients.

To evaluate the size differences of short (~250 bp) Kde rearrangement PCR products, they were size-separated by 15% polyacrylamide gel electrophoresis (PAGE) and visualized by ethidium bromide staining.

To obtain single-stranded PCR products for sequencing an asymmetric PCR reaction was performed followed by a double 50% ethanol extraction (44).

Fifty pmol sequence primer was used in each reaction (Table 1). The sequence reactions were performed with the T7-sequencing kit (Pharmacia) following manufacturer's instructions using ^{35}S radiolabeling, and run in a normal, denaturing 8% polyacrylamide gel. Sequencing was performed in both directions.

Specificity of Kde rearrangement PCR products and determination of the MRD-PCR detection limit

To determine the specificity of the Kde rearrangement PCR products, they were size-separated in 1.0%

agarose gels, transferred to Nytran-13N nylon membranes, and UV cross-linked as described (26,45). The PCR products were analyzed by hybridization using several [γ-^{32}P] dATP 5´ end-labeled junctional region leukemia/patient-specific oligonucleotide probes (Table 1).

To determine the MRD-PCR detection limit of Kde rearrangements a DNA dilution experiment was performed. DNA from a precursor B-ALL patient containing a VκII-Kde rearrangements was diluted in DNA from a healthy adult individual. The dilution steps were 1 to 10^{-8} patient DNA in control PB-MNC DNA. PCR reactions with the appropriate oligonucleotide primers were performed subsequently and the PCR products were analyzed by hybridization as described above.

RESULTS

PCR and sequencing analysis of junctional regions of Kde rearrangements

In all 121 selected B-lineage leukemia cases, the Southern blot data could be confirmed with PCR analysis of the two types Kde rearrangements with the designed oligonucleotide primers (Table 1). Difficulties in obtaining Kde rearrangement PCR products could be overcome by digestion of DNA with *Bgl*II in six patients.

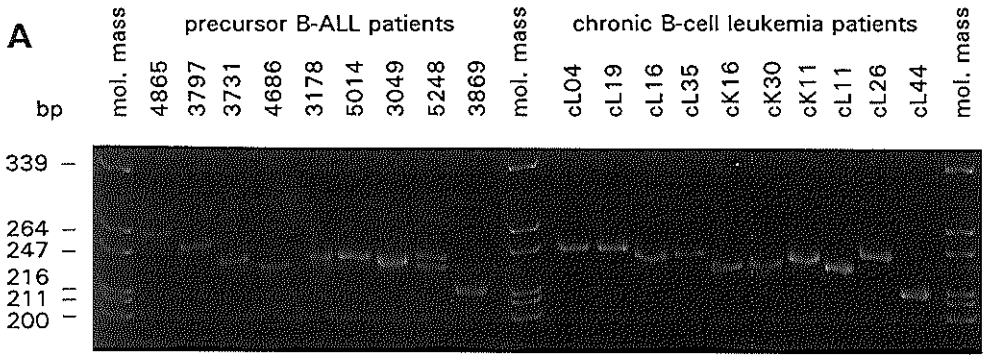
The relative size of the short PCR products was evaluated by 15% PAGE (Figure 2A). More variation in size was found in the precursor B-ALL group as compared to the chronic B-cell leukemia group. CLL patient cL44 represented an exception because of a very short PCR product due to extensive trimming of both rearranged segments (see also Figure 2B).

Direct sequencing of the Kde rearrangement PCR products from 82 B-lineage leukemias (34 in precursor B-ALL and 48 in chronic B-cell leukemias) revealed the junctional region sequences of 86 Kde rearrangements. A representative part of the sequences is shown in Figure 2B. Similar rearrangements on both alleles of one patient (e.g. two Kde to the intron RSS rearrangements or two Kde to Vκ gene rearrangements) resulted in a mixed PCR product as well as mixed sequences. In such cases, we unraveled the mixed sequences by matching information about sequences read from both directions. In this way, cloning of PCR products was unnecessary in all patients. Table 2 summarizes the characteristics of the junctional region sequences of Kde rearrangements in B-lineage leukemias. The sizes of the junctional regions differed from 0 to maximally 18 nucleotides with an average of 4.9 nucleotides in the precursor B-ALL group. The sizes of the junctional regions differed from 0 to maximally 8 nucleotides with an average of 1.6 nucleotides in the chronic B-cell leukemia group. In a large part of Kde rearrangements, especially in case of Vκ-Kde rearrangements, no randomly inserted N-region nucleotides were found in chronic B-cell leukemias. More N-region nucleotides and trimming of nucleotides was found in the junctional regions of Kde rearrangements to the intron RSS, whereas more P-region nucleotides were found in the junctional region sequences of Kde rearrangements to Vκ gene segments (Table 2).

Specificity of patient-specific oligonucleotide probes and primers

To determine the specificity of the patient-specific oligonucleotide probes the Kde rearrangement PCR products of several patient DNA samples were size-separated, blotted and successively hybridized with the various patient-specific oligonucleotide probes (Figure 3). In all cases analyzed, these patient-specific oligonucleotide probes were proven to detect exclusively the corresponding Kde rearrangement PCR products out of all tested

A



B

Precursor B-ALL (intron RSS-Kde)

intron Jκ-Cκ		Kde	
GCTTTCCTGATG	junct. reg.	GGAGCCCTAGTG	
GCTTTCCTG	CCCTCGGG	GCCCTAGTG	2308
GCTTTCCTG	G	TAGTG	2789
GCTTTCCTGA	AA	GAGCCCTAGTG	3049
GCTTTCCT	CAATCCTACT	GCCCTAGTG	3178
GCTTT	ATACCGA	CCCTAGTG	3264
GCTTTCCTGATG	ATGGACT	CCTAGTG	3779
–16	CCCGGGA	–17	3869
GCTTTCCTGATG	ATTGGG	CCTAGTG	3899
GCTTTCCTG	CTGGTAGCGAG	GTG	4711
GCTTTCCTGATG	GTTGGG	CCTAGTG	5014
GCTTTCCTGATG	AAAAA	G	5162
GCTTTCCTGAT	CCCG	TAGTG	5236
GCTTTCCTGAT		AGTG	5248
GCTTTCCTGATG		GCCCTAGTG	5248

Chronic B-cell leukemias (intron RSS-Kde)

intron Jκ-Cκ		Kde	
GCTTTCCTGATG	junct. reg.	GGAGCCCTAGTG	
GCTTTCCTGATG	A	AGCCCTAGTG	cK11
GCTTTCCTGATG	CCC	GCCCTAGTG	cK13
GCTTTCCT	CCCCCTGT	GTG	cK30
GCTTTCCTGATG	CT	CCCTAGTG	cK37
GCTTTCCTGATG		GAGCCCTAGTG	cK42
GCTTTCCT	CCCC	GGAGCCCTAGTG	cL03
GCTTTCCTGATG	AG	G	cL11
GCTTTCCTGATG		CCCTAGTG	cL17
GCTTTCCTG	CT	GAGCCCTAGTG	cL20
GCTTTCCTGA	XGA	AGCCCTAGTG	cL26
GCTTTCCTGATG		AGCCCTAGTG	cL29
GCTTTCCTGA		GCCCTAGTG	cL29
GCTTTCCTG	TCC	GAGCCCTAGTG	cL33
GCTTTCCTG		AGCCCTAGTG	cL41
–19	TTTGC	–15	cL44
GCTTTCCTG		GTG	cL47
GCTTTCCTG	CT	CCCTAGTG	cL48

Precursor B-ALL (Vκ-Kde)

VκI	TAGTTA..CT..			
VκII	.CA.T.T.CT..			
VκIII	TAACTGG.CT..		Kde	
VκIV	AGTACTCCTCCC	junct. reg.	GGAGCCCTAGTG	
B3	AATTTTCCTTCC	T	GAGCCCTAGTG	3178
A17	ACACTGGCCTCC	TCGCA	GAGCCCTAGTG	3264
L9	TAGTTACCCTC	A	GGAGCCCTAGTG	3510
A27/A11	-40	GAGG	GTG	3725
A27/A11	TAGCTCACC		GAGCCCTAGTG	3731
L8	TAGTTACCCTC	AGC	CCCTAGTG	3750
018/08	TAATCTCCCTC	GGGTCGAA	GGAGCCCTAGTG	3797
A17/A1	ACACTGCC	TCC	GAGCCCTAGTG	3869
L12	T	TCCCTCTGG	-20	4533
L9	TAGTTACC	GTGAACG	-15	4686
L16	TAACTGGCC	CCCGAG	CCTAGTG	4842
L2	TAACTGGC	TCCGGGGACGGCTAACCA	GAGCCCTAGTG	4865
A17	ACACTGGCCTCC	A	CTAGTG	5014
A30	TAGTTACCCTCC	ggACGG	GGAGCCCTAGTG	5162

Chronic B-cell leukemias (Vκ-Kde)

VκI	TAGTTA..CT..			
VκII	.CA.T.T.CT..			
VκIII	TAACTGG.CT..		Kde	
VκIV	AGTACTCCTCCC	junct. reg.	GGAGCCCTAGTG	
A2	ACAGCTTCCTC	T	GAGCCCTAGTG	cK07
A27	TAGCTCACCTCC		GTG	cK16
A19	ACAAACTCC		GGAGCCCTAGTG	cK47
L2	TAACTGGCCCCC	ggA	CCTAGTG	cL04
L12	TAGTTATTCTC	T	CCTAGTG	cL06
L1	TAGTTACCCTC		AGCCCTAGTG	cL12
014	CAATGCCCCT		GAGCCCTAGTG	cL16
B3	AGTACTCC		GAGCCCTAGTG	cL17
L4	TAGTTACCCTCC	CC	GAGCCCTAGTG	cL19
A18	ATACGCCTTCC		GAGCCCTAGTG	cL24
A30	TAGTTACCC		GCCCTAGTG	cL31
A27	TAGCTCACCT		AGCCCTAGTG	cL33
B3	AGTACTCCTCC		GAGCCCTAGTG	cL35

Figure 2. PCR and sequencing analysis of junctional regions of Kde rearrangements. (A) PAGE analysis of Kde rearrangement PCR products of several precursor B-ALL and chronic B-cell leukemias. The PCR-mediated amplification of the Kde rearrangements was performed using the Vκ-family-fr1 and IGKDE3 oligonucleotide primers in case of Vκ-Kde rearrangements or the IGKI3 and IGKDE3 oligonucleotide primers in case of Kde rearrangements to the intron RSS (Table 1). (B) Sequences of the junctional regions of Kde rearrangements are aligned with the known Vκ germline sequences, with the germline sequences of the Jκ-Cκ intron, and with the Kde germline sequences (double underlined). Lower-case characters at the end of a junctional region represent P-region nucleotides (30). All other nucleotides at the junctional region represent N-region nucleotides. The used patient-specific oligonucleotide probes for hybridization analysis are indicated (underlined).

TABLE 2. Characteristics of the junctional region sequences of Kde rearrangements in B-lineage leukemias[a].

B-lineage leukemias	Kde rearrangements to Vκ gene segments: junctional nucleotides	N-region nucleotides	P-region nucleotides	deleted nucleotides	Kde rearrangements to the intron RSS: junctional nucleotides	N-region nucleotides	P-region nucleotides	deleted nucleotides
precursor B-ALL (n=34)	4.6 (0-18)	4.5 (0-18)	0.2 (0-2)	3.2 (0-20)	5.3 (0-11)	5.3 (0-11)	0	4.6 (0-17)
chronic B-cell leukemias (n=48)	0.5 (0-3)	0.4 (0-2)	0.2 (0-2)	2.0 (0-9)	2.0 (0-8)	1.9 (0-8)	0.1 (0-1)	3.2 (0-19)

a. Average number of nucleotides per rearranged allele is given. The range is given between brackets.

patients. In addition, neither in normal BM nor in normal PB positive hybridization signals were detected (Figure 3).

Detection limit of the MRD-PCR technique

To establish the detection limit of the MRD-PCR technique, DNA of a precursor B-ALL

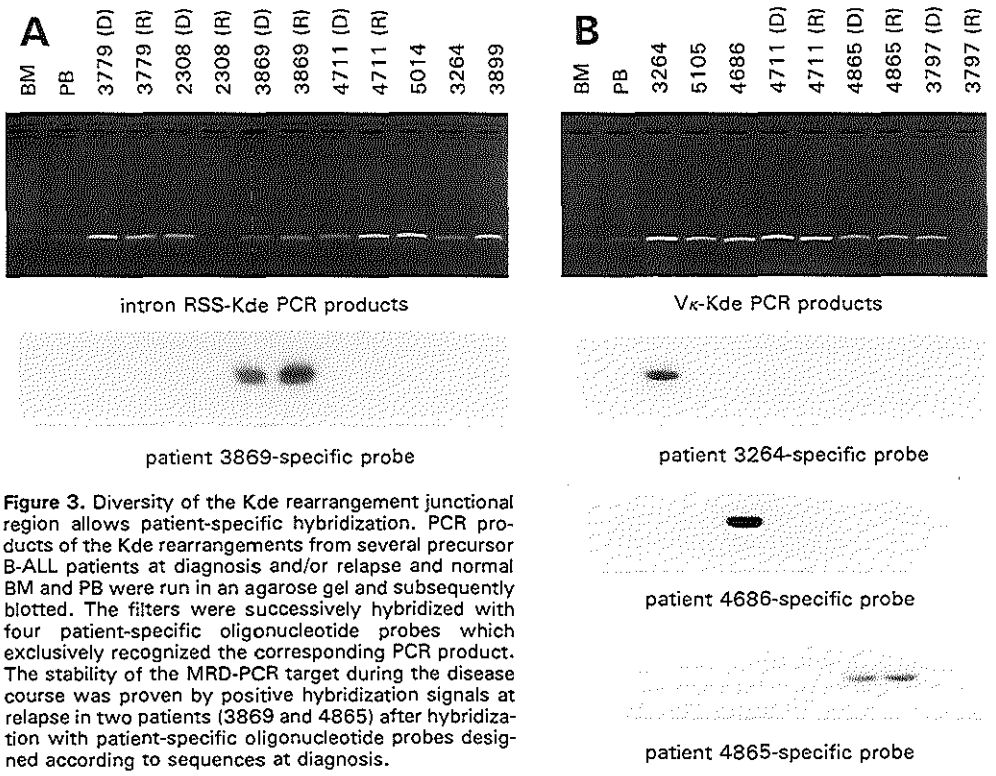


Figure 3. Diversity of the Kde rearrangement junctional region allows patient-specific hybridization. PCR products of the Kde rearrangements from several precursor B-ALL patients at diagnosis and/or relapse and normal BM and PB were run in an agarose gel and subsequently blotted. The filters were successively hybridized with four patient-specific oligonucleotide probes which exclusively recognized the corresponding PCR product. The stability of the MRD-PCR target during the disease course was proven by positive hybridization signals at relapse in two patients (3869 and 4865) after hybridization with patient-specific oligonucleotide probes designed according to sequences at diagnosis.

patient with a Vκ II-Kde rearrangement was diluted in DNA from PB-MNC of a healthy individual. PCR analysis of the Vκ II-Kde rearrangement in each DNA mixture and subsequently hybridization of the blotted PCR product with the patient-specific oligonucleotide probe revealed hybridization signals down to the $\sim 10^{-4}$ dilution mixture (Figure 4).

Stability of the Kde rearrangement

The stability of Kde rearrangements at diagnosis and at subsequent relapse can be studied in five ways: first, Southern blot analysis reveals identically rearranged Igκ gene bands upon hybridization with the IGKDE probe in at least two different digests (23,41); second, in case of Vκ-Kde rearrangements, PCR analysis shows identical Vκ family usage; third, after blotting of the PCR products at relapse positive hybridization signals are detected upon hybridization with the patient-specific junctional region oligonucleotide probe, designed accordingly to the sequences of the DNA sample at diagnosis; fourth, when using the relapse DNA sample as substrate, a PCR product is obtained with a patient-specific junctional region primer obtained at diagnosis and the general IGKDE3 primer; and finally, sequencing analysis of the junctional region PCR products at diagnosis and relapse results in identical sequences. The stability of the Kde rearrangement was proven, when beside the first two at least one of the latter three criteria resulted in identical outcome at diagnosis and relapse.

DNA samples of seven precursor B-ALL patients were analyzed at diagnosis and relapse by PCR, subsequent hybridization with patient-specific oligonucleotide probes, or sequencing analysis. In five precursor B-ALL patients identical results were obtained proving

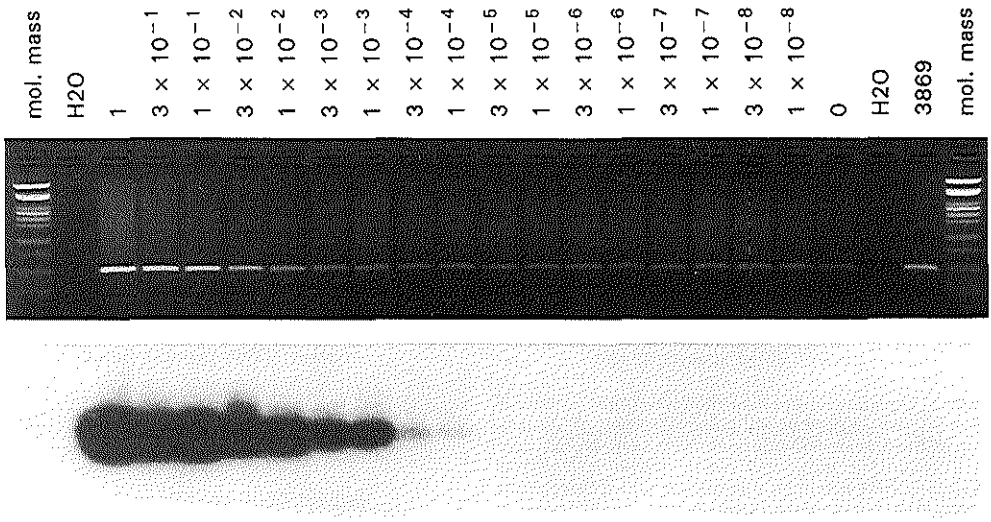


Figure 4. Dilution experiment in which DNA from a precursor B-ALL patient with a Vκ II-Kde rearrangement was diluted in PB-MNC from a healthy individual. PCR products obtained after amplification with the IGKVII3 and IGKDE3 oligonucleotide primers were run in agarose gel (**A**) and blotted to a nylon membrane, which was hybridized with the ^{32}P-labeled 3264 (Vκ II) patient-specific junctional region probe (**B**). Kde rearrangement PCR products were detectable down to the $\sim 10^{-4}$ dilution mixture. The precursor B-ALL patient 3869 was used as a positive control.

the stability of the Kde rearrangements (Figure 3) and in two patients, the occurrence of a secondary leukemia (i.e. acute myeloid leukemia) (patient 3979) and the selection of a subclone without Kde rearrangements at relapse (patient 2308) was confirmed (41).

Vκ gene segment usage in B-lineage leukemias

In 69% of Kde rearrangements a Vκ-Kde rearrangement was found in precursor B-ALL and in 46% in chronic B-cell leukemias (23). The usage of the different Vκ families estimated by PCR analysis is summarized in Table 3. VκI family members are more frequently used in Vκ-Kde rearrangements in chronic B-cell leukemias, whereas in Vκ-Kde rearrangements in precursor B-ALL more VκII family members were utilized. On the other hand VκIII and VκIV family members are equally used by both B-lineage leukemia groups. Kde rearrangements to the VκV, VκVI, and VκVII families are rare (Table 3).

All Vκ gene segment sequences obtained after direct sequencing analysis of Vκ-Kde rearrangements, had significant homology to known germline sequences (13,14). In the majority of Vκ-Kde rearrangements (93%;28/30) in precursor B-ALL and chronic B-cell leukemias, proximal Vκ gene segments were used. In precursor B-ALL preferential usage of L1,L9, and A30 as VκI family members, A17 as VκII family member, and A27 as VκIII family member was found (Figure 2B). Only in chronic B-cell leukemias preferential usage of A27 as VκIII family member was found (Figure 2B).

TABLE 3. Frequency of Vκ family usage in Vκ-Kde rearrangements in B-lineage leukemias.

Vκ family (number of members)[a]	Precursor B-ALL (n=51)	Chronic B-cell leukemias (n=47)
VκI (23)	39%	47%
VκII (14)	27%	17%
VκIII (8)	22%	21%
VκIV (1)	10%	11%
VκV (1)	0%	0%
VκVI (2)	0%	2%
VκVII (1)	2%	2%

a. Number of Vκ gene segments belonging to a Vκ family which are potentially functional or have minor defects but still have the potention to become functional due to mutations (13,14).

DISCUSSION

Sequence analysis of the Kde rearrangements either to the intron RSS or to Vκ gene segments revealed a striking difference in junctional region diversity between precursor B-ALL and chronic B-cell leukemias (Figure 2 and Table 2). Three times more insertion of N-region nucleotides and nearly two times more deletion of nucleotides was found in the junctional regions of Kde rearrangements in precursor B-ALL as compared to chronic B-cell leukemias. Apparently in precursor B-ALL, TdT is still active at the time Kde rearrangements take place, whereas in chronic B-cell leukemias, TdT activity was decreased or absent at the time Kde rearrangements occurred. Also in normal B-cells junctional regions of Vκ-Jκ gene rearrangements are small due to limited N-region insertion (average 1-2 nucleotides)

(46-50). Even in normal precursor B-cells, which rearrange Igκ genes at the same time as they undergo VH to D-J rearrangements, no much N-region diversity (average <1 nucleotides) is found (42). Our findings are in line with the finding that the recombinase system as well as TdT are still active in precursor B-ALL (46-48). This is probably related to the malignant transformation and maturation arrest in the precursor B-cell stage. The continuous recombinase and TdT activity in precursor B-ALL cells explains the frequently occurring continuous gene rearrangements (7,49,50), including the high frequency of Igκ gene rearrangements and deletions (23), as well as large N-regions in Kde rearrangements (Figure 2B).

Detection of MRD by use of the PCR technique is a powerful tool for monitoring leukemic cells during and after treatment, because these techniques are potentially able to detect frequencies of 10^{-5} to 10^{-6} leukemic cells (35). However, the applicability of the MRD-PCR technique is highly dependent on the presence of suitable targets and the detection limit of the technique, which is influenced by the occurrence of the involved MRD-PCR target in normal cells. Nowadays, chromosome aberrations and IgH, TcR-γ, and TcR-δ gene rearrangements are the most commonly used MRD-PCR targets. In this study, we investigated the usage of Kde rearrangements as new MRD-PCR targets.

We evaluated whether it was possible to use Kde rearrangements in precursor B-ALL as leukemia/patient-specific MRD-PCR target, despite the occurrence of these rearrangements in normal cells. We anticipated that normal cells would not cause a false-positive background, because in normal B-cells the junctional diversity of Vκ-Jκ rearrangements and probably also the junctional diversity in Kde rearrangements is low (42,51-56). Hybridization of patient-specific oligonucleotide probes to Kde rearrangement PCR products of various sources indeed proved the specificity of the probes, because they were able to detect their corresponding PCR products without distinct hybridization to Kde rearrangement PCR products from other patients or from normal BM and PB (Figure 3). The sensitivity of this new MRD-PCR target was determined by use of a dilution experiment, in which DNA of a precursor B-ALL with VKII-Kde rearrangement was diluted in DNA of PB-MNC from a healthy individual. This dilution experiment revealed a detection limit of $\sim 10^{-4}$ (Figure 4).

Igκ gene rearrangements as well as Kde rearrangements are not found in T-ALL and are rare in AML (5,57). In contrast, Kde rearrangements occur in ~50% of precursor B-ALL (23), which makes it a potential important MRD-PCR target in these leukemias.

The stability of the Kde rearrangement can be studied in five ways as mentioned in the Result section. The stability of the Kde rearrangement was proven, when beside the first two at least one of the latter three criteria mentioned succeeded in identical results at diagnosis and relapse. In five of the seven studied precursor B-ALL cases, the stability of Kde rearrangements at relapse was proven. In the two other precursor B-ALL cases a secondary leukemia to AML and a selection of a subclone without Kde rearrangements at relapse was confirmed (41). The stability of Kde rearrangements seem to be in contrast to the continuing rearrangements in precursor B-ALL (7,50). However, it should be noted that Kde rearrangements are 'end-stage' rearrangements, which can not undergo further rearrangements.

Until recently, only a few studies about Vκ gene usage in Igκ gene rearrangements in normal and malignant (precursor) B-cells were reported (58-62). But with the discovery of the PCR technique (63,64), the analysis of antigen specific receptor gene rearrangements,

as well as the analysis of Igκ gene rearrangements, is moving rapidly (51-56,65-67). However, reports about Vκ gene usage in Kde rearrangements are still scarce (56). Vκ family usage (VκI-IV) in our Kde rearrangements were compared with Vκ family usage in Vκ-Jκ rearrangements as reported in the literature and summarized in Table 4 (58,61,62,66,67). This comparison revealed that Vκ family usage in Vκ-Jκ gene rearrangements and Vκ-Kde rearrangements is comparable except for a higher tendency of VκII family usage in Vκ-Kde gene rearrangements, especially in precursor B-ALL. The most frequently used Vκ gene segments of each Vκ family found in functional Vκ-Jκ gene rearrangements are also used in Vκ-Kde gene rearrangements. (52-54,66,67). Distal Vκ gene segments are not frequently used in Vκ-Kde gene rearrangements. Apparently, the usage of Vκ gene segments is not clearly dependent on the type of rearrangement (Vκ-Jκ or Vκ-Kde), but particular Vκ gene segments (e.g. VκI: L1,L9, and A30, VκII: A17, and VκIII: A27) are probably more prone to rearrange than other Vκ gene segments.

In conclusion, sequencing analysis of Kde rearrangements revealed a striking difference in size of junctional regions between precursor B-ALL and chronic B-cell leukemias. This might be related to the ongoing activity of the recombinase system and TdT in precursor B-ALL, due to the malignant transformation and the maturation arrest in the precursor B-cell stage. The relatively large size of the junctional regions of Kde rearrangements and the limited number of primers necessary for optimal PCR analysis of Kde rearrangements makes the Kde rearrangement a potential MRD-PCR target in precursor B-ALL. The suitability of this MRD-PCR target was further established by proving its specificity by using leukemia/patient-specific oligonucleotide probes. In addition, the sensitivity of this target was analyzed by use of a dilution experiment, which revealed a detection limit of $\sim 10^{-4}$ and the stability was proven by use of patient-specific oligonucleotide probes in precursor B-ALL at diagnosis and at relapse. Therefore, junctional regions of Kde rearrangement represent sensitive and stable MRD-PCR targets in precursor B-ALL.

TABLE 4. Vκ family usage in Vκ-Jκ rearrangements in normal and malignant lymphoid cells as compared to Vκ family usage in Vκ-Kde rearrangements in B-lineage leukemias.

	Vκ-Jκ rearrangements		Vκ-Kde rearrangements	
Vκ family	Normal B-cells[a]	Malignant lymphoid cells[b] (n=106)	Chronic B-cell leukemias (n=45)	Precursor B-ALL (n=50)
VκI	50%	58%	49%	40%
VκII	8%	13%	18%	28%
VκIII	25%	19%	22%	22%
VκIV	14%	10%	11%	10%

a. Unfortunately, only percentages of Vκ family usage in functional Vκ-Jκ rearrangements were reported in the literature; data are obtained from references (58,61,62).

b. The malignant lymphoid cells concern precursor B-ALL (n=6), non-Hodgkin lymphoma (n=20), CLL (n=52), PLL (n=6), HCL (n=5), Waldenström macroglobulinemia (n=7), and Multiple myeloma (n=10) and are obtained from references (65-67).

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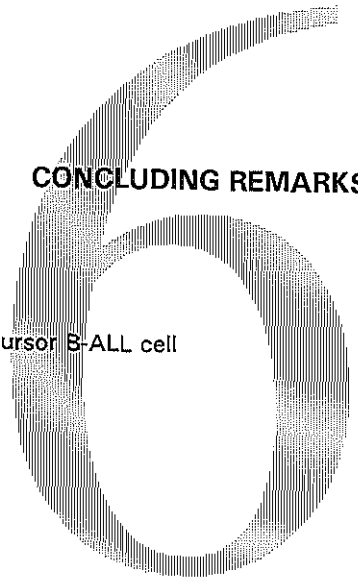

CONCLUDING REMARKS

CHAPTER 6

CONCLUDING REMARKS

6.1 INTRODUCTION

During the last decades, extensive studies in acute lymphoblastic leukemia (ALL) are performed at diagnosis, during follow-up, and at relapse. These studies include analysis at the single cell level: analysis of cell morphology, immunophenotype, and karyotype; analysis at the DNA level: immunoglobulin (Ig) and T-cell receptor (TcR) gene rearrangement studies; and *in vitro* cell culture analysis. These extensive analyses provided more insight into the development of ALL and more knowledge about the characteristics of the leukemic cell as well as the occurrence of relapse. Furthermore, leukemias are generally used as a model to study normal B- and T-cell differentiation, since they can be regarded as clonal malignant counterparts of normal (immature) B- or T-cells.

The main purpose of this study was to get more insight into the factors and mechanisms which influence the development of relapse in childhood ALL. In this thesis, we have focussed on the analysis of Ig and TcR gene rearrangements at diagnosis and at subsequent relapse in ALL, especially in precursor B-ALL. The different findings of the immunogenotypic studies have been discussed extensively in the individual chapters of this thesis. Therefore, the most significant findings will only be briefly summarized. First, to improve the detection of Ig heavy-chain (IgH) and kappa Ig light-chain (IgL) gene rearrangements by Southern blot analysis for diagnostic purposes, we carefully determined the precise restriction map of the relevant IgH and Igκ regions and evaluated the appropriate combination of restriction enzymes with newly developed optimal DNA probes (Chapter 2). With these optimal Southern blot conditions, Ig and TcR gene rearrangements have been analyzed in detail in a large group of precursor B-ALL patients. The results revealed in 30-40% multiple rearranged IgH gene bands and in 6% multiple rearranged Igκ gene bands in precursor B-ALL. These multiple rearranged IgH and Igκ gene bands are most probably due to subclone formation. In the majority of precursor B-ALL cases, this subclone formation (bi-/oligoclonality) is caused by continuing and/or secondary gene rearrangements. In addition, cross-lineage TcR gene rearrangements were found in 94% of precursor B-ALL at diagnosis (Chapter 3). Not only multiple rearranged Ig and cross-lineage TcR gene rearrangements were observed in precursor B-ALL at diagnosis, but also differences in Ig and TcR gene rearrangement patterns between diagnosis and relapse were found. These differences (clonal evolution), were detected in ~65% of precursor B-ALL and ~50% of T-ALL, and could be explained in nearly all cases by continuing and/or secondary gene rearrangement processes (Chapter 4). The observed heterogeneity in Ig and TcR gene rearrangement patterns (subclone formation and clonal evolution) has important implications for the detection of minimal residual disease (MRD) by the polymerase chain reaction (PCR)-mediated amplification of tumor-specific IgH, TcR-γ, and TcR-δ gene junctional regions,

since subclone formation at diagnosis and clonal evolution at relapse will lead to false-negative results. It is therefore that a new potentially 'reliable' MRD-PCR target has been found and studied in the junctional regions of kappa deleting element (Kde) rearrangements, which can be detected in ~50% of precursor B-ALL cases (Chapter 5).

In this Chapter 6, two related questions which can be raised when combining the various immunogenetic results described in this thesis, will be discussed. What is the origin of the precursor B-ALL cell and can precursor B-ALL serve as a model for normal B-cell differentiation? Before discussing these questions, the relevant data from the literature concerning normal B-cell differentiation and our data concerning immunogenetic findings in B-lineage leukemias will be summarized.

6.2 THE ORIGIN OF THE PRECURSOR B-ALL CELL

The development of the normal B-cell

The differentiation of B-lymphocytes from committed precursor cells to antibody-secreting plasma cells proceeds through multiple steps that are defined by changes in the expression pattern of lineage-specific genes (1-4). The most important process during B-cell differentiation in the bone marrow (BM) from immature pro-B-cell to mature B-lymphocyte is the expression of unique Ig molecules on the cell membrane, which are specialized in antigen recognition (1,5). Each of the Ig chains consists of a C (constant) region as well as a variable region which is involved in antigen recognition. These variable regions of the Ig chain are encoded by combinations of gene segments (V (variable), (D (diversity),) and J (joining) gene segments) which are different in each lymphocyte (1,6). The different gene segments are joined during rearrangement processes, which occur in a programmed manner (first IgH and subsequently IgL gene rearrangements) and are regulated and influenced by several proteins, such as products of the recombination activating genes: RAG1 and RAG2 and terminal deoxynucleotidyl transferase (TdT) (7-10). During B-cell differentiation RAG expression is found from pro-B-cell to immature B-cell stage, whereas TdT expression is found from the lymphoid stem cell to the pre-B-cell stage (defined by cytoplasmic Igμ staining) (see also Chapter 2.1) (8,11-15).

Once the immune system has been built, it contains around 10^{12} cells of the B-lineage of which 5-10% are precursors that are active in regeneration, whereas 90-95% are resting, mature B-lymphocytes (16). Large numbers of B-lymphocytes are produced daily from lymphoid stem cells within the BM (17-19). During this production of B-lymphocytes a distinct percentage (50-60%) of immature B-cells and self-reactive B-cells will be aborted, in which the latter B-cells are eliminated through a two-step process of developmental arrest and apoptotic cell death (16-21). The reason for the waste of (immature) B-cells is unclear. Although the chance of failures during Ig gene rearrangement processes is high, each (immature) B-cell has the opportunity to overcome these failures in recombination by secondary rearrangements (D-J replacements, V-J replacements, or V replacements), besides the presence of two IgH and IgL alleles (6,22-26). A self-reactive B-cell has the opportunity to replace its Igκ chain by a new Igκ chain (via V-J replacement) or by a newly assembled Igλ chain. These latter processes are a form of receptor editing (27-29).

Immunogenotypic findings in precursor B-ALL cells

From the literature and our own data we know that TdT and RAG expression is found in all precursor B-ALL cells (11-14,30-33). IgH gene rearrangements are found in 96% in precursor B-ALL, whereas in 4% of cases the IgH genes are in germline configuration or (partly) deleted on both alleles (see Chapter 3.2). Furthermore, biclonality and/or oligoclonality at diagnosis has been found in 30-40% of precursor B-ALL cases at the IgH gene level and in ~6% at the Igκ gene level (see Chapter 3.2 and Table 1). This form of subclone formation can be explained by continuing rearrangement processes (see above and Chapter 3.2). These continuing rearrangement processes are also (partly) responsible for the changes in Ig gene rearrangement patterns (so-called clonal evolution) at relapse in ALL (see Chapter 4.3). The development of changes in Ig and TcR gene rearrangement patterns was related to remission duration, suggesting an increasing chance of continuing rearrangement processes with time.

Igκ gene rearrangements and/or deletions are found in 62% of precursor B-ALL cases, which is a higher percentage than would be expected in normal immature B-cells because

TABLE 1. Immunogenetic characteristics of B-lineage leukemias[a].

	Precursor B-ALL[b]	Chronic B-cell leukemias[c]
IgH genes		
rearrangements (R/G, R/R, D/R)	96%	100%
biallelic deletions (D/D)	4%	0%
bi-/oligoclonality	30-40%	<1%
N-region insertion[d]	~10-30 bp	~10-30 bp
Igκ genes[e]		
rearrangements (R/G, R/R, D/R)	29%	Igκ^+ B-cells: 100% Igλ^+ B-cells: 83%
deletions (Kde-mediated) (D/G, D/D)	50%	Igκ^+ B-cells: 32% Igλ^+ B-cells: 100%
N-region insertion[d] (Kde)	~5 bp	~1 bp
TcR genes		
rearrangements and/or deletions	94%	0-5%
N-region insertion[d]	~6 bp[f]	?

a. The immunogenotypic characteristics found in chronic B-cell leukemias are believed to be comparable with the immunogenotypic findings in normal mature B-cells, because these leukemias encompass cells which are assumed to have gone through the normal B-cell differentiation pathway.

b. The precursor B-ALL include null ALL, common ALL, and pre-B-ALL and their data are obtained from the various chapters of this thesis.

c. The chronic Smlg$^+$ B-cell leukemias include chronic lymphocytic leukemias, hairy cell leukemias, and prolymphocytic leukemias and their data are obtained from Chapters 2.2, 2.3, and 5.2.

d. N-region insertion is found at the junctions of V-D and D-J gene rearrangements in case of IgH, TcR-β, and TcR-δ genes. The N-region insertion is found at the junctions of V-J gene rearrangements in case of TcR-γ genes and at the junctions of V-Kde and intron RSS-Kde in case of Igκ genes.

e. In 62% of precursor B-ALL cases, Igκ gene rearrangements and/or deletions are found. Vκ-Jκ rearrangements and Kde-mediated Igκ gene deletions (Kde rearrangement to the intron RSS (heptamer)) can be found on one allele simultaneously resulting in a decrease of the total number of Igκ gene rearrangements and/or deletions.

f. Data are obtained from references (43-46).

in 10-20% of immature B-cells Igκ gene rearrangements are present (Table 1) (see also Chapter 2.1 and 2.3) (34-37). Moreover, in normal (immature) B-cells the junctional regions formed by Vκ and Jκ gene rearrangements are small due to limited N-nucleotide insertion (N-region) (~1 bp; range: 0-5) (38-41). As soon as IgH gene rearrangements are completed during B-cell differentiation, TdT activity starts to decrease, resulting in limited N-regions in IgL gene rearrangements. Our data comparing junctional regions formed by Kde rearrangements in precursor B-ALL and chronic B-cell leukemias show that TdT is still active in precursor B-ALL as shown by an average N-region of 5 bp (range 0-18 bp), whereas in the chronic B-cell leukemias an average N-region of 1 bp (range 0-8 bp) was found, which appeared to be comparable with the N-regions found in junctional regions formed by Vκ-Jκ gene rearrangements in the normal B-cell (Table 1) (see above and Chapter 5.2) (38-41).

Another striking difference between precursor B-ALL and normal (immature) B-cells is the presence of cross-lineage TcR gene rearrangements, occurring in 94% of precursor B-ALL and in only ~5% in chronic B-cell leukemias and probably not in normal precursor B-cells (see also Chapter 3.4 and Table 1) (30,42). The various immunogenotypic findings in precursor B-ALL and chronic B-cell leukemias are summarized in Table 1. The immunogenotypic characteristics found in chronic B-cell leukemias are believed to be comparable with the immunogenotypic findings in normal mature B-cells, because these leukemias encompass cells which are assumed to have gone through the normal B-cell differentiation pathway (47). Although it is not clear where in the B-cell differentiation the malignant transformation occurs, apparently the maturation arrest of the 'malignant' B-cell takes place in a later stage when the recombinase system is inactive.

The development of the precursor B-ALL cell

Although the precursor B-ALL cell is comparable with its normal counterpart at the morphologic and immunophenotypic level, in the majority of cases (>90%) its immunogenotype differs completely from normal (immature) B-cells. What are the explanations for these findings in precursor B-ALL and what can be learned from it, considering the origin and the development of precursor B-ALL?

A. Are immature B-cells with an aberrant immunogenotype leukemia-target cells?
B. Are (pre-)abortive B-cells leukemia-target cells?
C. Represent immunogenotypic abnormalities a genetic deregulation as a result of the malignant transformation and maturation arrest of an immature B-cell?

These three possibilities are visualized in Figure 1. In this hypothetical diagram of the origin of the precursor B-ALL and chronic B-cell leukemia cells, two compartments are indicated: one populated with non-leukemic (precursor) B-cells, divided in 'normal' (precursor) B-cells and (pre-)abortive B-cells and one populated with leukemic cells (monoclonal or bi-/oligoclonal cell populations). These two compartments are separated by the malignant transformation (oncogenic event). As shown, immunogenotypic aberrations (e.g. early Igκ gene rearrangements and/or deletions, large N-regions in the junctional regions of Kde rearrangements, cross-lineage TcR gene rearrangements, and the increasing chance of continuing rearrangement processes with time) occur either before (possibilities A and B) or as result of the malignant transformation (possibility C). The fact that these immunogenotypic aberrations, untill now, are only found in precursor B-ALL and not in normal

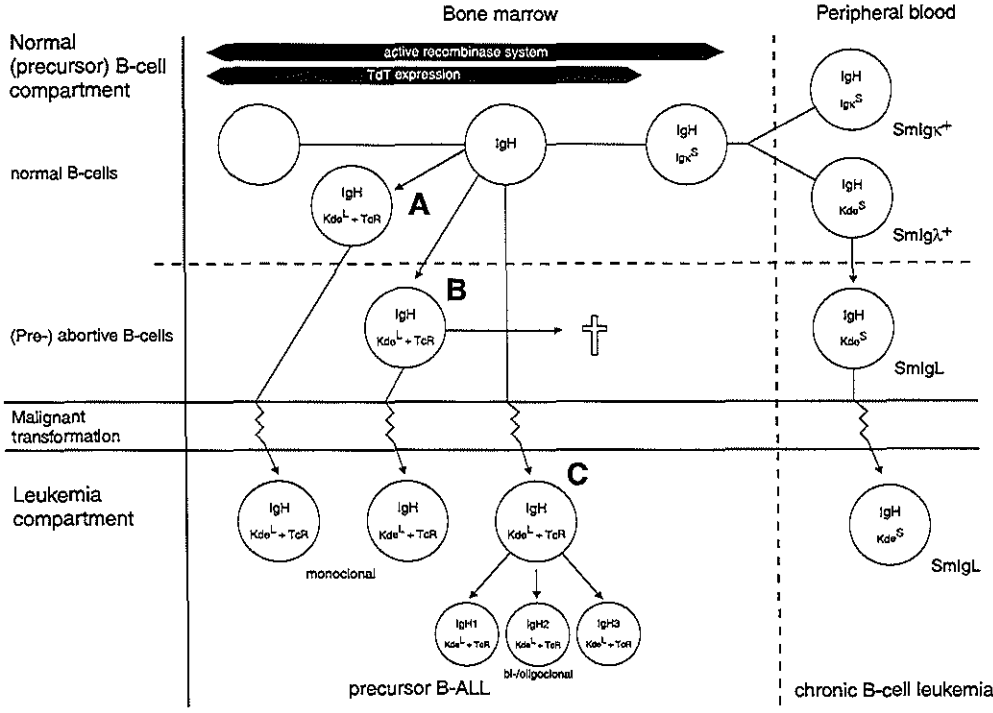

Figure 1. Hypothetical diagram of the origin of the precursor B-ALL and chronic B-cell leukemia, based on Ig and TcR gene rearrangement studies. Indicated are two compartments: one populated with non-leukemic (precursor) B-cells, divided in 'normal' (precursor) B-cells and (pre-)abortive B-cells and one populated with leukemic cells (monoclonal or bi-/oligoclonal cell populations). These two compartments are separated by the malignant transformation. The explanation of the three possibilities in development of (precursor)B-cell leukemia are discussed in the text. In case of chronic B-cell leukemias it is not clear where in the B-cell differentiation the malignant transformation occurs, apparently the maturation arrest of the 'malignant' B-cell takes place in a later stage when the recombinase system is inactive. Abbreviations used: IgH, IgH gene rearrangements; Igκ^{S}, Igκ gene rearrangements with small N-regions; Kde^{L}, Kde rearrangements with large N-regions; Kde^{S}, Kde rearrangements with small N-regions; Smlgκ^{+}/Smlgλ^{+}/SmlgL, expression of Ig molecules on the cell membrane.

(immature) B-cells or chronic B-cell leukemias and the fact that RAG and TdT are continuously expressed in precursor B-ALL cells, give strong support for possibility C. This means that the ongoing activity of the recombinase system as result of the malignant transformation and maturation arrest is the cause for these immunogenetic abnormalities. Unfortunately, definitive conclusions are difficult to draw.

The solution to this dilemma may be found in the immunogenotypic characterization of normal precursor B-cells. Although currently no indications for the presence of immunogenetic abnormalities in normal precursor B-cells have been observed, the knowledge of immunogenetic characteristics of normal precursor B-cells is limited.

Precursor B-cells can easily be identified by the presence of TdT expression on the nuclear membrane and simultaneously CD10 expression on the cell membrane, unfortunately their number in normal BM is low (children 5-10%; adults 2-5%) and sufficient cells for

immunogenotypic analysis are therefore difficult to obtain. This problem may be overcome by using regenerating BM from children treated by chemotherapy for malignancies (e.g. solid tumors or brain tumors). Because, in between the therapy protocols, the BM is often loaded with high numbers of CD10/TdT double positive cells (20-50% of mononuclear cells) due to regeneration.

After cell sorting, DNA can be isolated from the precursor B-cells and subsequently analyzed. Using the presence of preferential gene rearrangements, which can be easily evaluated due to known restriction fragments, can probably answer the raised questions. These preferential gene rearrangements are: e.g. Kde rearrangements to the recombination signal sequences in the Jκ-Cκ intron (intron RSS) and Vδ2-Dδ3 or Dδ2-Dδ3 gene rearrangements. Within a few years, single precursor B-cell analysis by use of laser microscopy and single cell PCR will be possible, providing a new dimension in this field of research.

B-lineage leukemias as a model for normal B-cell differentiation

As mentioned above, B-lineage leukemias are generally considered as clonal malignant counterparts of normal (immature) B-cells. The clonal cells of these leukemias are thought to be frozen in a specific stage of the B-cell differentiation. Precursor B-ALL are assumed to originate in the BM from malignantly transformed precursor B-cells. This is confirmed by the fact that the precursor B-ALL and its normal counterpart have comparable morphological and immunophenotypic characteristics. In addition, although aberrant (e.g. cross-lineage) immunological marker expression can be found on precursor B-ALL cells, it cannot be excluded that these 'aberrant' markers are also found on normal precursor B-cells.

The immunogenotypic findings in precursor B-ALL described and discussed in this thesis are in the far majority not comparable with the immunogenotypic characteristics of normal immature B-cells. This raises the question whether it is permitted to use leukemias as a model for studying normal lymphoid differentiation, especially in case of Ig and/or TcR gene rearrangement studies. We know that leukemias are clonal cell populations, in which generally each cell has identical morphological, immunophenotypic, karyotypic, and immunogenotypic features. This phenomenon provides an unique tool for studying the 'single' cell, which can be easily distinguished from the background of 'normal' cells. This is also the case with immortalized ALL cells, the so-called cell lines. Still, the the use of ALL or cell lines as a model for normal lymphoid differentiation has its limitations, because it is difficult to distinguish between processes occurring as a result of the malignant transformation and maturation arrest, and processes due to physiological processes occurring in normal (precursor) B-cells. Fortunately, these limitations (especially immunogenetic abnormalities) can be recognized and explained easily.

6.3 PERSPECTIVES

Although treatment results in childhood ALL have improved considerably during the last two decades, still 20-30% of ALL patients develop a relapse. Unfortunately, in adult ALL the prognosis is worse, because 60-75% of adult ALL patients will eventually develop a relapse. For the most optimal choice of ALL treatment in children and adults, accurate

diagnosis and follow-up of ALL and a reliable prognosis of the disease course is important. Besides morphological and immunophenotypic analysis at diagnosis, immunogenotypic characterization of the malignant cell (population) with the Southern blot technique can provide additional information (e.g. subclone formation), important for prognosis and follow-up of the patient. Still to prevent unnecessary extensive analyses and needless expense, careful evaluation of the various possible analyses in different patients or patient groups have to be done. Furthermore, the clinical relevance (lower relapse rates and higher survival rates due to adaptation of treatment protocols) of early detection of MRD should be investigated in prospective multicenter studies. The costs of these analyses will be high and should be considered in the advantages of early detection of MRD.

Although extensive studies in ALL have given more insight in the origin of ALL, explanations for its cause remain ambiguous. Our immunogenotypic studies helped to raise a corner of the veil. Future studies especially in normal precursor B-cells will hopefully elucidate the relation between the malignant cell and its normal counterpart.

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SUMMARY

In children the majority (~85%) of acute leukemias consists of acute lymphoblastic leukemia (ALL), while a minority (~15%) consists of acute non-lymphocytic leukemia (ANLL). ALL is a disorder characterized by an uncontrolled proliferation of immature lymphoid cells originating either in the bone marrow (BM) or in the thymus. In general, each ALL cell has identical characteristics, thereby proving that ALL is a clonal disease. Although treatment results in childhood ALL have improved considerably during the last two decades, still 20-30% of ALL patients develops a relapse. To increase insight into the factors and mechanisms which influence the development of a relapse in childhood ALL, extensive analyses have been performed on the leukemic cells at diagnosis and subsequent relapse. These analyses concerned morphological, immunophenotypic, karyotypic, and immunogenotypic analysis.

Chapter 1 summarizes the present knowledge on epidemiology, etiology, diagnosis, treatment, and relapse of childhood ALL. Each year 75-85 children with ALL are diagnosed in the Netherlands, with an incidence rate of ~3.7 per 100,000 children (<16 years). Inherited, environmental, and endogenous factors are assumed to be responsible for the induction of ALL by various mechanisms and through a multi-step process. Still, in most cases the etiology is unknown. Immature B- and T-cells are due to its intrinsic developmental program at higher risk of (spontaneous) mutation than other somatic cells, particularly at early stages of development. This probably explains the relative high frequencies of leukemia in young children. Still, leukemias will only develop through mutational events when these mutations effect critical genes, such as proto-oncogenes or suppressor genes. The findings of non-random specific chromosomal abnormalities associated with ALL supports the important pathogenetic role of genetic alterations.

The diagnosis of ALL is traditionally based on cytomorphology and cytochemistry of BM smears and classified according to the French American British (FAB) classification system. With the application of more advanced techniques, such as immunological marker analysis, cytogenetic analysis, and molecular analysis, the diagnosis of ALL has become more accurate. In addition, these advanced techniques have given the possibility to further subcategorize the ALL. The latter appeared to be important for the determination of prognostic factors at diagnosis and for stratification in treatment protocols. Chemotherapy is the treatment of first choice in childhood ALL. Presently, a complete remission is achieved in ~97% of childhood ALL cases with the applied chemotherapy. Bone marrow transplantation (BMT) will be done after first relapse during second remission, or in first remission in case of the occurrence of special translocations, such as t(9;22) and t(4;11), which are associated with a poor prognosis. Nevertheless, still 20-30% of ALL patients relapse. The most common site of ALL relapse is the BM, but extramedullary relapses (e.g. in the central nervous system, testis, or other isolated sites) are not unusual. Although relatively little is known about the cause of relapse, in the majority of relapse cases a relation with the ALL at initial presentation can be proven by identical morphology, immunophenotype, karyotype, or immunogenotype. Apparently, despite of the applied

cytostatic treatment, residual ALL cells persist. This emphasizes the importance of methods to detect residual ALL cells to determine the effectiveness of the applied treatment.

In general, ALL are regarded as clonal malignant counterparts of normal immature B- and T-cells. Immunoglobulin (Ig) and T-cell receptor (TcR) gene rearrangements, necessary for obtaining antigen specific receptor molecules on the cell membrane of mature B- and T-lymphocytes, respectively, occur early during lymphoid differentiation. Therefore, ALL cells will have identically rearranged Ig or TcR genes. Chapter 2.1 describes the basic aspects of these antigen specific receptor genes and gene rearrangements. Analysis of Ig and TcR gene rearrangements by Southern blotting or by the polymerase chain reaction (PCR) technique can be used for diagnostic studies. To improve the detection of Ig heavy-chain (IgH) and kappa Ig light-chain (IgL) gene rearrangements by Southern blot analysis for diagnostic purposes, the precise restriction map of the relevant IgH and Igκ regions were carefully determined. In addition, the appropriate combinations of restriction enzymes with newly developed optimal DNA probes were evaluated (Chapter 2.2 and 2.3). Our extensive Southern blot analysis revealed that rearrangements in the JH and Jκ region are optimally detectable by use of J probes, which are complementary to the 3´ flanking sequences of the J region, such as the IGHJ6 and IGKJ5 probes for detection of IgH and Igκ gene rearrangements, respectively. Furthermore, at least two different restriction enzyme digests which are not affected by polymorphisms, and which produce small restriction fragments (generally <10 kb), are recommended. Small restriction fragments will reduce the chance of comigration of germline and/or rearranged bands (Chapter 2.2 and 2.3).

Igκ gene deletions occur in $\sim 50\%$ of precursor B-ALL and in the far majority ($>98\%$) are mediated via a site-specific rearrangement of a so-called kappa deleting element (Kde). Two types of Kde-mediated Igκ gene deletions (Kde rearrangements) can occur: i.e. Kde rearrangements either to heptamer recombination signal sequences in the Jκ-Cκ intron (intron RSS), thereby deleting the Cκ region, or to a Vκ gene segment, thereby deleting the Jκ and Cκ regions. The two types of Kde rearrangements are easily detectable with a newly developed Kde probe (IGKDE). This is in contrast to the inaccurate information obtained with the Jκ and Cκ probes, because these probes detect deletions only in the form of decreased densities of Jκ and/or Cκ germline bands in the absence of rearranged bands (Chapter 2.3).

With these optimal Southern blot conditions, Ig gene rearrangements were analyzed in a large group of precursor B-ALL patients at diagnosis (Chapter 3). The results show that IgH, Igκ, and Igλ gene rearrangements and/or deletions occur in 96%, 62%, and 21% of cases, respectively, suggesting a hierarchical order in IgH and IgL gene rearrangements. When excluding hyperdiploidy of chromosome 14, multiple IgH and Igκ genes were found in 30-40% and 6% of cases, respectively. These multiple IgH and Igκ gene rearrangements are most probably due to subclone formation (bi-/oligoclonality), caused by continuing or secondary gene rearrangements in the majority of precursor B-ALL. It has been suggested that precursor B-ALL with multiple IgH gene rearrangements have a higher tendency to relapse. Although higher relapse rates were found in the oligoclonal group (53%) compared with the monoclonal group (20%), the log rank trend test showed no significance (Chapter 3.2).

In five of ten bi-/oligoclonal precursor B-ALL patients differences in IgH gene rearrangement patterns between BM and peripheral blood samples at diagnosis were found.

These differences could be interpreted as the presence of an extra subclone in two cases and differences in size of the subclones in three cases. In all five cases, the extra subclones or the relatively larger sized subclones were found in the BM, suggesting that subclone formation in precursor B-ALL starts in this tissue compartment from which the precursor B-ALL cells are thought to originate (Chapter 3.3).

Chapter 3.4 describes the Southern blot analysis of cross-lineage TcR gene rearrangements in precursor B-ALL. In 94% (101/108) of the precursor B-ALL group, at least one TcR gene was rearranged. TcR-β, TcR-γ, and TcR-δ gene rearrangements and/or deletions were found in 36%, 57%, and 91% of cases, respectively. TcR-β gene rearrangements were monoallelic V(D)Jβ2 rearrangements in 54% and incomplete Dβ-Jβ2 rearrangements in 37% of cases. TcR-γ gene rearrangements most frequently contained monoallelic rearrangements to Jγ1 gene segments and ~80% of TcR-δ gene rearrangements represented incomplete Vδ2-Dδ3 or Dδ2-Dδ3 gene rearrangements. The most likely explanation for the occurrence of these cross-lineage TcR gene rearrangements in precursor B-ALL is that these rearrangements represent a genetic deregulation due to the ongoing activity of the recombinase system after malignant transformation and maturation arrest. In this way the virtual absence of cross-lineage TcR gene rearrangements in normal precursor B-cells and in mature B-cell malignancies can be explained. Comparison of our results with over 400 cases reported in the literature revealed a higher percentage of cross-lineage TcR gene rearrangements in our group of precursor B-ALL. This discrepancy is probably due to our larger panels of TcR DNA probes (especially for the TcR-β and TcR-δ genes).

One of the important aims of the study was to obtain insight into the mechanisms which influence the development of relapse in childhood ALL. For this purpose, several comparative analyses at diagnosis and relapse have been performed. Chapter 4.1 summarizes the relevant comparative cytogenetic data reported in the literature. Karyotypic shifts were found in 33% (81/246 cases) at relapse. In only a minority of these shifts (5%;4/81) no clonal relation was found. Our comparative morphological and immunophenotypic analyses at diagnosis and relapse in 40 childhood ALL (32 precursor B-ALL and eight T-ALL) are described in Chapter 4.2. Morphological differences between diagnosis and relapse were detected in 34% of precursor B-ALL and in only one case of T-ALL. Differences in immunological marker expression were found in 69% of precursor B-ALL and in 63% of T-ALL. No obvious relation was found between the morphological shifts and the marker-shifts. In 10%, the marker-shifts resulted in an intra-lineage shift and in 5% in an inter-lineage shift to ANLL in precursor B-ALL. In T-ALL no intra- or inter-lineage shifts were found. Chapter 4.3 summarizes the comparative analysis of Ig and TcR genes in 40 childhood ALL (30 precursor B-ALL and 10 T-ALL) at diagnosis and relapse. Differences in rearrangement patterns between diagnosis and relapse were found in 67% (20 cases) of precursor B-ALL (including all eight analyzed bi-/oligoclonal cases) and 50% (five cases) of T-ALL. In all analyzed Ig (IgH, Igκ, and Igλ) and TcR (TcR-β, TcR-γ, and TcR-δ) genes, changes were found in precursor B-ALL at relapse. In T-ALL, changes at relapse were detected in all Ig and TcR genes except for the IgL genes, which remained in germline configuration. Two precursor B-ALL showed completely different Ig and TcR genes rearrangement patterns at relapse, suggesting the absence of a clonal relation between the leukemic cells at diagnosis and relapse and the development of a secondary leukemia (i.e.

ANLL). The clonal evolution in the other 23 ALL patients was based on continuing rearrangement processes and selection of subclones. The development of changes in Ig and TcR gene rearrangement patterns was related to remission duration, suggesting an increasing chance of continuing rearrangement processes with time.

Ig and TcR gene rearrangement patterns in leukemias and malignant lymphomas have been used as markers for clonality, which are unique for each malignancy. This especially concerns the junctional regions of rearranged Ig and TcR genes, because they are regarded as tumor-specific markers. Based on this assumption, the PCR technique has been used for amplification of the tumor-specific junctional regions of rearranged IgH, TcR-γ, and TcR-δ genes to detect minimal residual disease (MRD) during follow-up of ALL patients, as summarized in Chapter 5.1. The junctional regions of IgH, TcR-γ, and TcR-δ gene rearrangements can be identified in $\sim$80% of precursor B-ALL and in >90% of T-ALL (Chapters 3.4 and 5.1). The heterogeneity in Ig and TcR gene rearrangement patterns found at diagnosis and relapse might hamper PCR-mediated MRD detection. However in 75-90% of ALL cases, at least one IgH, TcR-γ, or TcR-δ allele remains stable at relapse. Therefore, two or more junctional regions of different Ig/TcR genes should be monitored for optimal MRD detection during follow-up of ALL patients (Chapters 4.3 and 5.1). ALL are also characterized by high frequencies of clonal chromosome aberrations (e.g. translocations). These translocations result in fusion genes and fusion transcripts, such as t(9;22), t(1;19), and t(4;11) in precursor B-ALL or aberrations with site-specific breakpoints such as *TAL1* deletions in T-ALL. So far such breakpoint fusion region can be identified in 15-20% of childhood ALL and 25-30% of adult ALL. The main advantage of chromosome aberrations as PCR-MRD target is its stability during the disease course (Chapter 5.1).

Chapter 5.2 describes the analysis of junctional regions formed by Kde rearrangements either to the intron RSS or to a Vκ gene segment in B-lineage leukemias (precursor B-ALL and chronic B-cell leukemias). Subsequent PCR and sequencing analysis of the junctional regions revealed a striking difference between both groups of B-lineage leukemias. An average insertion and deletion of 4.9 and 4.2 nucleotides, respectively, resulting in a large junctional diversity in precursor B-ALL was found, whereas in chronic B-cell leukemias this junctional diversity was essentially lower (1.0 and 2.5, respectively), especially in the junctional regions formed by Vκ-Kde rearrangements. This junctional diversity as well as the limited number of primers necessary for PCR analysis of Kde rearrangements make Kde rearrangements a potential PCR target for MRD detection in precursor B-ALL. Kde rearrangements were found in $\sim$50% in precursor B-ALL (Chapter 2.3). A detection limit of $\sim 10^{-4}$ was observed in a dilution experiment and the specificity of the leukemia/patient-specific oligonucleotide probes was proven by hybridization analysis. The stability of the MRD-PCR target was proven in five precursor B-ALL at relapse.

Chapter 6 addresses the significance of the various immunogenotypic findings in precursor B-ALL for the development of ALL and the implications for the use of precursor B-ALL as a model for normal B-cell differentiation. The immunogenetic characteristics of the precursor B-ALL (e.g. early Igκ gene rearrangements and/or deletions, substantial N-nucleotide insertion in the junctional regions of Kde rearrangements, and cross-lineage TcR gene rearrangements) are generally not found in normal immature B-cells. The most likely explanation for these 'aberrant' immunogenetic findings is that these 'aberrations' represent a genetic deregulation due to ongoing activity of the recombinase system as a result of the

malignant transformation and maturation arrest in an immature B-cell stage. The use of precursor B-ALL as a model for normal B-cell differentiation has its limitations, because it is difficult to distinguish between processes occurring as a result of the malignant transformation and maturation arrest, and processes due to physiological mechanisms occurring in normal (immature) B-cells.

We conclude that accurate diagnosis and follow-up of leukemias and lymphomas is important for optimal choice of treatment in children and adults. Nowadays, extensive analysis (morphological, immunophenotypic, cytogenetic, molecular, and cell culture analysis) is possible in almost every leukemia or lymphoma patient at diagnosis to obtain all relevant data of the leukemia or lymphoma. In this way a reliable prognosis of the disease course can be made and the most optimal treatment can be applied. Still to prevent unnecessary extensive analyses and needless expense, careful evaluation of the various possible analyses in different patients or patient-groups has to be done. In addition, the significance of MRD studies with PCR during follow-up has to be validated in prospective multicenter studies. Moreover, the extra costs of these analyses have to be considered against the advantages of these MRD-PCR studies for the treatment of ALL and lymphoma patients.

SAMENVATTING

Acute leukemie op de kinderleeftijd is in ±85% van de gevallen een acute lymfatische leukemie (ALL) en in ±15% een acute niet-lymfatische leukemie (ANLL). ALL is een vorm van bloedkanker die wordt gekenmerkt door een ongecontroleerde groei van onrijpe lymfatische cellen in het beenmerg of in de thymus. In het algemeen worden de cellen van een ALL beschouwd als kwaadaardige tegenpolen van normale onrijpe B- en T-cellen. Deze ALL cellen blijken vergelijkbare kenmerken te hebben, waarmee het ontstaan uit één cel (klonaliteit) van de ALL bevestigd wordt. Ondanks de verbeterde behandelingsresultaten bij ALL op de kinderleeftijd gedurende de laatste twintig jaar, ontwikkelt 20 tot 30% van de kinderen een recidief. Om het inzicht te vergroten in de factoren en mechanismen welke het ontstaan en de verdere ontwikkeling van een recidief van ALL op de kinderleeftijd beïnvloeden, hebben wij de leukemiecellen uitgebreid geanalyseerd bij zowel diagnose als recidief. Dit betreft morfologische, immunofenotypische, cytogenetische en immunogenotypische analyses.

Hoofdstuk 1 beschrijft de huidige kennis van de epidemiologie, etiologie, diagnose, behandeling en ontstaan van recidief bij ALL op de kinderleeftijd. Elk jaar worden in Nederland 75 tot 85 kinderen met een ALL gediagnostiseerd, met een incidentie van 3,7 nieuwe patiënten per 100.000 kinderen (leeftijd <16 jaar). Men veronderstelt dat erfelijke, omgevings- en endogene factoren verantwoordelijk zijn voor het ontstaan van ALL via een meerstaps tumor-inducerend proces. Toch is van de meeste ALL de oorzaak onbekend.

Vooral in de eerste levensjaren worden B- en T-cellen massaal aangemaakt, omdat deze cellen een centrale rol spelen in het afweersysteem, dat zich juist op die leeftijd snel ontwikkelt als reactie op de (eerste) contacten met de vele micro-organismen. Onrijpe B- en T-cellen hebben tijdens hun ontwikkeling een hoger risico op (spontane) mutaties in het DNA dan andere lichaamscellen. Dit verklaart waarschijnlijk de relatief hoge frequentie van ALL bij jonge kinderen. Een leukemie kan echter alleen ontstaan op basis van mutaties, wanneer deze mutaties plaatsvinden in specifieke genen, zoals proto-oncogenen of suppressiegenen. De rol van genetische veranderingen bij het ontstaan van leukemie wordt benadrukt door de associatie tussen "non-random" chromosoomafwijkingen en verschillende typen ALL, zoals voorloper B-ALL en T-ALL.

Van oudsher wordt ALL gediagnostiseerd en geklassificeerd volgens het Frans-Amerikaans-Britse (FAB) klassificatiesysteem, dat gebaseerd is op morfologische en cytochemische kenmerken van de leukemiecellen in een beenmerguitstrijkje. Door toepassing van meer geavanceerde technieken, zoals immunologische markeranalyse, cytogenetische analyse en immunogenetische analyse, is nadere karakterisering van ALL mogelijk en kunnen verschillende ALL (sub)typen worden onderscheiden. Dit blijkt belangrijk te zijn voor de prognose en het vaststellen van het behandelingsprotocol.

Kinderen met ALL worden in het algemeen behandeld met chemotherapie, waarmee bij ongeveer 97% van de kinderen een complete remissie (CR) wordt bereikt; dit betekent dat in bloed en beenmerg geen leukemiecellen aantoonbaar zijn met morfologische technieken. Een tweede mogelijke therapie, de beenmergtransplantatie (BMT) waarbij

beenmerg (BM) van een donor (vaak broer of zus) of "schoon" BM van de patiënt zelf gegeven wordt aan de leukemie patiënt zal veelal pas worden uitgevoerd na het eerste recidief tijdens tweede CR. In bijzondere gevallen, zoals bij specifieke chromosoom translocaties met een slechte prognose bijvoorbeeld t(9;22) en t(4;11)) kan BMT worden toegepast in eerste CR. Tegenwoordig recidiveert 20 tot 30% van de kinderen met ALL. Meestal ontstaat een recidief in het beenmerg, hoewel het niet ongewoon is dat recidieven buiten het beenmerg ontstaan, zoals in het centraal zenuwstelsel, in de testis of op andere geïsoleerde locaties. Hoewel nog weinig bekend is over de oorzaak van een ALL recidief, wordt in de meerderheid van de recidieven een relatie met de oorspronkelijke leukemiecellen bij diagnose gevonden: de morfologische, immunofenotypische, cytogenetische en immunogenotypische kenmerken zijn geheel of gedeeltelijk identiek. Hoewel de patiënten in CR waren, heeft de chemotherapie blijkbaar niet alle leukemiecellen kunnen doden. Het is daarom belangrijk om technieken te ontwikkelen voor het aantonen van kleine aantallen resterende leukemiecellen, zodat de effectiviteit van de chemotherapie beter vast gesteld kan worden.

Immunoglobuline (Ig) en T-cel-receptor (TcR) genen coderen voor de antigeen-specifieke receptoren op de celmembraan van respectievelijk rijpe B- en T-cellen. Deze Ig en TcR genen zullen vroeg tijdens de lymfatische differentiatie herschikken met behulp van een genherschikkings-enzymsysteem, waarbij V, (D) en J gensegmenten van Ig en TcR genen aan elkaar gekoppeld worden. Door de klonaliteit van de ALL zal elke leukemiecel identiek herschikte Ig en/of TcR genen hebben. Hoofdstuk 2.1 beschrijft de basale aspecten van deze antigeen-specifieke receptorgenen en genherschikkingen. Analyse van de Ig en TcR genherschikkingen met behulp van de "Southern blot" techniek en de zogenaamde polymerase ketting reactie (PCR) techniek kan gebruikt worden voor onderzoek en diagnostiek van leukemieën en lymfomen. Om herkenning van de Ig zware keten (IgH) en kappa Ig lichte keten (IgL) genherschikkingen met behulp van de "Southern blot" techniek te verbeteren, werd een gedetailleerde kaart met knipplaatsen van verschillende relevante restrictie-enzymen (restrictie kaart) van de IgH en Igκ genen samengesteld. Tevens werden optimale combinaties van restrictie-enzymen en nieuw gemaakte DNA probes geselecteerd en geëvalueerd (Hoofdstuk 2.2 en 2.3). Uitgebreide "Southern blot" analyse maakt duidelijk dat herschikkingen in de IgH en Igκ genen het best aangetoond kunnen worden met J probes die het gebied pal achter de J regio's herkennen zoals onze IGHJ6 en IGKJ5 probes. Wij raden hierbij aan om twee verschillende restrictie-enzym digesten te gebruiken, die niet beïnvloed worden door polymorfismen en die resulteren in kleine restrictiefragmenten (<10 kb). Kleine restrictiefragmenten verkleinen namelijk de kans op co-migratie van kiemlijn en/of herschikte banden zodat problemen bij de interpretatie van de analyse veelal voorkomen worden (Hoofdstuk 2.2 en 2.3).

Igκ gendeleties komen in ongeveer 50% van de voorloper B-ALL voor. Deze Igκ gendeleties worden in de meerderheid ($>98\%$) gereguleerd door een specifieke herschikking van het zogenaamde kappa deletie element (Kde). Er zijn twee typen Kde herschikkingen bekend: een Kde herschikking naar een heptameer recombinatie signaal sequentie in het Jκ-Cκ intron (intron RSS), waarbij de Cκ regio verdwijnt en een Kde herschikking naar een Vκ gensegment, waarbij naast de Cκ regio ook de Jκ regio verdwijnt. Deze twee typen Kde herschikkingen zijn gemakkelijk te herkennen met een door ons nieuw ontwikkelde Kde probe (IGKDE). Het aantonen van deze Kde herschikkingen met de Jκ en

Cκ probes is veel lastiger of onmogelijk omdat deze probes de Kde herschikkingen alleen kunnen herkennen als een zwakker signaal van de Jκ en/of Cκ kiemlijn banden zonder dat er herschikte banden aanwezig zijn (Hoofdstuk 2.3).

De optimale "Southern blot" condities werden gebruikt voor analyse van Ig genherschikkingen bij een grote groep voorloper B-ALL patiënten bij diagnose (Hoofdstuk 3). De resultaten lieten zien dat in de voorloper B-ALL patiënten IgH, Igκ, en Igλ genherschikkingen en/of deleties voorkomen in respectievelijk 96%, 62% en 21%. Meer dan twee IgH en Igκ genherschikkingen werden gevonden bij respectievelijk 30 tot 40% en 6% van voorloper B-ALL patiënten. Deze meerdere IgH en Igκ genherschikkingen worden veroorzaakt door subkloonvorming (bi-/oligoklonaliteit) binnen de voorloper B-ALL. In de meerderheid van de voorloper B-ALL wordt deze subkloonvorming veroorzaakt door doorgaande of secundaire genherschikkingen. In de literatuur werd gesuggereerd dat voorloper B-ALL patiënten met meerdere IgH genherschikkingen een grotere kans hebben op een recidief. Hoewel in onze oligoklonale patiënten relatief meer recidieven (53%) werden gevonden in vergelijking met de monoklonale patiënten (20%), was dit verschil toch niet significant (Hoofdstuk 3.2).

In vijf van de tien onderzochte bi-/oligoklonale voorloper B-ALL patiënten werden bij diagnose verschillen in IgH genherschikkingspatronen gevonden tussen beenmerg en bloed celmonsters. Deze verschillen konden bij twee patiënten worden geïnterpreteerd als de aanwezigheid van een extra subkloon en bij drie patiënten als een verschil in omvang van de verschillende subklonen. In elke patiënt werd de extra subkloon of relatief grotere subkloon in het beenmerg gevonden. Dit zou kunnen betekenen dat subkloonvorming in voorloper B-ALL start in het weefselcompartiment waar normale voorloper B cellen ontstaan, nl. het beenmerg.

Hoofdstuk 3.4 beschrijft de "Southern blot" analyse van "cross-lineage" TcR genherschikkingen in voorloper B-ALL. "Cross-lineage" genherschikkingen worden gedefinieerd als genherschikkingen die gevonden worden buiten hun eigen normale differentiatielijn. In 94% (101/108) van de voorloper B-ALL bleek tenminste één TcR gen herschikt te zijn. TcR-β, TcR-γ en TcR-δ genherschikkingen en/of deleties werden in respectievelijk 36%, 57% en 91% gevonden. TcR-β genherschikkingen bestonden voornamelijk uit monoallelische V(D)Jβ2 herschikkingen (54%) of incomplete Dβ-Jβ2 herschikkingen (37%). De TcR-γ genherschikkingen waren voornamelijk Jγ1 herschikkingen, terwijl de meeste TcR-δ genherschikkingen (±80%) Vδ2-Dδ3 of Dδ2-Dδ3 herschikkingen waren. De belangrijkste verklaring voor de aanwezigheid van de ongebruikelijke "cross-lineage" TcR genherschikkingen in voorloper B-ALL is dat deze genherschikkingen een genetische deregulatie vormen, veroorzaakt door een doorgaande aktiviteit van het genherschikkings-enzymsysteem ten gevolge van de kwaadaardige transformatie en de uitrijpingsblokkade. In normale voorloper B-cellen staat het genherschikkings-enzymsysteem tijdelijk aan en is de activiteit van dit enzymsysteem sterk gereguleerd, hetgeen waarschijnlijk verklaart dat de ongebruikelijke "cross-lineage" TcR genherschikkingen niet of nauwelijks voorkomen in normale onrijpe en rijpe B-cellen en ook niet in rijpe B-celmaligniteiten, zoals chronische B-cel leukemieën. In vergelijking met de "cross-lineage" TcR genherschikkingen bij meer dan 400 voorloper B-ALL patiënten die in de literatuur gepubliceerd zijn, bleek dat in onze voorloper B-ALL groep meer "cross-lineage" TcR genherschikkingen werden gevonden. Dit verschil wordt waarschijnlijk veroorzaakt door

onze gedetailleerde analyses met een groot aantal DNA probes, vooral voor analyse van de TcR-β en TcR-δ genen.

Eén van de belangrijkste doelen van ons onderzoek was om meer inzicht te krijgen in de mechanismen die het ontstaan en de verdere ontwikkeling van een recidief bij kinderen met ALL beïnvloeden. In de literatuur zijn reeds verscheidene vergelijkende analyses bij diagnose en recidief gepubliceerd. Hoofdstuk 4.1 vat informatie samen van relevante vergelijkende cytogenetische studies. Veranderingen in karyotype bij recidief werden bij 33% (81/246) van de ALL patiënten gevonden. In een minderheid van deze veranderingen (5%;4/81) werd geen klonale relatie aangetoond. Onze vergelijkende morfologische en immunofenotypische analyses bij diagnose en recidief van 40 kinderen met ALL (32 voorloper B-ALL en 8 T-ALL) zijn beschreven in Hoofdstuk 4.2. Veranderingen in de morfologie bij recidief werden gevonden bij 34% van de voorloper B-ALL en bij slechts één T-ALL. Verschillen in immunologische marker expressie werden gevonden bij 69% van de voorloper B-ALL en 63% van de T-ALL. Er werd geen duidelijke relatie aangetoond tussen de morfologische veranderingen en veranderingen in de expressie van immunologische markers. Verandering in de expressie van immunologische markers gaat bij 10% van de voorloper B-ALL gepaard met een intra-lineage verandering en bij 5% met een inter-lineage verandering naar een ANLL. Hoofdstuk 4.3 vat de informatie samen van de vergelijkende analyse van Ig en TcR genen bij diagnose en recidief van 40 kinderen met ALL (30 voorloper B-ALL en 10 T-ALL). Verschillen in Ig en TcR genherschikkingspatronen tussen diagnose en recidief werden gevonden bij 67% (20/30) van de voorloper B-ALL patiënten, inclusief de acht geanalyseerde bi-/oligoklonale voorloper B-ALL, en bij 50% (5/10) van de T-ALL patiënten. Hoewel de veranderingen in herschikkingspatronen ieder gen bleken te kunnen treffen, werden bij voorloper B-ALL vooral veranderingen in IgH en TcR-δ genen gevonden. Bij T-ALL werden veranderingen gevonden in IgH en de TcR genen, maar de IgL genen bleken in kiemlijnconfiguratie te blijven. In twee voorloper B-ALL patiënten werden geheel andere Ig en TcR genherschikkingspatronen gevonden bij het recidief vergeleken met de diagnose. De afwezigheid van een klonale relatie tussen diagnose en recidief bij deze twee patiënten kon worden verklaard door de ontwikkeling van een secundaire leukemie (ANLL). De klonale evolutie in de overige 23 kinderen met ALL was gebaseerd op doorgaande genherschikkingsprocessen en selectie van subklonen. De kans op veranderingen in Ig en TcR genherschikkingspatronen bij recidief bleek deels gerelateerd aan de remissieduur. Dit suggereert dat er een grotere kans bestaat op doorgaande genherschikkingen naarmate de ALL cellen langer overleven en het genherschikkings-enzymsysteem langer actief kan zijn.

De Ig en TcR genherschikkingspatronen worden gebruikt als unieke klonaliteitsmarkers in leukemieën en kwaadaardige lymfomen. Met name de verbindingsplaatsen tussen de verschillende, aan elkaar gekoppelde gensegmenten (de zogenaamde "junctional regions") van herschikte Ig en TcR genen worden gebruikt. Dit berust op het feit dat de "junctional regions" van herschikte Ig en TcR genen sterk kunnen variëren als gevolg van het samenkomen van V, (D) en J gensegmenten, het verlies van nucleotiden van deze gensegmenten en het toevoegen van extra nucleotiden op de verbindingsplaatsen van de gensegmenten. Deze "junctional regions" zijn identiek in elke ALL cel en worden daarom als tumor-specifieke markers beschouwd. Op basis van dit gegeven worden met behulp van de PCR techniek tumor-specifieke "junctional regions" van herschikte IgH, TcR-γ en TcR-δ

genen vermeerderd om kleine aantallen leukemiecellen tijdens follow-up van ALL patiënten aan te tonen (Hoofdstuk 5.1). Deze "junctional regions" van IgH, TcR-γ en TcR-δ genherschikkingen kunnen in ongeveer 80% van de voorloper B-ALL en meer dan 90% van de T-ALL precies worden geïdentificeerd (Hoofdstukken 4.3 en 5.1). De mogelijke verschillen tussen Ig en TcR genherschikkingspatronen bij diagnose en recidief, kan de PCR-gemedieerde detectie van kleine aantallen leukemiecellen hinderen. Gelukkig blijft in 75 tot 90% van de ALL patiënten tenminste één IgH, TcR-γ of TcR-δ gen stabiel bij het recidief. Voor optimale detectie van kleine aantallen leukemiecellen is het daarom belangrijk om twee of meer "junctional regions" van verschillende Ig en/of TcR genen te gebruiken bij het vervolgen van ALL patiënten (Hoofdstukken 4.3 en 5.1).

ALL worden ook gekenmerkt door klonale chromosoomafwijkingen zoals translocaties. Fusiegenen en fusie-transcripten zijn het gevolg van deze chromosoomafwijkingen, zoals bij t(9;22), t(4;11) en t(1;19) in voorloper B-ALL en bij *TAL1* deleties in T-ALL. Tot nu toe kunnen deze breukpunt-fusie regio's in 15 tot 20% van kinderen met ALL en in 25 tot 30% van volwassenen met ALL geïdentificeerd worden. Deze breukpunt-fusie regio's van de chromosoomafwijkingen kunnen ook als tumor-specifieke markers gebruikt worden en zijn dus geschikt om met behulp van de PCR techniek vermeerderd te worden om kleine aantallen leukemiecellen aan te tonen. De stabiliteit van deze chromosoomafwijkingen gedurende het ziekteverloop is hierbij het belangrijkste voordeel (Hoofdstuk 5.1).

Hoofdstuk 5.2 beschrijft de analyse van "junctional regions" gevormd door Kde herschikkingen naar de intron RSS en naar Vκ gensegmenten in voorloper B-ALL en chronische B-cel leukemieën. Achtereenvolgende PCR en sequentie analyse van deze "junctional regions" bracht een belangrijk verschil aan het licht tussen beide groepen leukemieën. Een forse junctionele diversiteit werd gevonden als gevolg van een gemiddelde toevoeging en verlies van respectievelijk 4,9 en 4,2 nucleotiden in de "junctional regions" van voorloper B-ALL, terwijl bij chronische B-cel leukemieën deze junctionele diversiteit aanmerkelijk minder was (respectievelijk 1,0 en 2,5 nucleotiden), vooral in de "junctional regions" gevormd door de Vκ-Kde herschikkingen. De omvangrijke junctionele diversiteit in voorloper B-ALL en het beperkte aantal oligonucleotide primers dat nodig is voor de PCR analyse van Kde herschikkingen, maken deze Kde herschikkingen een potentieel doelwit voor de detectie van kleine aantallen leukemiecellen bij patiënten met voorloper B-ALL. Deze Kde herschikkingen worden in ongeveer 50% van de voorloper B-ALL gevonden (Hoofdstuk 2.3). Een detectielimiet van $\pm 10^{-4}$ (1 leukemiecel tussen 10.000 normale cellen) werd gevonden in een verdunningsexperiment waarbij leukemiecellen werden verdund in normale witte bloedcellen. De specificiteit van de leukemie/patiënt-specifieke oligonucleotide probes werd bewezen door patiënt-specifieke hybridisatie resultaten. De stabiliteit van dit PCR doelwit werd bewezen door identieke PCR, hybridisatie of sequentie-analyse resultaten bij diagnose en recidief bij de vijf onderzochte voorloper B-ALL.

Hoofdstuk 6 bediscussieert de betekenis van de verschillende immunogenetische bevindingen in voorloper B-ALL voor het ontstaan en de verdere ontwikkeling van ALL. Tevens worden de implicaties voor het gebruik van voorloper B-ALL als model voor normale B-cel ontwikkeling besproken. De immunogenetische kenmerken van voorloper B-ALL, zoals vroege Igκ genherschikkingen en/of deleties, verlies en toevoeging van nucleotiden in de "junctional regions" van Kde herschikkingen en "cross-lineage" TcR genherschikkingen, werden niet of nauwelijks in normale voorloper B-cellen gevonden. De meest waarschijnlijke

verklaring voor deze ongebruikelijke immunogenetische bevindingen is dat ze een genetische deregulatie weerspiegelen, veroorzaakt door een doorgaande aktiviteit van het genherschikkings-enzymsysteem na kwaadaardige transformatie en de uitrijpingsblokkade in een voorloper B-cel stadium. Het gebruik van voorloper B-ALL als model voor normale B-cel ontwikkeling blijkt beperkingen te hebben omdat het moeilijk is om onderscheid te maken tussen processen ten gevolge van de kwaadaardige transformatie en de uitrijpingsblokkade en normale fysiologische processen.

Wij concluderen dat nauwkeurige diagnose en nauwgezette follow-up van leukemieën en kwaadaardige lymfomen belangrijk zijn voor de optimale keuze van behandeling, zowel bij kinderen als bij volwassenen. Met de huidige veelheid aan diagnostische technieken (morfologie, immunofenotypering, cytogenetica, immunogenetica, cel en weefselkweek, etc.) is dit mogelijk bij de meeste patiënten met een leukemie of kwaadaardig lymfoom. Hierdoor kan een betrouwbare prognose van het ziekteverloop verkregen worden en de meest optimale therapie worden toegepast. Om echter onnodig diagnostisch onderzoek en daarmee onnodige uitgaven te voorkomen is een nauwkeurige evaluatie van de diverse analyses per patiënt of patiëntengroep noodzakelijk. Tevens dient het belang van de PCR techniek om kleine aantallen leukemiecellen op te sporen bevestigd te worden in prospectieve "multicenter" studies, zodat de extra kosten van deze analyse afgewogen kunnen worden tegen de voordelen van deze vorm van diagnostiek voor de behandeling van patiënten met een leukemie of een maligne lymfoom.

ABBREVIATIONS

ABMT	:	autologous bone marrow transplantation
ALL	:	acute lymphoblastic leukemia
AML	:	acute myeloid leukemia
ANLL	:	acute non-lymphoblastic leukemia
ARA-C	:	cytosine arabinoside
B-ALL	:	B-cell acute lymphoblastic leukemia
BM	:	bone marrow
BMT	:	bone marrow transplantation
bp	:	base pair
BSA	:	bovine serum albumine
c	:	copy
C	:	constant
CD	:	cluster of differentiation/cluster of designation
CLL	:	chronic lymphocytic leukemia
CNS	:	central nervous system
CR	:	complete remission
Cy	:	cytoplasmic
CyCD3	:	cytoplasmic expression of CD3 antigen
Cyl$g\mu$	:	cytoplasmic immunoglobulin heavy-chain μ
D	:	diversity
DCLSG	:	dutch childhood leukemia study group
DGGE	:	denaturing gradient-gel electroforese
DNA	:	deoxyribonucleic acid
F	:	female
FAB	:	French American British cytomorphological classification of acute leukemias
FC	:	flow cytometer
FISH	:	fluorescence in situ hybridization
FITC	:	fluorescein isothiocyanate
GAP	:	GTPase activating protein
GF	:	growth factor
GFR	:	growth factor receptor
HCL	:	hairy cell leukemia
HRG	:	high risk group
HVP	:	hypervariable (or length) polymorphism
IF	:	immunofluorescence
Ig	:	immunoglobulin
IgH	:	immunoglobulin heavy-chain
IgL	:	immunoglobulin light-chain
Igκ	:	immunoglobulin kappa-chain
Igλ	:	immunoglobulin lambda-chain
intron RSS	:	heptamer recombination signal sequence located in the Jκ-Cκ intron
IP	:	immunoperoxidase
J	:	joining
kb	:	kilobase
Kde	:	kappa deleting element
LN	:	lymph node
m	:	messenger

M : male
McAb : monoclonal antibody
MDR : multidrug resistance
MNC : mononuclear cells
mo : month(s)
MPO : myeloperoxidase
MRD : minimal residual disease
MRG : medium risk group
N : nucleotide
ND : not determined
NHL : non-Hodgkin lymphoma
NT : not tested
PAGE : poly-acrylamide gel electroforese
PAS : periodic acid-Schiff
PB : peripheral blood
PBS : phosphate buffered saline
PCR : polymerase chain reaction
PL : pleura exudate
PLL : prolymphocytic leukemia
pre-B-ALL : acute lymphoblastic leukemia of pre-B cell origin
RAG : recombination activating gene
RFLP : restriction fragment lenght polymorphism
RNA : ribonucleic acid
RSS : recombination signal sequence
RT : room temperature
Sm : surface membrane
SmCD3 : surface membrane expression of CD3 antigen
SmIg : surface membrane immunoglobuline
SmIgμ : surface membrane immunoglobuline heavy-chain μ
SNS : symphathetic nervous system
SRG : standard risk groep
T-ALL : T-cell acute lymphoblastic leukemia
TcR : T-cell receptor
TcR-β : T-cell receptor bèta
TcR-γ : T-cell receptor gamma
TcR-δ : T-cell receptor delta
TK : tyrosine kinase
SSCP : single-stranded confirmation polymorphism
TdT : terminal deoxynucleotidyl transferase
TRITC : tetramethylrhodamine isothiocyanate
V : variable
w : weak
WBC : white blood cells
y : year(s)

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Auke

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- november 1992 : Oxford Higher certificate of proficiency in English.

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